ORIGINAL ARTICLE

Interactions between egg parasitoids and predatory ants for the biocontrol of the invasive brown marmorated stink bug *Halyomorpha halys*

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Abstract

The brown marmorated stink bug *Halyomorpha halys* is an Asian species that has become a major agricultural pest in North America and Europe. Ants from the genus *Crematogaster* are predators of *H. halys* nymphs in Asia, as well as in the Mediterranean, where known native predators are still few. At the same time, ants usually do not harm *H. halys* eggs, which are the target of the main biological control agents, the scelionid parasitoids of the genus *Trissolcus*. However, ants, as generalist predators and territorial organisms, may kill or displace a variety of other insects, potentially interfering with parasitoids and biological control programmes. We conducted laboratory experiments to investigate the interactions between the Mediterranean ant *Crematogaster scutellaris* and the parasitoids *T. japonicus* and *T. mitsukurii*, evaluating the possibility that the ants could damage the parasitized eggs, attack the parasitoids during emergence or interfere with the egg-laying behaviour of female parasitoids. Our results demonstrate that *C. scutellaris* is not able to damage parasitized eggs and is not aggressive towards adult parasitoids at any stage. The presence of ants can slow down the parasitization rate in *T. mitsukurii* females in the smallest laboratory setups; however, this has not been observed in a more natural setting. We suggest that ants may play a complementary role together with egg-parasitoids in the control of *H. halys* without interfering with each other.

KEYWORDS

Crematogaster scutellaris, intraguild relationships, *Trissolcus japonicus*, *Trissolcus mitsukurii*

1 | INTRODUCTION

The brown marmorated stink bug *Halyomorpha halys* (Stål), native to Eastern Asia and with invasive populations throughout the European, northern and southern American continents, is currently one of the most dangerous pests of fruit and seed crops (Leskey & Nielsen, 2018). Its high invasive capacity is facilitated by human activities and trade (Maistrello et al., 2018) and by high polyphagy (Rice et al., 2014), high mobility of the adults (Lee & Leskey, 2015), and high reproductive potential (Costi et al 2017). In Italy, where it

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was first officially detected in 2012 (Maistrello et al., 2016), *H. halys* quickly became a key pest of fruit orchards (Maistrello et al., 2017); and in 2019, the estimated damage to fruit production in northern Italy was € 588 million, with yield losses of up to 80%–100% in orchards (CSO Italy, 2020). To counter this invasive pest, the use of broad-spectrum insecticides has increased dramatically, resulting in a major disruption to previous integrated pest management (IPM) programmes with negative consequences on the environment (Maistrello et al., 2017). Long-term and more sustainable management strategies include conservation and classical biological control.

In native Asia, *H. halys* egg masses are attacked by different species of egg parasitoids, among which the Scelionidae *Trissolcus japonicus* (Ashmead) and *T. mitsukurii* (Ashmead) have the highest specificity and parasitization efficiency, ranging between 50% and 90% (Qiu, 2010; Yang et al., 2009; Zhang et al., 2017). In Northern Italy, adventive populations of *T. mitsukurii* and *T. japonicus* were first detected in 2016 (Scaccini et al., 2020) and 2018 (Sabbatini Peverieri et al., 2018), respectively. A large-scale survey conducted throughout northern Italy and Switzerland in 2019, showed that both species had rapidly spread into all types of habitats where *H. halys* is present, with a wide distribution, continuous expansion and high levels of parasitism (Zapponi et al., 2021). Furthermore, in 2020, *T. japonicus* was selected by the Italian Ministry of Environment and the Protection of the Land and Sea as a candidate for classical biocontrol of the invasive pest (MATTM, 2020) and thousands of these parasitoids were released in the northern Italian regions for 3 years, leading to one of the largest biocontrol projects ever attempted in Italy and Europe. Meanwhile, laboratory studies conducted to verify the potential of generalist antagonists showed that ants are among the most efficient predators of *H. halys* (Bulgarini, Badra, et al., 2021; Bulgarini, Castracani et al., 2021; Castracani et al., 2017). Specifically, experiments with the two European ants most frequently encountered in agroecosystems, *Crematogaster scutellaris* (Olivier) and *Lasius niger* (Linnaeus), demonstrated their ability to kill *H. halys* nymphs without damaging eggs or adult stink bugs (Bulgarini, Castracani, et al., 2021; Castracani et al., 2017). Further studies conducted with the Japanese ant *Crematogaster matsumurai* Forel, 1901 and *C. osakensis* Forel, 1900 as well the cosmopolite invasive Argentine ant *Linepithema humile* (Mayr, 1868), had a similar outcome (Kamiyama et al., 2021).

This study aims to investigate the interactions between the native European ant *C. scutellaris* and the exotic egg parasitoids *T. japonicus* and *T. mitsukurii* in terms of the outcome on the efficiency of biological control of *H. halys*.

Ants are ubiquitous across most terrestrial ecosystems of the world, where they often play a significant ecological role as generalist predators (Hölldobler & Wilson, 1990; Lach et al., 2010; Parker & Kronauer, 2021). Their predatory abilities against insect pests can not only make them good key biological control agents (Choate & Drummond, 2011; Offenberg, 2015) but also may negatively affect other important biocontrol agents, including both parasitoids and predators (e.g. Appiah et al., 2014; Jiggins et al., 1993; Mgocheki & Addison, 2009).

We hypothesized that ants may attack *H. halys* egg parasitoids as they do with *H. halys* nymphs (Bulgarini, Castracani, et al., 2021; Castracani et al., 2017). In particular, we investigated two moments in the life of adult parasitoids in which they could be particularly vulnerable: the moment in which the female parasitoid lays her eggs, which requires her to stand still on the stink bug egg mass for an extended time, and the moment of emergence of the newly metamorphosed individuals, as they need time to break an opening in the stink bug egg to free themselves. We also tested whether parasitized eggs might be more susceptible to ant attack than non-parasitized eggs, which are usually not attacked, and whether their attractiveness to ants could vary over time.

2 | MATERIALS AND METHODS

2.1 | Insect rearing and equipment

Adults of *Halyomorpha halys* were collected during the spring and summer of 2020–2021 from urban parks in Modena and Reggio Emilia provinces (Emilia-Romagna, Italy) using the tree-beating technique. Stink bugs were set in BugDorm cages (17.5×17.5×17.5 cm) and placed in climatic chambers at 26°C and L16: D8. Each cage contained up to 50 adults with a sex ratio of 50:50. The stink bugs were fed twice a week with fresh organic fruits and peanuts. Sheets of filter paper were placed in the cage as egg-laying substrates. Freshly laid egg masses of *H. halys* (<24 h old) with 27–28 eggs were used for the experiments. Rarer egg masses with different numbers of eggs were excluded.

Trissolcus japonicus and *T. mitsukurii* adults were obtained from field-collected *H. halys* egg masses and were reared in BugDorm cages (12×12×12 cm) in climatic chambers at 23°C and L16: D8, and fed with drops of a honey-water solution (70% organic honey solution). Every 3 days, freshly laid egg masses of *H. halys* (<24 h old) were offered to the parasitoids. The parasitized egg masses were individually transferred to empty vials and stored at 26°C and L16: D8 pending the emergence of the parasitoids. The newly emerged parasitoids of each species were mated (one female and one male) for 1 week in vials (Falcon 50 mL, the lid of which was replaced by a piece of pantyhose fixed with an elastic band) and supplied with drops of the honey-water solution. After the mating period, the females of each species were used in the experiments.

Colony fragments consisting in several hundred workers of *Crematogaster scutellaris* (Olivier, 1792) were collected in the wild from Parma (Italy) and reared in plastic cages under the following conditions: T: 25°±1 C, RH: 55±10%, L16: D 8. They were fed with the same honey-water solution used for parasitoids and with *Tenebrio molitor* Linnaeus larvae. Ants endured a 48 h starvation period prior to the experiments.

All tests were conducted in a climatic chamber at 26°C and L16:D8 in the Laboratory of Applied Entomology of the University of Modena and Reggio Emilia.

All video recordings were performed using an HC-V380 Panasonic camera. A binocular microscope Zeiss Stemi 508 was used to verify if ants and/or parasitoids were alive after the experiments.

2.2 | Experimental procedure

We carried out three experiments. Experiment I aimed at verifying whether parasitized eggs and emerging parasitoids can be attacked and damaged by ants. Experiments II and III aimed at evaluating whether ants and adult parasitoids behave aggressively in a simplified context (one-to-one interactions in a Petri dish) and a more complex system (a parasitoid couple, a larger number of ants, and a plant) respectively. In the simplified context of Petri dishes, single workers of *C. scutellaris* retain their basic foraging behaviours, killing and carrying away prey insects (e.g. Giannetti et al., 2022; Schifani, Giannetti, & Grasso, 2023; Schifani, Peri, Giannetti, Alınç, et al., 2023; Schifani, Peri, Giannetti, Colazza, & Grasso, 2023). In all experiments, we counted the number of sting bugs and parasitoids that emerged from the eggs, and the number of surviving parasitoids.

2.3 | Experiment I: Interactions between ants and parasitized eggs or emerging parasitoids

To verify whether parasitized eggs attracted the interest of ants, we prepared egg masses in which parasitization of all eggs by *T. japonicus* or *T. mitsukurii* was established during preliminary observations. Specifically, after introducing a parasitoid female to each egg mass, its activities were video-recorded and the number of markings was checked. The following behaviours have been observed: probing the host, inserting the ovipositor and performing head-pumping movements and body vibrations associated with egg-release, partially extracting the ovipositor and sweeping it over the surface of the host egg with 'figure 8'-shaped movements, as described by Field (1998).

Each egg mass was transferred in the centre of a Petri dish (∅ = 9 cm), which was followed by the introduction of a single ant worker. The petri dish was then filmed for 40 min to collect behavioural data, after which the ant was removed. Egg masses were exposed to ants after either 0, 2, 4, 6 or 9 days after parasitization, to test the behaviour of ants towards parasitized eggs at different development stages, or during parasitoid emergence, to test ant behaviour towards emerging adults. Six replicates were performed for each developmental stage of each parasitoid species, both for the treatment (presence of the ant) and for the control (no ant).

2.4 | Experiment II: 1 versus 1 interactions in petri dishes (40 min)

Tests were conducted by placing a non-parasitized egg mass in the centre of a Petri dish (∅ = 9 cm) and introducing a single female parasitoid. As soon as the parasitoid made its first contact with the egg mass, we introduced an ant worker. Once the ant was introduced, we filmed the petri dish for 40 min to collect behavioural data. No ants were introduced into the control replicates, and filming started as soon as the parasitoid made its first contact with the egg mass. At the end of each test, we checked under the microscope whether the

ant and the parasitoid were still alive and if any of them had suffered visible injuries. We conducted 10 treatment replicates with ants and 10 control replicates (no ants) for each of the two parasitoid species.

2.5 | Experiment III: Interactions in insect cages (24 h)

Tests were conducted using a 30 × 30 × 30 cm insect cage. At the centre of each cage, we placed the following items: (i) a *Capsicum annuum* L. plant (approximately 15 cm tall); (ii) a Falcon vial containing a female and a male parasitoid of either *T. japonicus* or *T. mitsukurii*; (iii) a plastic jar (∅ = 4 cm, height = 7 cm) containing a group of 50 ant workers, partially filled with small wood pieces, and with the inner upper edge covered with an ant repellent substance (50% glycerine oil, 50% petroleum jelly) to prevent their escape. To start the experiments, we performed the following steps: (i) on an apical leaf of each plant we clipped a 1 × 3 cm filter paper with a single egg mass previously attached with a glue stick; (ii) we placed a 12 cm wooden stick to connect the plant on one hand and the wood pieces in the plastic jar on the other, allowing the ants to get out of the jar and visit the plant; (iii) we opened the lid of the vial, allowing the two parasitoids to move freely inside the cage. Each experimental test lasted 24 h, after which we removed the egg masses and the parasitoids and checked whether the latter were alive or dead. The egg masses were incubated until they hatched, or parasitoids emerged.

We conducted 24 replicates per parasitoid species (*T. japonicus* or *T. mitsukurii*), equally divided between replicates with ants and control replicates without ants.

2.6 | Behavioural data

The behaviour of ants and parasitoids was analysed by video-recording the experiments and analysing the resulting videos with the software Solomon Coder (https://solomon.andraspeter.com/).

Concerning ants, we recorded the time between their entry into the experimental arena and their first contact with the eggs or parasitoids (contact latency), and the number of times the following six behaviours, directed towards the eggs or the parasitoids as targets, were observed: (i) *antennation* (making contact with the antennae); (ii) *biting with mandibles*; (iii) *licking*; (iv) *walking over* the female parasitoid; (v) *threatening with open mandibles* (assuming a motionless posture with open mandibles); (vi) *threatening with the stinger* by directing it in the direction of the target at close range, as typical of the spatulate stinger of *Crematogaster* ants; (vii) *gaster rising*, consisting in an alarm posture typical of *Crematogaster* ants in which the gaster is raised above in a position perpendicular to the body plane.

Concerning parasitoids, we recorded the number of times the following three behaviours were observed: (i) *oviposition* (*including marking*), which consists of probing the host, inserting the ovipositor, and making head-pumping movements and body vibrations
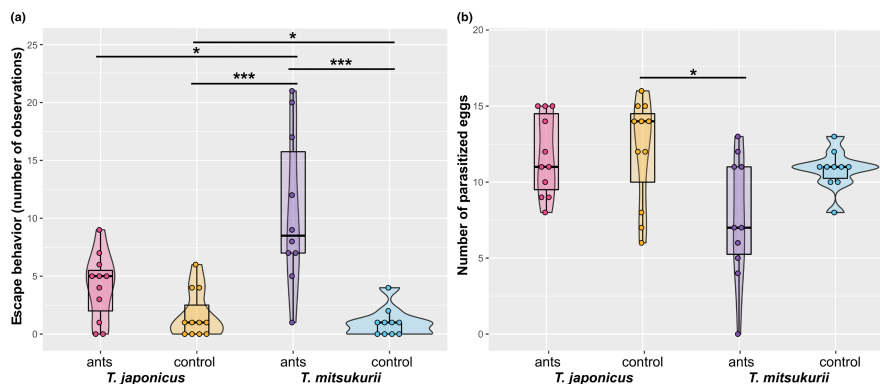
FIGURE 1 Most significant results of the experiments II, in which the effects of *Crematogaster scutellaris* ants on the behaviour of *Trissolcus japonicus* and *T. mitsukurii* females parasitizing *Halyomorpha halys* eggs were observed in the restricted setting of a petri dish for 40 min. (a) The number of times female parasitoids moved away from the egg-masses (escape behaviour); (b) the number of stink bug eggs the parasitoids were able to parasitize. According to pairwise comparisons, significantly different treatments are connected by black horizontal lines, and the above asterisks indicate significance levels (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

associated with the egg-release, partially exerting the ovipositor and sweeping it across the surface of the host egg in ∞-shaped movements as described by Field (1998); (ii) *chase-off*, consisting in running directly towards the ant, sometimes lunging with raised wings, making contact and biting it as described by Field (1998); (iii) *escape*, i.e. moving away from the egg mass; (iv) *resting*, i.e. stopping the oviposition and standing immobile.

2.7 | Statistical analyses

Statistical analyses were conducted using the software R 4.1.2 (R Core Team, 2020). We used Wilcoxon rank-sum tests to analyse differences between two groups, and Kruskal–Wallis tests followed by Dunn's post hoc tests with Benjamini–Hochberg p-value adjustment to analyse differences between multiple groups. Statistical tests were not run for behaviours occurring in less than 15% of the trials. For each test, $\alpha = 0.05$.

3 | RESULTS

Data collected in all experiments are provided in Table S1.

3.1 | Experiment I: Interactions between ants and parasitized eggs or emerging parasitoids

Regardless of the *Trissolcus* species tested in the trials, ants never caused any noticeable harm to parasitized eggs, and never attacked emerging parasitoids.

The number of emerged parasitoids did not differ among treatments (distinguishing between *T. japonicus* or *T. mitsukurii*, eggs exposed to ants 0, 2, 4, 6 or 9 days after parasitization, and eggs never exposed to ants before emergence) ($0.09 < p < 1.000$, Dunn's test).

In trials with ants, antennation was always observed and no significant differences were detected between treatments with the two parasitoid species ($p = 0.09 < p < 1.000$, Dunn's test). The escape behaviour was recorded in 58% of the trials with emerging parasitoids and was not significantly different between the two parasitoid species ($p = 0.23$, Wilcoxon rank-sum test). The following behaviours were extremely rare across the 72 trials that were run (<15%): biting (10 trials), licking (1 trial), threatening with open mandibles (6 trials), gaster rising (0 trials) and threatening with the stinger (2 trials).

3.2 | Experiment II: 1 versus 1 interactions in petri dishes (40 min)

Ants were never observed to attack and harm either of the two parasitoid species. Approaching ants often caused *T. mitsukurii* females to temporarily leave the egg masses, slowing their overall parasitization rate. On the contrary, *T. japonicus* females remained on the egg masses even when ants touched them, and their parasitization rate was not affected by the ants' presence.

The escape behaviour of parasitoids was significantly different based on treatment ($p < 0.001$; Kruskal–Wallis test): it was higher for *T. mitsukurii* in presence of ants compared to the other three treatments ($0.035 < p < 0.001$; Dunn's post hoc test), and more frequently observed for *T. japonicus* with ants compared to *T. mitsukurii* without ants ($p = 0.038$; Dunn's post hoc test), while no significant differences were detected in the remaining comparisons (Figure 1). Treatment affected the number of eggs that were parasitized ($p = 0.033$; Kruskal-Wallis test): in presence of ants *T. mitsukurii* parasitized a significantly lower number of *H. halys* eggs compared to *T. japonicus* trials with no ants ($p = 0.023$; Dunn's test), while no significant differences were detected in the remaining comparisons. The parasitoids managed to parasitize 11 eggs on average (39% of all eggs in the egg masses).

Contact latency of ants approaching parasitized eggs was not significantly different between replicates with *T. japonicus* and with *T. mitsukurii* (*p* = 0.123; Wilcoxon rank-sum tests). Walking over was observed in half of the *T. japonicus* trials, threatening with the gaster in one *T. japonicus* trial, while both behaviours were not observed in trials with *T. mitsukurii*. Resting behaviour was only observed once per each parasitoid species. Gaster rising and chase-off behaviours were never observed.

3.3 | Experiment III: Interactions in insect cages (24 h)

Ants had no impact on the parasitization activity and mortality of either *T. mitsukurii* or *T. japonicus*.

There were no statistically significant differences in the number of parasitoids hatched from the eggs in relation to the parasitoid species or the presence of ants (*p* = 0.821; Kruskal–Wallis test) nor any significant difference in the number of parasitoids found alive after the experiments (*p* = 0.424; Kruskal–Wallis test) (Figure 2).

4 | DISCUSSION

In our experiments, ants never directly attacked *Trissolcus* parasitoids, including in the potentially vulnerable moment of their emergence. Furthermore, parasitization did not alter the ant's ability to attack *H. halys* eggs, suggesting that parasitized and non-parasitized eggs are equally unlikely to suffer any damage by this ant. Stink bug eggs are rarely successfully attacked by ants, and the few known examples refer to cases of relatively large ants capable of considerable biting force, while chemical cues (or their absence) may also contribute to avoiding ant attacks (Castracani et al., 2017; Schifani, Giannetti, & Grasso, 2023).

Crematogaster scutellaris still affected the behaviour of *T. mitsukurii* in the confined space of Petri dishes, even if it did not perform any direct attack against the parasitoid. Notably, in the presence of an ant worker, female *T. mitsukurii* significantly more often stopped the egg-laying process and moved away, which diminished the number of stink bug eggs parasitized during the observation time, albeit not significantly. The same did not occur with *T. japonicus*, since the latter mostly ignored the approach of an ant, and even upon contact, it normally avoided abandoning the eggs. However, such interesting behavioural differences did not appear to play a role when ant-parasitoid interactions were observed in the more complex and larger cage environment, where *T. japonicus* and *T. mitsukurii* had similar parasitization success, regardless of the presence of *C. scutellaris* workers. Since *C. scutellaris* ants never harmed the parasitoids in direct encounters and had only a slight disturbance effect when artificially enclosed with *T. mitsukurii* in a very confined space, it is highly unlikely that interactions between *C. scutellaris* and *Trissolcus* parasitoids play a significant role under field conditions. *Crematogaster scutellaris* and the two non-native egg parasitoids *T. japonicus* and *T. mitsukurii* are currently co-occurring and rapidly spreading across the northern Italian regions invaded by *H. halys* (Zapponi et al., 2021). Multiparasitism laboratory experiments with *T. japonicus* and *T. mitsukurii* indicate that the order of arrival on the host's eggs is crucial to ensure the most successful parasitization, and that, competition between the two species did not result in reduced *H. halys* egg mortality (Costi et al., 2022).

As biological control agents, ants are appreciated for their polyphagy, territorial aggressiveness, resistance to starvation, and the possibility to manipulate their behaviour (Choate & Drummond, 2011; Offenberg, 2015). Negative effects are mainly observed when ants have a mutualistic relationship with pest insects, usually, honeydew-producing hemipterans, which they can defend against predatory insects and parasitoids used to control them (e.g. Jiggins et al., 1993; Mgocheki & Addison, 2009). The relationship of ants with parasitoids of ant-mutualistic hemipterans is generally antagonistic but not always relevant to biocontrol (Schifani, Peri, Giannetti, Colazza, & Grasso, 2023), and there are a few exceptions of myrmecophilous parasitoids adapted to exploit ant's presence (Pierce & Mead, 1981; Völkl, 1992). However, as generalist predators, ants may also attack parasitoids that do not interact with their mutualistic networks
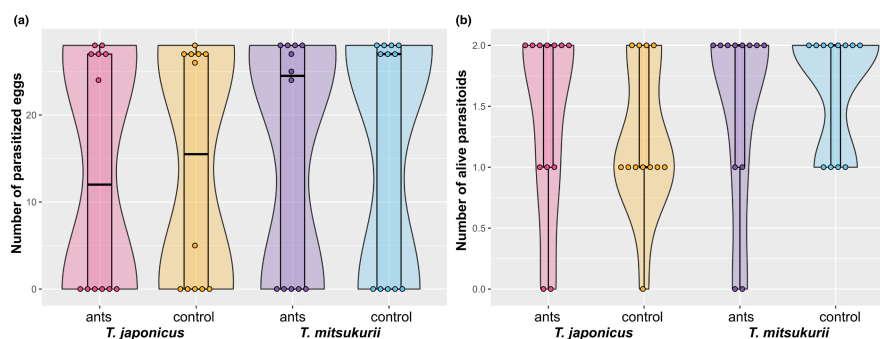
FIGURE 2 Most significant results of the experiments III, in which the effects of *Crematogaster scutellaris* ants on the behaviour of *Trissolcus japonicus* and *T. mitsukurii* females parasitizing *Halyomorpha halys* eggs were studied in the more natural setting of an insect cage with a plant, in which the insects were released for 24 h. (a) The number of stink bug eggs the parasitoids were able to parasitize; (b) the number of alive parasitoids verified at the end of the experiment. In both cases, no statistically significant differences between treatments were detected.

(Appiah et al., 2014). We have observed substantial neutrality between ants and parasitoids in our experiments. *Crematogaster scutellaris* is an ant that may play a useful role in pest management thanks to its common presence in agroecosystems and its predatory abilities against other pests such as the codling moth *Cydia pomonella* (L.), the ambrosia beetle *Xylosandrus compactus* (Eichoff, 1876) or the stink bug *N. viridula* (Giannetti et al., 2022; Schifani, Giannetti, & Grasso, 2023; Schifani, Peri, Giannetti, Alınç, et al., 2023). Notably, both stink bugs and parasitoids are attracted by sugary nectars, whose provision may serve the purpose of manipulating their behaviour or enhancing their efficacy as biocontrol agents (Colazza et al., 2022; McIntosh et al., 2020; Schifani et al., 2020).

By revealing that ants do not interfere with egg parasitization nor they attack egg parasitoids, our study encourages the possibility that ants and parasitoids may be integrated in the control of *H. halys*, with a combined effect on both eggs and nymphs that needs to be evaluated in field assessments (Bulgarini et al., 2022; Campolo et al., 2015; Castracani et al., 2017; Offenberg, 2015; Wright & Diez, 2011).

AUTHOR CONTRIBUTIONS

Enrico Schifani: Conceptualization; methodology; data curation; validation; formal analysis; visualization; resources; writing – original draft; investigation. **Daniele Giannetti:** Conceptualization; methodology; investigation; validation; formal analysis; resources; data curation. **Elena Costi:** Conceptualization; methodology; validation; formal analysis; investigation; resources; data curation. **Giulia Franconi:** Investigation; data curation; resources. **Arianna Campostrini:** Investigation; resources; data curation. **Lara Maistrello:** Conceptualization; validation; project administration; supervision; funding acquisition; resources. **Donato Grasso:** Conceptualization; validation; project administration; supervision; resources; funding acquisition.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

Data supporting the reported results can be found at https://doi.org/10.5281/zenodo.8082046.

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SUPPORTING INFORMATION

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