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DEVELOPMENT OF THE STERILE INSECT TECHNIQUE FOR THE INTEGRATED MANAGEMENT OF THE BROWN MARMORATED STINK BUG *HALYOMORPHA HALYS* STÅL (HEMIPTERA: PENTATOMIDAE): BASIC REQUIREMENTS AND NEW KNOWLEDGE ACQUISITIONS TO SET UP THE NEW TOOL

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LIST OF SYMBOLS AND ABBREVIATIONS

AW-IPM	Area-Wide Integrated Pest Management
BCA	Biological Control Agent
BMSB	Brown Marmorated Stink Bug
CBC	Classical Biological Control
IPM	Integrated Pest Management
LINAC	Linear Accelerator
SIT	Sterile Insect Technique

ABSTRACT

The brown marmorated stink bug (BMSB), *Halyomorpha halys* (Stål) (Hemiptera: Pentatomidae), is an invasive phytophagous species native to eastern Asia. Due to its wide host plant range, it is considered among the most harmful agricultural pests in the invaded areas, including Europe, North and South America. In addition to its impact on crops, this insect causes a nuisance in urban areas, by invading buildings for overwintering using their aggregation pheromones and emitting an unpleasant smell when disturbed. The current management of this species is mainly based on the use of chemical insecticides, which show efficacy when applied frequently, with consequent negative impact on beneficial insects in the agroecosystems. As a result, there is a desire for more environmentally friendly solutions for the management of BMSB. A valid alternative is the use of exotic and coevolved biocontrol agents, for instance, the Samurai wasp, *Trissolcus japonicus* (Ashmead) (Hymenoptera: Scelionidae), which is already showing promising medium and long-term results against BMSB in classical biocontrol projects. Within the frame of the least-toxic alternatives, the use of the Sterile Insect Technique (SIT) in an Area-Wide management approach is potentially a valid strategy to be included in integrated control and eradication programs.

The main objective of this research, therefore, was to develop a SIT for potential use in the eradication of BMSB in newly introduced areas and/or as an additional tool to reduce its impact in areas where invasive populations have been already established. However, the effective use of SIT requires producing a high number of insects, and mass rearing for such a species is considered too complicated and expensive. To overcome this limitation, we evaluated a harvest, irradiation, and release strategy using wild overwintering adults. New live traps baited with high-load pheromones have been developed for mass trapping, exploiting the natural phenomenon ruled behaviour of aggregation of BMSB in preparation for overwintering. The captures of the new pheromone traps were compared with those of identically baited sticky panels through daily checks lasting two weeks. Following winter diapause, the collected males were irradiated with high-energy photons at four different dosages: 16, 24, 32 and 40 Gy. The effects of irradiation on the biology i.e., fertility, longevity, and mating behaviour of overwintering BMSB males were evaluated with respect to unirradiated males. To encourage the use of SIT, a linear accelerator from a hospital facility was used for irradiation instead of the classical method involving radioactive materials. A second control 0 Gy was adopted to evaluate the possible negative effects on the biology (longevity, fertility, and fecundity) of insects due to the preparation for the irradiation.

Moreover, as part of an integrated pest management (IPM) strategy, the combined use of the egg parasitoid *T. japonicus* and SIT was considered. The suitability of sterile eggs as oviposition substrate for *T. japonicus* was evaluated by comparing irradiated BMSB eggs at 40 Gy, eggs obtained by fertile female mated with sterile males irradiated at 50 Gy, and refrigerated sterile eggs currently used for laboratory rearing and field monitoring.

The results of the research are encouraging for SIT applications in IPM approach. 1) The new traps caught up to 15-times more adult BMSB than the sticky panels in the two weeks of collections. 2) The developed irradiation protocol that involved a 6 MV medical linear accelerator has allowed the achievement of a high level of sterility of overwintering males (over 95%) already at 32 Gy without compromising males' longevity and mating behaviour (in no-choice conditions). No adverse effects due to the irradiation preparation protocol were observed. 3) Sterile eggs obtained by mating sterile irradiated males and fertile females (SIT eggs), showed a higher parasitoid emergence rate (%) than both those directly irradiated and refrigerated. The difference in the emergence rate is even more evident when the eggs are 20 days old, with a parasitoid emergence rate of (75.56 %) for SIT eggs compared to those irradiated (44.68 %) and refrigerated (37.57%).

In conclusion: 1) the new live traps are effective in mass trapping, 2) wild harvested males of BMSB can be used in SIT, 3) the use of linear accelerators for irradiation of BMSB is a feasible alternative to classical radioactive sources and 4) sterile eggs obtained by mating sterile males and fertile females are a valid substrate for *T. japonicus* oviposition, supporting the potential use of SIT on BMSB.

CHAPTER 1. INTRODUCTION

1.1 Global Change and Biological Invasions

Human activities have led the Earth into a new era characterized by significant alterations in the global environment. Global change has introduced unprecedented conditions that will have long-lasting effects for years to come (Sage, 2019).

One of the primary challenges related to human activities is the climate change (Rădoi, 2020), exhibiting interconnected effects on insect pests and the physiology of host plants (Boullis et al., 2015; War et al., 2016). Alterations in climatic parameters result in changes to various aspects of insect populations, including voltinism, survival, fecundity, and dispersal, while also impacting the fitness of natural enemies and parasitism (Caselli & Petacchi, 2021). The most compelling current evidence supporting the potential influence of climate change on the emergence of novel non-indigenous species resides primarily within the domain of terrestrial arthropods (Hulme, 2016). This can be attributed to their ectothermic physiological nature, frequent rapid dispersal capabilities, significant involvement in commercial activities, and symbiotic connections with human-inhabited environments (Hulme, 2016). Indeed, human activities have facilitated the transportation of over 120,000 species worldwide. These introductions can occur intentionally for agricultural or horticultural purposes, or unintentionally through the transportation of exotic species in cargo or ballast (Sage, 2020). Under these circumstances, invasive phytophagous insects result in a yearly global economic loss of no less than 70 billion USD (Bradshaw et al., 2016).

As a pervasive component of global change, biological invasions pose a considerable challenge to the preservation of biodiversity and natural resources (Simberloff et al., 2013). The

Mediterranean basin will face escalating risks across multiple levels in the forthcoming decades (Caselli & Petacchi, 2021; Fraga et al., 2021). Therefore, this situation necessitates enhanced risk assessment and restructuring of control strategies to adapt to these changes.

1.2 New Awareness in Agriculture: The Integrated Pest Management

Historically, the control of pests relied heavily on the use of synthetic chemical compounds generally associated with negative consequences on the environment and beneficial organisms. In addition, they often induce pesticides resistance on target species (Damos et al., 2015). Most pest management programs still rely primarily on this control strategy with conventional insecticides (Pretty & Bharucha, 2015; Damos et al., 2015). However, a complete prohibition of pesticides could impose significant costs on the economy, crop yields, and food security (Pretty & Bharucha, 2015).

Fortunately, the current global challenges are accompanied by a growing awareness of the growers (Nobre, 2019) and there is a desire to limit the use of broad-spectrum insecticides in favour of alternative sustainable techniques. Over the past three decades, significant progress has been made towards achieving a more sustainable form of agriculture (Gaffney et al., 2019). Despite the intricate nature of the subject (Gaffney et al., 2019), efforts have advanced to safeguard the environment and conserve its resources, and this trend is now steadily and irreversibly unfolding.

Integrated pest management (IPM) exemplifies a sustainable intensification system achieved by redesigning agroecosystems (Damos et al., 2015; Pretty & Bharucha, 2015; Pretty et al., 2018). By employing various tactics, IPM facilitates the reduction of economic losses caused by pests and diseases while minimising the reliance on synthetic pesticides, thereby generating a range of associated benefits (Pretty & Bharucha, 2015). Particularly, an area-wide IPM approach is a long-term strategy based on preventive management of the entire pest population instead of field-by-field management and requires well-coordinated implementation. This approach typically employs sophisticated methods like geographic information systems, remote sensing, mathematical models, and population genetics, demanding a significant level of specialized knowledge and expertise (Klassen, 2005).

1.3 Biological control

Biological control is a pest management strategy that aims to reduce the population of a species (commonly an insect pest) by releasing living organisms that have been chosen for that purpose. Classical biological control (CBC) involves the release of exotic natural enemies and establishing a new equilibrium by controlling the targeted pests; conservation biocontrol aims to preserve and enhance the effectiveness of natural enemies), and augmentative biocontrol requires the release of natural enemies and augment their natural populations (Greene et al., 2018).

CBC requires to deliberately import and introduce exotic natural enemies of the target species, nonetheless, the development of CBC programs need regulatory approvals in many countries (Bigler et al., 2006; Heimpel & Cock, 2018). Indeed, in Europe the use of exotic biological control agents (BCAs) is limited by the “Habitats Directive” (Council Directive 92/43/EEC, 21/05/1992, on the conservation of natural habitats and wild fauna and flora). CBC can also help reduce the use of chemicals in pest management (Bigler et al., 2006; Heimpel & Cock, 2018).

In the context CBC, egg parasitoids exhibit a greater rate of establishment than other types of parasitoids, except for Trichogrammatidae; however, they have not demonstrated the same level of efficacy in significantly reducing the population of the desired pest (Mills, 2010). By definition, egg parasitoids are parasitoids organisms that both oviposit and complete their development inside a host egg (Mills, 1994). Egg parasitoids used in CBC are categorised within seven families of parasitic

Hymenoptera. Among these, three families (Mymaridae, Scelionidae, and Trichogrammatidae) consist exclusively of egg parasitoids, while the remaining four families (Elasmidae, Encyrtidae, Eulophidae, and Platygastridae) include species that include egg parasitoids (Mills 2010).

Probably the most known example of CBC with scelionid egg parasitoids is the introduction of *Trissolcus basalis* (Wollaston) from Egypt for the control of *Nezara viridula* (L.) in Australia, New Zealand and Hawaii (Clarke, 1990; Jones, 1995). Even if it was reported as a complete success (Waterhouse & Norris 1987), *N. viridula* remains a pest of annual concern for legumes and macadamia nuts (Loch and Walter 1999, Knight and Gurr 2007) respectively in Australia and in Hawaii (Jones, 1995). Moreover, *T. basalis* has also impacted on non-target pentatomids in Australia (Loch & Walter 1999) and Hawaii (Johnson et al., 2005).

1.4 Pheromone traps

Pheromones and other semiochemicals are used to monitor the presence and abundance of insects and in animal and plant protection (Witzgall et al., 2010). They are species-specific, and even in synthetic incomplete blends, generally affect only the target species, except some taxonomically close species (Cardé & Haynes, 2004).

The main application of sex pheromones is their use in insect detection and population monitoring. The use of traps baited with synthetic pheromone lures can show whether a specific insect is present, and when its seasonal flight period starts, suggesting the timing of action with insecticide sprays accordingly and monitoring their efficacy (Levinson & Levinson, 1979; Witzgall et al., 2010). Pheromone traps exhibit the necessary sensitivity to identify populations with low density. As a result, they prove to be efficient in monitoring invasive species during their establishment. (El-Sayed et al. 2006; Liebhold & Tobin 2008).

Over the years, many types of traps have been developed; among the most common are adhesive traps and funnels (Levinson & Levinson, 1979). Detection and monitoring are just some of the potential uses of pheromones in insect pest management, indeed, the control of insect populations using pheromones can be also achieved by mating disruption and mass annihilation (Witzgall et al., 2010).

Mating disruption induces disorientation and disrupts the communication between the sexes. As a result, delays, reduces, or prevents mating and the fertilization of females; it is probably the main utilized semiochemical-based technique for insect population control in IMP (Baker & Heath, 2004; Witzgall et al., 2008; Rizvi et al., 2021).

Mass annihilation, by mass trapping or attract and kill, relies on the attraction of one or both sexes and nymphs (or larvae) to a lure, in combination with a large-capacity trap or an insecticide-impregnated target (Witzgall et al., 2010; Morrison et al., 2016). Unlike detection and monitoring, where only a small proportion of a population needs to be sampled, mass annihilation requires the use of the most attractive lure (Witzgall et al., 2010), however in long-term pest management, attract and-kill can reduce the insecticides usage by diminishing the male adult population and their reproduction rate (El-Sayed et al., 2009; Morrison et al, 2016; Rizvi et al., 2021).

1.5 The sterile insect technique

Among the frame of the least toxic alternatives, another potential control tactic for insect pest management is the sterile insect technique (SIT).

This environmentally friendly and species-specific method involves mass rearing, sterilization, and release of predominantly male sterile insects in large numbers (Klassen & Curtis, 2021). To induce insect sterility, mass-reared insects are commonly exposed to gamma rays or X-rays, which prevents them from producing viable offspring (Klassen & Curtis, 2021). By releasing enough sterile males able to mate with wild females, populations of the targeted species decline (Lance & McInnis, 2021). To guarantee a successful outcome of SIT, many assumptions must be met. Some critical points are the absence of parthenogenesis in the target species, the possibility of mass rearing of the insects and little or no damage to crops by released insects (Klassen & Curtis, 2021).

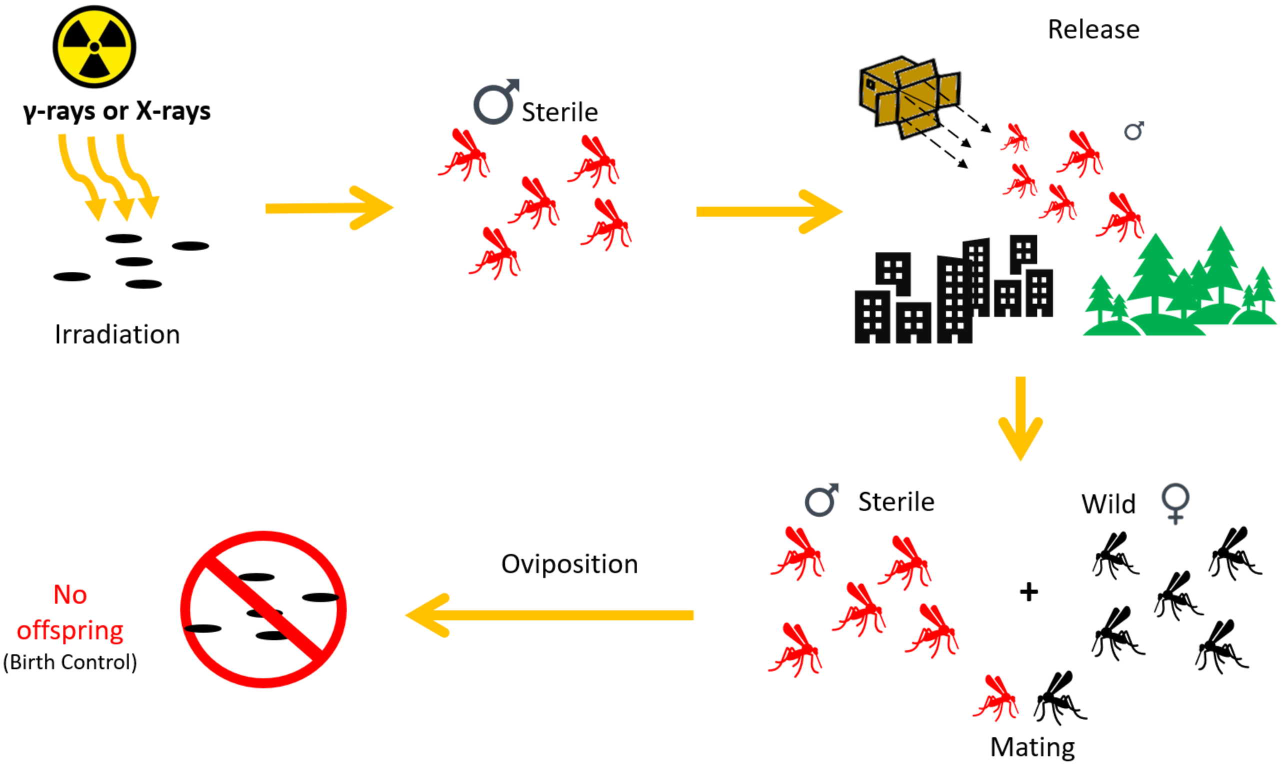

Figure 1.1. Simplified diagram of the operation of the SIT: Mass reared insects, or pupae, are irradiated with gamma- or X-rays, then released into the environment, where they mate with wild females without generating progeny.

The sterile insect technique has effectively implemented area-wide IPM (AW-IPM) strategy (Klassen & Vreysen, 2021). It has been utilized to suppress, control, and prevent the (re)introduction, and even achieve local eradication of insect pests and disease vectors (Klassen & Curtis, 2021). The horticultural industry of countries such as Guatemala, Belize, Mexico, and the United States has been protected by *Ceratitits capitata* (Wiedemann) thanks to its application (Enkerlin et al., 2017); also applied against the tsetse fly *Glossina austeni* (Wiedemann) made possible its eradication from

Unguja Island, Zanzibar where represented a problem for livestock (Vreysen et al., 2000). Also, for applications affecting human health, there is much interest, and SIT is used in various programs to control different species of mosquitoes, which are vectors of serious pathogens of human diseases such as malaria and dengue (Lees et al., 2021).

The use of SIT in AW-IPM strategy has been well accepted over the years end has demonstrated the possibility of suppression or eradication for Lepidoptera and Diptera (Arnone et al., 2017; Vreysen et al., 2006). The success of AW-IPM can also be supported by the synergism of SIT and classical biological control approaches (Knipling, 1979; Liebhold et al., 2016).

1.6 Invasive stink bugs

Among the ecological and agricultural pest that have surfaced in the past few decades, pentatomoids unquestionably assume a significant role because most of them are phytophagous, feeding on a diverse array of crops such as fruits, vegetables, nuts, grains, in addition to various wild host plants (McPhearson et al., 2018) The feeding activity mainly impacts developing seeds, fruits or growing shoots, but involves also other plant parts such as roots, stems and leaves. The main damage that ensues may involve the development of discoloration surrounding the puncture location and the formation of powdery voids resulting from the extraction of cell contents, leading to spoilage (Panizzi, 1997).

Exacerbating the adverse impacts of their feeding behavior is their capacity to serve as vectors for plant pathogens. Pentatomids have been identified or are under suspicion for their role in transmitting a diverse range of plant pathogens (Mitchell et al., 2018) responsible for inducing various diseases. These include the pathogens responsible for seed and boll rot, yeast spot, leaf spot and vein necrosis, stem canker, stigmatomycosis, panicle and shoot blight, witches' broom, hartrot, and marchitez. (Mitchell et al., 2018)

Among stink bugs, some species are extremely polyphagous or impact important crops and emerged as insect pest in many continents:

a) *Piezodorus guildini* (Westwood), originally from the Lesser Antilles, it currently extends from Argentina and Brazil to the southern United States (Panizzi, 2015). It is an important pest of soybean wherever the crop is grown in warmer regions of the American continent (Panizzi et al. 2000; Artabe and Martínez 2003). In South America, it is also a pest of other legumes including alfalfa, *Medicago sativa* (L.) and various forage legumes (Zerbino et al. 2015).

b) *Nezara viridula* (L.) is an important worldwide pest that is highly polyphagous, with a preference for legumes and brassicas (Esquivel & Ward 2014) and is also adapted to a wide host range of wild plant species when preferred food sources are absent or during overwintering. These abilities facilitate its wide distribution and expansion (Grozea et al., 2012).

c) *Bagrada hilaris* (Burmeister), native to Africa, the Middle East, and Central Asia (Singh et al., 1993; Verma et al., 1993), invaded USA, Mexico (Reed et al., 2013a; Conti et al., 2021), Europe (Pantelleria Island, Italy, and Malta), Australia, Chile, and Southeast Asia (Bundy et al., 2018; Carvajal et al., 2019). It is a polyphagous species that feeds on 96 different host plants belonging to 25 families, although most of its hosts belong to the Brassicaceae family (n = 30) which includes many plants of agricultural interest and on which it causes most economic damage (Bundy et al., 2018).

d) *Halyomorpha halys* (Stål) is an invasive phytophagous species native to Eastern Asia (Lee et al., 2013). It has successfully invaded various regions, including Europe and the United States (Costi et al., 2017; Leskey et al., 2018), where is among the most damaging pests for many agricultural crops (Costi et al., 2017); apples, peaches, nectarines, pears, grapes, sweet corn, soybeans and hazelnuts are some of the most susceptible crops (Leskey & Nielsen, 2018).

Control of pentatomids in invaded regions relies heavily on insecticides, most of which are disruptive to beneficial insects (Knight & Gurr, 2007; Reed et al., 2013b; Leskey et al., 2014). Therefore, the development of more environmentally friendly and species-specific control methods for the management of stink bugs in general is desirable (Knight & Gurr 2007). Some strategies include CBC and the use of pheromon or multistimolous traps (Knight & Gurr, 2007; Short et al., 2017; Conti et al., 2021; Zapponi et al., 2023).

In the native range of stink bugs, their main natural enemies are egg parasitoids (Hymenoptera: Scelionidae, Encyrtidae, and Eupelmidae); in the invasive range, also the native parasitoids of the adult stink bugs (Diptera: Tachinidae), egg parasitoids (Hymenoptera: Scelionidae, Encyrtidae, and Eupelmidae) and the native predators -also prey on the various instars- have in some cases adapted to the new hosts (Conti et al., 2021).

CBC using the most effective exotic egg parasitoids against stink bugs is considered the long-term solution, (Conti et al., 2021). However, the real impact of CBC is often overestimated (Abram et al., 2020), and could not represent a definitive solution if applied alone (Abram et al., 2020; Conti et al., 2021).

Among the environmentally friendly solutions, considering targeting Hemiptera with SIT is unusual, as all developmental stages and potential release stages can damage crops (Bourtzis & Vreysen, 2021). Nonetheless, efforts have been made to evaluate whether certain pest species within this group (*H. halys, N. viridula,* and *B. hilaris*) can be effectively controlled using this technique (Welsh et al., 2017; Horrocks et al., 2020; Cristofaro et al., 2022).

1.7 The challenge of *Halyomorpha halys*

Among invasive stink bugs, the brown marmorated stink bug (BMSB), *Halyomorpha halys* (Stål) (Hemiptera: Pentatomidae) stands out for its rapidly expansion around the world (Zhu et al. 2011) and its extremely wide host range, with an elevated damage potential (Leskey & Nielsen 2018).

Native to China, Japan, Korea, and Taiwan (Lee et al., 2013), BMSB is currently invasive in many regions including the USA (Hoebeke & Carter 2003), Europe (Costi et al., 2017), Chile (Faúndez & Rider, 2017) and the Caucasus (Musolin et al., 2018). BMSB emerged as an insect pest in the United States in the 1990s and in Europe in the 2000s. Since then, it has further expanded its invasive range (Haye et al., 2015), however, the full extent of its native realized niche in invaded ranges is not yet colonized (Zhu et al., 2017). BMSB potential for further expansion is substantial, particularly in the southern hemisphere (Haye et al., 2015a; Kritickos et al., 2017). Zhu et al. (2012) indicated that the most optimal conditions for BMSB establishment were present in Mediterranean nations across Europe and the Middle East, Northern Algeria, Northern Cameroon, the western coastline of the United States, eastern regions of North America, central Chile, Northeastern Argentina, as well as Uruguay and Southern Brazil. Other potential zones for encompass crucial fruit-growing locales like the South Island of New Zealand and the southern region of Africa. The bioclimatic modelling utilized distribution data within Asia and climate information sourced from CliMond (Kriticos et al. 2012).

Currently, BMSB has already invaded a large part of the regions at risk of establishment as shown by the EPPO Global Database, (2023) (Figure 1.2). In future scenarios, climate change may shift the distribution of BMSB northwards, particularly in North America and Europe, while contracting from its southern temperature range limits in the US due to increased heat stress. Consequently, the horticultural areas most at risk are in Europe, the north-eastern United States and south-eastern Canada, both with the current and future climate conditions (Kistner, 2017).

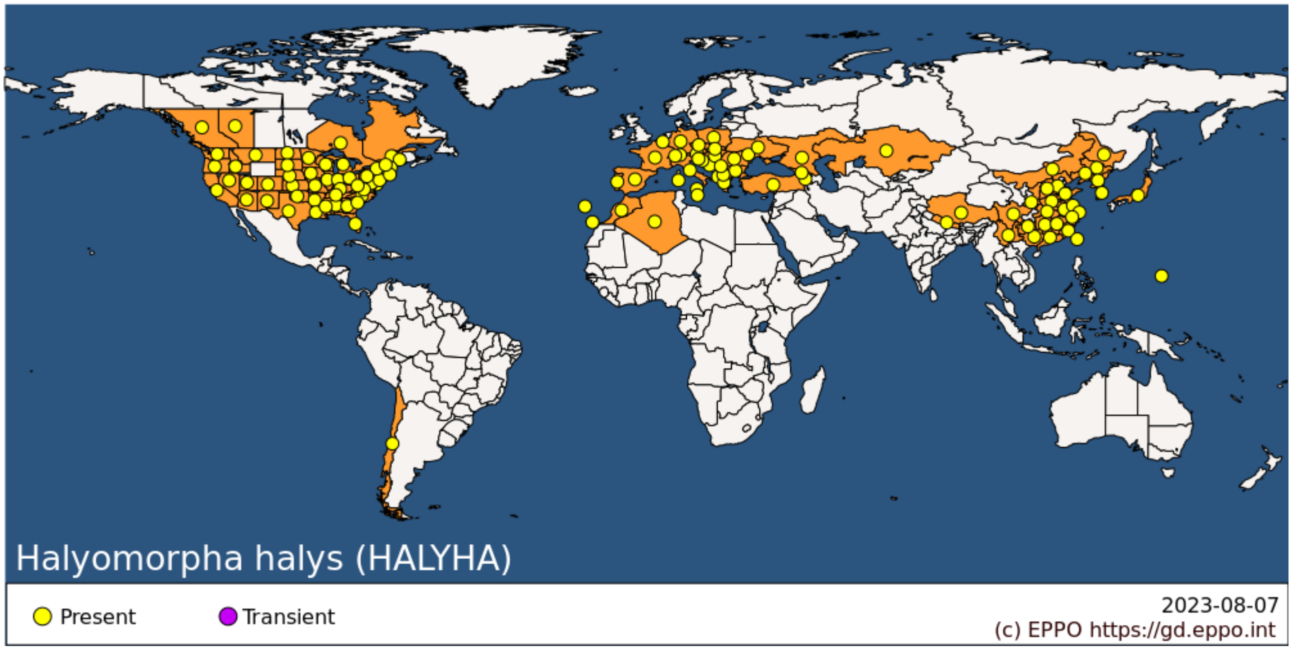

Figure 1.2. *Halyomorpha halys* world distribution (EPPO Global Database, 2023).

In the native areas, BMSB is considered an occasional pest of fruit orchards and soybean (Lee et al., 2013), but in invaded areas exhibits a wide plant host range, feeding on more than 170 plant species, among which many of significant agricultural value (Leskey & Nielsen, 2018); for this reason, is considered an important pest species in the new invaded areas, causing significant crop losses (Rice et al., 2014; Leskey & Nielsen, 2018). The feeding activity by punctures on fruits and plant tissue (Haye & Weber, 2017), usually leads to suberification, malformation, and necrosis (Zhang et al. 1993; Yu et al. 2002). It causes significant damage to fruit crops (Lee et al., 2013), whether in terms of early-season yield losses or late-season quality loss (Nielsen & Hamilton, 2009). For instance, in 2010, the apple cultivation region of the Mid-Atlantic area in the United States experienced high population densities of BMSB, which led to a loss of 37 US million dollars (Leskey & Nielsen, 2018). In Italy, a substantial increase in the population of BMSB occurred during the summer of 2015 with the species spreading extensively in the fruit-growing areas in the Emilia-Romagna region. This resulted in more than 50% of certain early-ripening pear cultivars being damaged during the harvest (Bariselli et al., 2016).

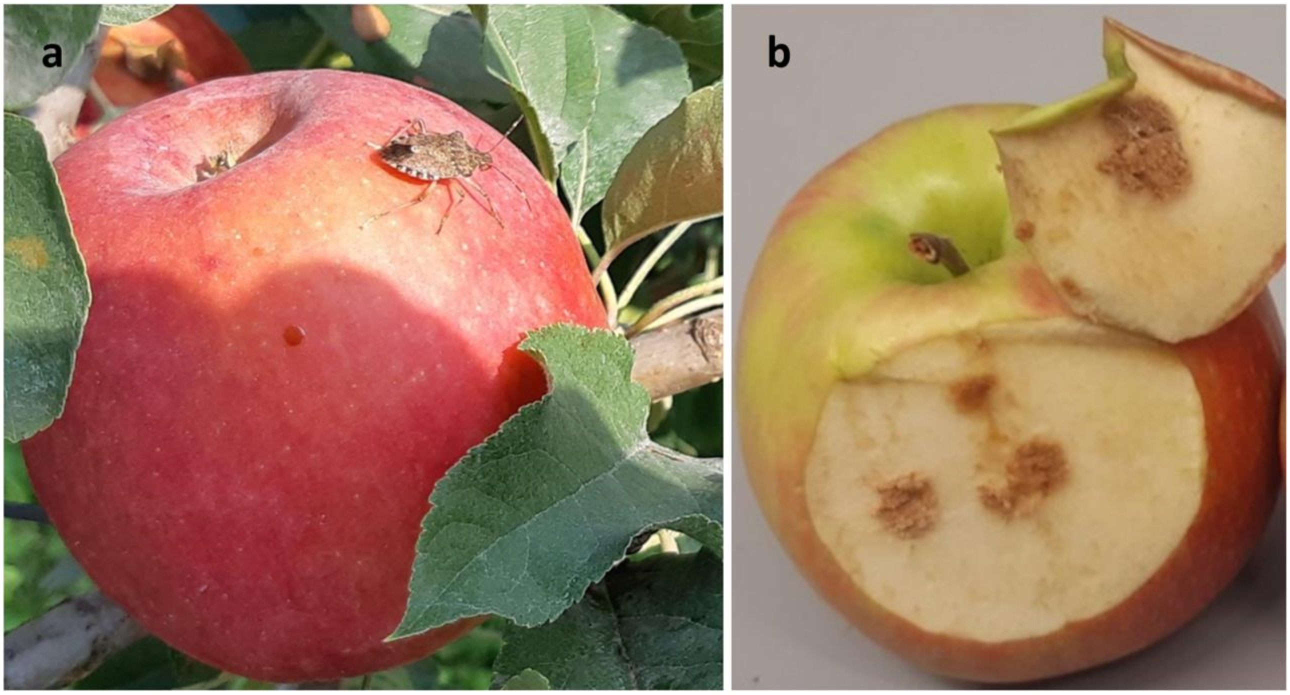

Figure 1.3. a) *Halyomorpha halys* on an apple in an orchard; b) Apple damaged by the feeding activity of *H. halys.*

Another problem caused by this insect is related to its aggregation behaviour in preparation for winter diapause, causing disturbance in urban areas invading buildings and emitting an unpleasant smell when disturbed (Inkley et al., 2012; Rice et al., 2014; Malek et al., 2018).

This species also showed an excellent flying ability (Lee & Leskey, 2015), especially in June after the overwintering period. When the temperature is between 25 and 30 °C, 87% of insects take flight, then fall to 3% when temperatures are between 10 and 15 °C, suggesting timing of action in its management (Lee & Leskey, 2015).

1.8 Management of *Halyomorpha halys*

The predominant approach to managing BMSB involves employing insecticides, specifically pyrethroids and neonicotinoids, along with other agronomic and biocontrol strategies (Kuhar et al., 2012; Leskey et al., 2014; Kuhar & Kamminga, 2017). Among pyretroids, the most effective insecticides are beta-cyfluthrin, bifenthrin, permethrin, fenpropathrin, lambda-cyhalothrin, zetacypermethrin; while the most effective neonicotinoids are dinotefuran, clothianidin, and thiamethoxam, and carbamates such as methomyl and oxamyl, organophosphates, and the organochlorine endosulfan (Kuhar & Kamminga, 2017). However, they are broad-spectrum insecticides, and a short-term solution. Their frequent use can induce insecticide resistance, sometimes lead to secondary pest outbreaks (Leskey et al., 2012a, Leskey et al., 2012b) and potentially be disruptive for natural enemies and pollinators (Kuhar & Kamminga, 2017). Therefore, they are not compatible with most IPM systems (Kuhar & Kamminga, 2017).

On the other hand, the release and/or the conservation of both indigenous and exotic natural biocontrol agents has evinced encouraging medium- and long-term outcomes in suppressing BMSB (Abram et al., 2017; Leskey & Nielsen, 2018; Conti et al., 2021).

The generalist egg parasitoid, *Anastatus bifasciatus* (Geoffroy) was selected as a candidate for augmentative releases against BMSB, resulting the predominant native egg parasitoid of BMSB (Haye et al., 2015b; Costi et al., 2019; Moraglio et al., 2020), and able to complete developing in viable BMSB eggs (Roversi et al., 2016; Abram et al., 2017) in most of the invaded countries.

In the native range of BMSB, two egg parasitoids stand out, in the micro-Hymenopteran family Scelionidae: *Trissolcus mitsukurii* and *Trissolcus japonicus* (Ashmead).

Trissolcus mitsukurii is mainly associated with *N. viridula* and has been released in Brazil and Australia as BCA (Kobayashi & Cosenza, 1987; Clarke, 1990). *T. mitsukurii* attack the eggs of several pentatomid species and has a significant parasitism rate on BMSB eggs (Yang et al., 2009). On the other hand, *T. japonicus* is the predominant natural enemy of BMSB in Asia with a parasitization rate from 50 to 90% (Zhang et al., 2017). Both *T. mitsukurii* and *T. japonicus*, have been found in Europe and appear to have comparable performance in terms of exploitation efficiency and impact on BMSB eggs (Rot et al., 2021; Zapponi et al., 2021). However, *T. japonicus* is considered the most promising CBC candidate as it is the main BMSB egg parasitoid in its native range (Zhang et al., 2017; Buffington et al., 2018; Leskey & Nielsen, 2018; Haye et al., 2020; Conti et al., 2021). Adventive populations of *T. japonicus* were first detected in the United States in 2014 (Talamas et al., 2015) and then in Europe in 2017 (Sabbatini et al., 2018; Stahl et al., 2019). In addition to the adventive populations of *T. japonicus*, a classical biological control programme involving many regions and institutions has recently started in Italy, which aims to accelerate the natural control of BMSB by *T. japonicus* and the reconstruction of a natural biological equilibrium. The release of this exotic egg parasitoid for classic biological control needed the obtainment of an approval by the competent authorities after the evaluation of a comprehensive risk assessment submitted by scientific institutions according to a new Italian regulation. However, according to some authors, it could not represent a definitive solution as a standalone strategy (Abram et al., 2020; Conti et al., 2021), especially in the context of monocultures and frequent insecticide applications (Lowenstein et al., 2019).

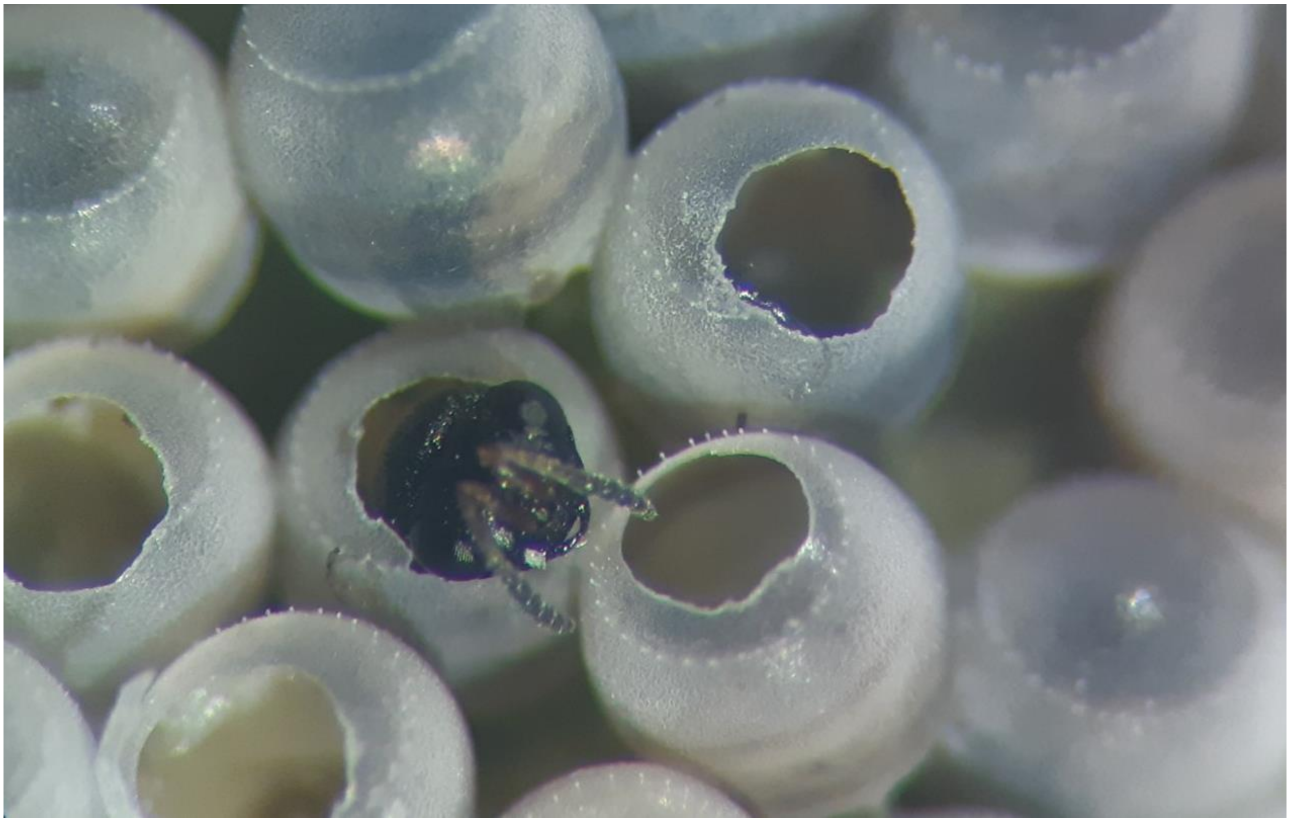

Figure 1.4. A *Trissolcus japonicus* female emerge from an egg of *H. halys*.

In response to these needs, the discovery of the aggregation pheromone of BMSB, as (3S,6S,7R,10S)-10,11-epoxy-1-bisabolen-3-ol and (3R,6S,7R,10S)-10,11-epoxy-1-bisabolen-3-ol (Khrimian et al., 2014), and his synergic effect with methyl (2E,4E,6Z)-decatrienoate (Weber et al., 2014) has led to new IPM approaches in which were tested many pheromone baited traps (Leskey et al., 2018) and their combined use with specific vibrational communication signals of BMSB (Zapponi et al., 2023). Attract and kill strategies have also shown good results (Suckling et al., 2019a; Leskey et al., 2020) suggesting that their introduction in IPM can make possible to reduce insecticide use in the orchard. Nevertheless, there are no real traps for the mass capture of BMSB (Anfora et al., 2019), and further environmentally friendly strategies need to be developed.

Some researchers have considered BMSB, as a potential candidate for SIT (Welsh et al., 2017; Suckling et al., 2019b; Nguyen et al., 2021), evaluating the possible crop damages tolerable if compared to the long-term benefits of its eradication or suppression. However, a critical point for the implementation of SIT against BMSB remains the mass rearing considered impossible for such a species. Indeed, according to Lance & McInnis (2021), the implementation of SIT on hemimetabolous insect is more complicated than on holometabolous. The high motility of nymphal stages and adults makes difficult to rear and irradiate the insects. Moreover, for BMSB, the frequent phenomenon of cannibalism on eggs and newly emerged adults, by nymphs and other adults (Medal et al., 2012) obliges to separate continuously egg masses, nymphs and adult to different cages or containers. Finally, the absence of an artificial diet further complicates the development of SIT on a species like BMSB. In order to overcome this limitation, Suckling et al. (2019b) proposed a strategy that involves the collection of wild insects during the aggregation phase linked to overwintering, irradiation, and their subsequent release.

1.9 Thesis Aim and Outline

This thesis aims to emphasize the potential benefits of adopting the sterile insect technique (SIT) to control BMSB, in an integrated pest management approach (IPM) considering its combination with the current most environmentally friendly solutions.

Specifically, considering the expansive and probably impossible mass-rearing of BMSB, we have devised an alternative strategy based on the mass trapping of wild insects during the aggregation for overwintering, cold storage, irradiation of males and their release in the spring.

As a first milestone, it was fundamental to provide an easy-to-obtain and cheap tool for mass trapping and field monitoring of BMSB by designing new effective traps. Subsequently, the feasibility of a SIT strategy involving overwintering wild-harvested males was evaluated. In addition, we tested the suitability of SIT in combination with classical biological control involving the exotic egg parasitoid *Trissolcus japonicus* in an integrated control strategy.

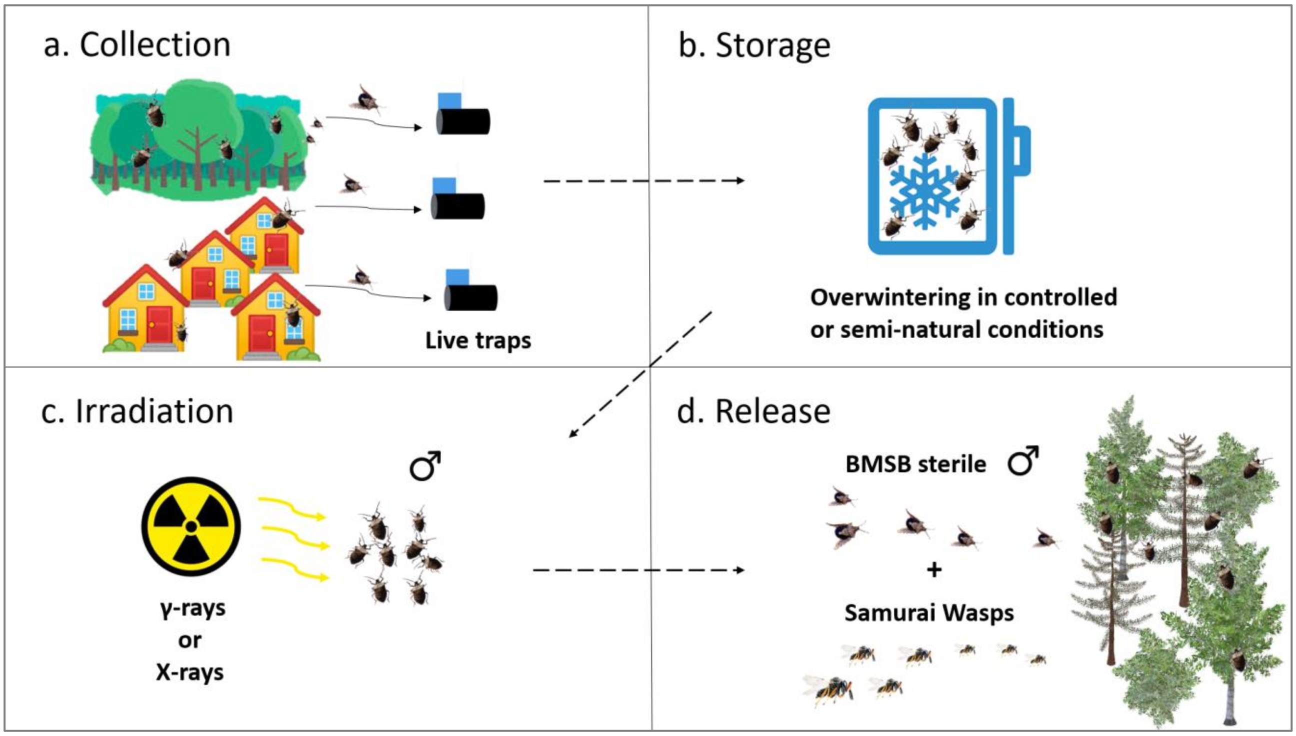

Figure 1.5. Potential control tactic, combining the use of live traps, SIT and CBC. a) Collection of BMSB from natural and urban areas using live traps; b) Storage of collected insects in climatic chambers (controlled conditions) or outside under semi-natural conditions; c) Irradiation of BMSB males with gamma- or X-rays; d) Release of BMSB sterile males and Samurai wasps (*Trissolcus japonicus*).

In **Chapter 2**, we tested the efficacy of novel pheromone live traps in the field collection of wild BMSB adults. The traps were designed to take advantage of the natural pheromone-ruled behaviour of aggregation of BMSB. In this chapter, we assessed the efficacy in collecting both males and females of BMSB compared to the sticky panels baited with an identical pheromone.

In **Chapter 3**, we evaluated a protocol based on wild harvesting, diapause in semi-natural conditions, and irradiation post-diapause of BMSB males. The response to male irradiation on fertility, fecundity, longevity, and mating behaviour was assessed through mating bioassays. Moreover, a hospital's medical linear accelerator (LINAC) was tested to support its use as an irradiator for the SIT.

Considering that LINACs are more widely available compared to gamma sources, they may reduce irradiation times and maintain good insect quality.

In **Chapter 4**, we evaluated sterile eggs as oviposition substrate for the exotic egg parasitoid *T. japonicus*, the main candidate for CBC of BMSB. We observed the effects on the success of parasitism of sterile clusters of BMSB eggs obtained by irradiation and mating between sterile males with fertile females and possible sub-lethal effects on the progeny of *T. japonicus*. The eggs were tested at various ages, up to 20 days. The aim was to understand if the BMSB eggs produced by SIT can implement biological control providing a good oviposition substrate or alternatively use them for monitoring of egg parasitoids in the field.

In **Chapter 5**, the results obtained and shown in all the previous chapters are discussed to obtain insights on the possible future of sustainable pest management of BMSB and further needed investigations to introduce SIT in an IPM strategy.

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CHAPTER 2.

LIVE TRAPS FOR ADULT BROWN MARMORATED STINK BUGS

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Abstract

Surveillance for detection of the brown marmorated stink bug, *Halyomorpha halys*, is reliant on sticky panels with aggregation pheromone, which are low cost, but very inefficient (est. 3%). Trapping for adults was conducted in Italy with novel live (or lethal) traps consisting of aggregation pheromone-baited cylinders with a wind vane, with the upwind end covered by mesh and the downwind end sealed by a removable entry-only mesh cone, admitting the attracted bugs. The novel traps caught up to 15-times more adult BMSB than identically-baited sticky panels in two weeks of daily checking (n = 6 replicates) (the new live traps were, in Run 1, 5-, 9-, 15-, 13-, 4-, 12-, 2-fold; and in Run 2, 7-, 1-, 3-, 7-, 6-, 6-, and 5-fold better than sticky traps, daily). The maximum catch of the new traps was 96 live adults in one trap in 24 h and the average improvement was ~7-fold compared with sticky panels. The rotating live traps, which exploit a mesh funnel facing the plume downwind that proved useful for collecting adults, could also be used to kill bugs. We expect that commercially-available traps could replace the crude prototypes we constructed quickly from local materials, at low cost, as long as the principles of a suitable plume structure were observed, as we discuss. The traps could be useful for the sterile insect technique, supporting rearing colonies, or to kill bugs.

Keywords: aggregation pheromone; *Halyomorpha halys*; trap; lure and kill; sterile insect technique; wild harvest

2. 1 Introduction

Halyomorpha halys (Stål, 1855), the brown marmorated stink bug (BMSB), is a highly invasive species from Southeast Asia, which feeds on field crops, vegetables, tree fruits, and nuts and ornamentals. Both nymphs and adults feed on developing and ripe fruits and seeds and can cause severe damage to crops (Bariselli et al., 2016; Leskey & Nielsen, 2018). In climate conditions such as Northern Italy or much of USA (Nielsen et al., 2016), BMSB is predicated to have two or more generations per year (Kriticos et al., 2017). Pre-reproductive adults over-winter inside houses and release an unpleasant odour when disturbed (Bergh & Quinn, 2018). The BMSB is increasingly widespread in Europe (Bariselli et al., 2016; Maistrello et al., 2016). In Northern Italy alone, the local associations of fruit production have estimated about €150 million/year losses in 2016–2018. Interceptions of this species have been reported in other countries (Cianferoni et al., 2018) including New Zealand (Vandervoet et al., 2019). No successful eradication attempts of established populations have been reported thus far.

A two-part aggregation pheromone ((3S,6S,7R,10S)-10,11-epoxy-1-bisabolen-3-ol and (3R,6S,7R,10S)-10,11-epoxy-1-bisabolen-3-ol) (Khrimian et al., 2014) has been synergized with the addition of methyl (E,E,Z)-2,4,6-decatrienoate (MDT) (Weber et al., 2014). Various trapping systems have been investigated for integrated pest management (IPM) (Leskey et al., 2015) and, based on these lure surveillance systems, have been tested for border protection, because of high interception rates in New Zealand (Vandervoet et al., 2019). For jurisdictions such as New Zealand that conduct surveillance, new and effective methods for both surveillance and suppression are important. Sticky panels are low cost, but could suffer from poor bug retention and loss of trap efficiency owing to the accumulation of dust and detritus. Other trap types have been investigated (Rice et al., 2018), although clear sticky panels were recently reported as suitable for BMSB monitoring and detection, by a New

Zealand government-supported study in the USA (Acebes-Doria et al., 2018). Most of the traps reported so far appear generally similar in functional concept as variations on a theme, and produce fairly similar results (Acebes-Doria et al., 2019). Simply hanging pyramid traps in trees did not result in an improvement, although placing the lures in such a way to increase airflow (outside the trap) did improve results about two-fold (Rice et al., 2018), which is fairly unsurprising. None of the treatments were particularly successful improvements.

Live traps for harvesting wild insects can support research in various ways, by offering efficient field collection (Ashby, 1976). A cylindrical trap design originally designed for live trapping tortricid moths in a mark-release recapture study (Suckling et al., 1990) was physically enlarged to take account of observations on BMSB behavior in Italy, and a prototype was built and tested. This proved successful (with a catch of 50 adult BMSB in the first 24 h), so a replicated trial was established to compare these traps with the proposed surveillance system for New Zealand, and this communication was produced to widen the debate about surveillance efficiency as well as suppression of this unwanted organism.

2.2 Materials and methods

2.2.1 Comparing the Live Traps and Sticky Panels

The aim of the research was to compare the catch efficiency of the highly mobile adult BMSB population from baited sticky panels and novel BMSB live traps, in the limited time available. Trapping studies for adult (and potentially nymph stages) of BMSB were conducted in late August 2019 at Fondazione Edmund Mach (46°11’43” N, 11°8’5” E), San Michele all’Adige, Trentino, Italy. We evaluated captures with two different kinds of traps, Pherocon sticky panels (Trécé, Adair, OK, USA), with high dose lures (Trécé, Adair, OK, USA), which contained 200 mg (i.e., 4-fold loading) of two component aggregation pheromones, (3S,6S,7R,10S)-10,11-epoxy-1-bisabolen-3-ol and (3R,6S,7R,10S)-10,11-epoxy-1-bisabolen-3-ol (Khrimian et al., 2014) plus methyl (E,E,Z)-2,4,6-decatrienoate (Weber et al., 2014). The lures were positioned on top of the sticky panels, on overhanging branches. This combination of lure and sticky panel trap has been under investigation for surveillance in New Zealand because of a high rate of interceptions (Vandervoet et al., 2019). The second trap type (Suckling et al., 1990) rotated using a wind vane, while trap cylinders were constructed from soft plastic plant pots (30 cm diameter x 40 cm high) with the bottoms cut off and the tops joined and sealed. The upwind end was covered with a flat panel of stainless gauze (1 mm mesh size) and sealed using hot glue, with the lure hung ~5 cm inside the opening of the center of the trap. The downwind end was sealed with a removable stainless mesh cone. The wind vane was added to the top of the downwind end as a vertical panel constructed from corflute (corrugated plastic), and the trap was suspended and able to pivot balanced on a string suspended from a tree branch (Figure 2.1).

Figure 2.1. Rotating live trap for *Halyomorpha halys* consisting of two large black flower pots joined in the middle, with bases removed and a removable mesh cone for bug entry and removal, an upwind mesh panel for airflow, and an aggregation pheromone lure, with a wind vane to generate the best plume from the trap.

We set the traps (n = 6 replicates, alternating trap types at 10 m spacings) at a sloping forest margin with adjacent vineyards of mixed grape varieties downhill. The experiment was set up on 22 August 2019 and checked daily for seven days (one run), before moving to a similar nearby location, 200 m along and 50 m higher, for a second identical run (traps were re-randomized). Catches in traps were sexed, counted, and removed daily. The large number of catches of live BMSB in the prototype live traps (up to 96 adult bugs per 24 h) required the trap contents to be emptied into a large plastic bag (80 cm x 1 m), and bugs were then individually sexed and removed around 9 am daily onto a second bag for transport to the laboratory culture. The smooth internal surface of the traps expedited emptying them. A short YouTube video accompanies this article to illustrate the new trap (Supplementary Materials).

2.2.2 Statistical Methods

On the basis of experience with two key factors affecting insect trapping, we balanced our design with replicates and days about evenly. Summed catches for each trap were log-transformed to generate an approximately normal distribution, in order to stabilize the variance ($p = 0.198$ for normality after transformation) (Akers, 2018). Catches were then compared for significant differences by type and replicate using a general linear model in Minitab (v18) (Akers, 2018).

2.3 Results

Both types of traps were successful at catching BMSB adults (Figure 2.2, Table S1), and the catches initially increased daily after installation of the live traps and, to a much lesser extent, in sticky traps, but varied day to day without obvious weather influence (see weather data, Supplementary Materials). Few nymphs were caught with either trap, so the results presented have been confined to adults for brevity. The analysis of variance (ANOVA) was highly significant for trap type, with a lower level of significance for replicate. Numbers caught reduced on days five to seven during Run 1. Numbers were lower at the location for the second run, located at about 50 m higher elevation and 200 m away, with a total of 1061 caught in Run 1 and 555 in Run 2.

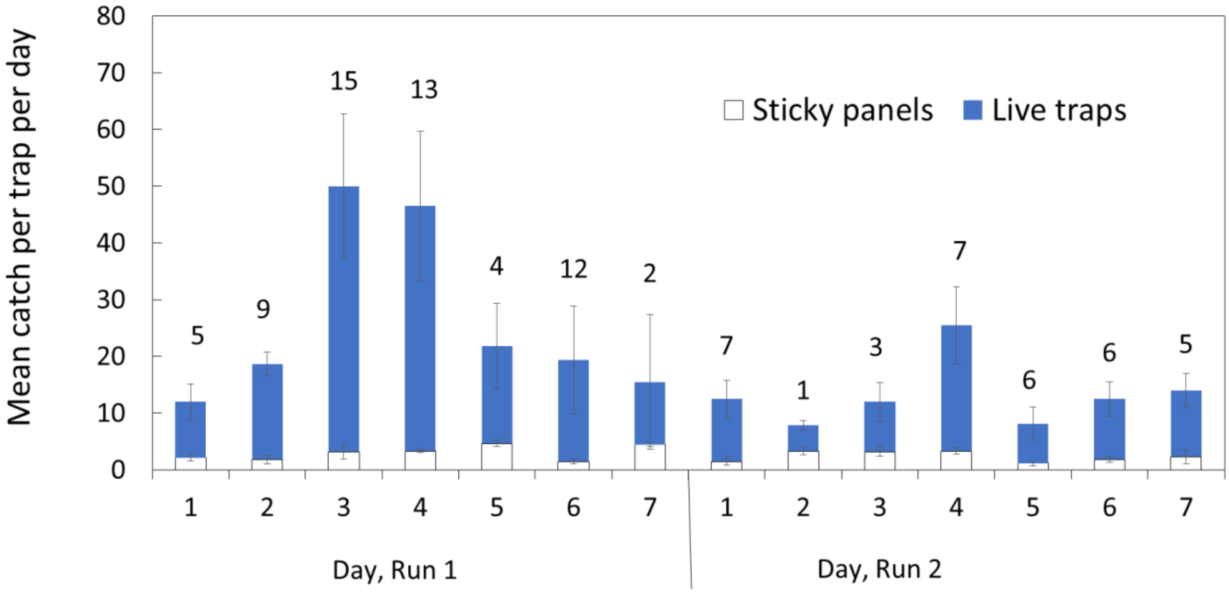

Figure 2.2. Mean daily catch per trap of adult *Halyomorpha halys* in alternating sticky panels and live traps, on a vineyard–forest margin at Fondazione Edmund Mach, San Michele all'Adige (TN), Italy. Error bars show one standard error (n = 6 replicates). Runs 1 and 2 were at different locations, 200 m apart. Labels indicate the mean improvement in catch from the live traps over sticky panels.

The difference in trap efficiency among traps varied daily, but the live trap was better than sticky panel traps on 13/14 of the days. Low catches or zero catches occurred with both trap types (Figure 2.3), and were frequent with the sticky traps, while the live traps caught up to 96 adults per day, with nine trap counts over 30 BMSB per day. The sex ratio was similar between trap types, and significantly biased (1.6 and 2.2 females per male for the live traps ($X^2 = 73.5$, $p < 0.0001$) and sticky panels ($X^2 = 55.6$, $p < 0.0001$), respectively).

Catch varied by transect position, with the lowest catches in internal traps (Figure 2.4), suggesting trap competition. Trap position appeared to have little effect on the sticky panels.

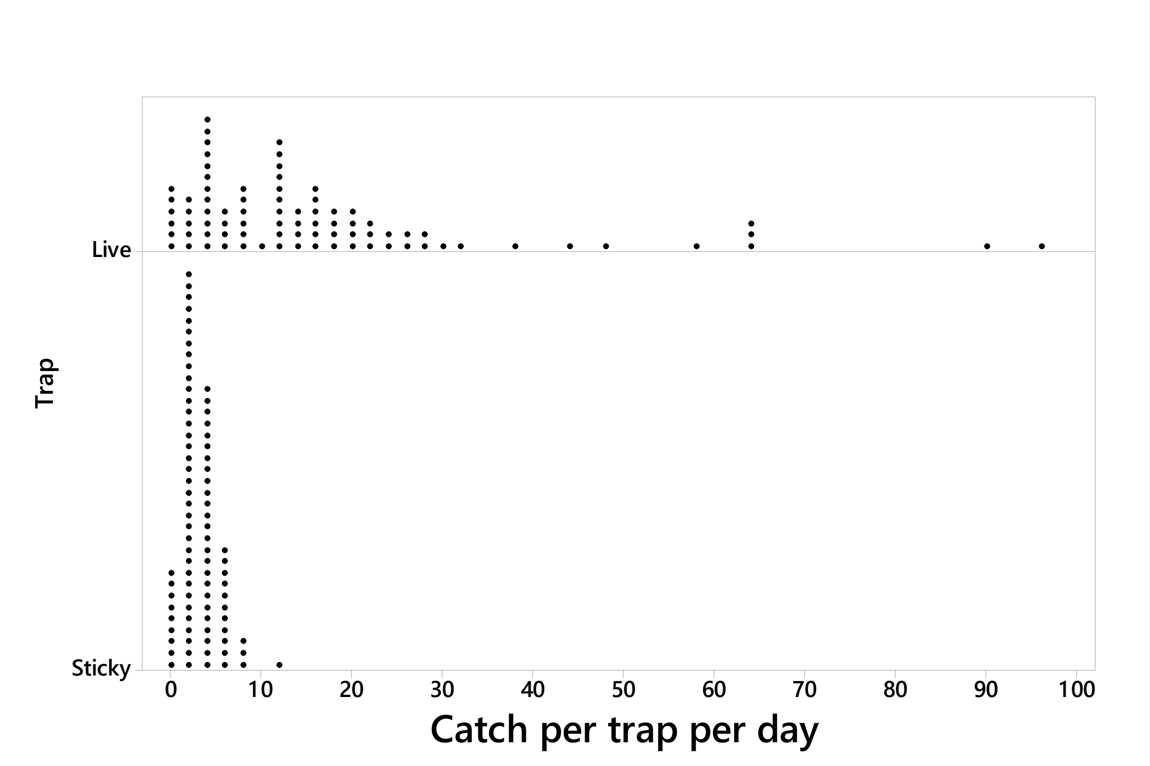

Figure 2.3. Dotplot of catch per trap per day with spatially-alternating live traps (live, upper) and sticky panels (lower) at the forest–vineyard margin in San Michele all'Adige (TN), Italy (n = 6 reps, operated daily for two runs of seven days, at 200 m spacings between runs).

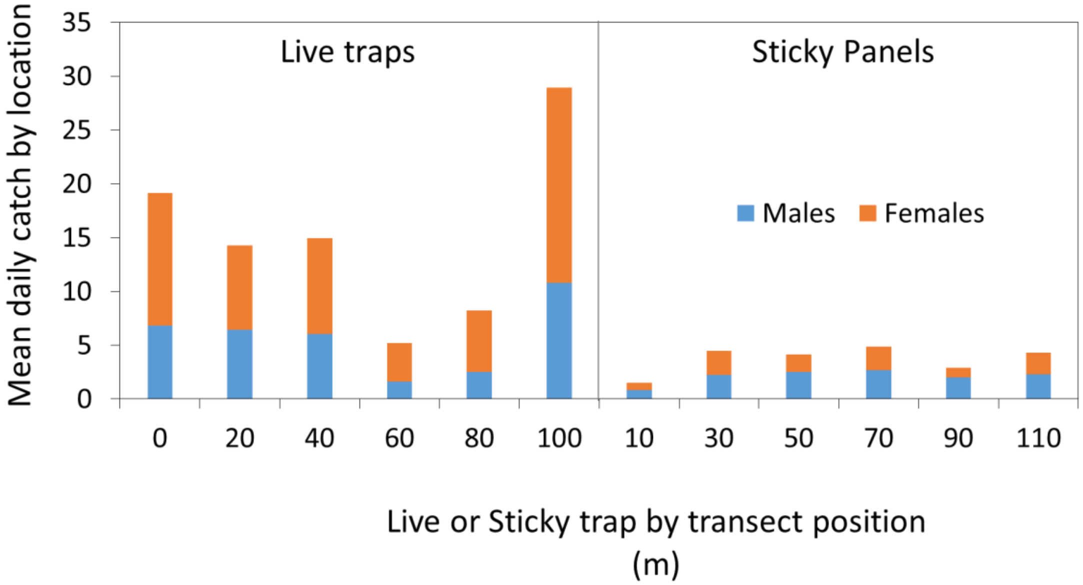

Figure 2.4. Effect of transect positions on mean daily catch of *Halyomorpha halys* by sex and trap type at the forest–vineyard margin in San Michele all'Adige (TN), Italy (n = 6 replicates, operated for 14 days (runs combined)).

2.4 Discussion

Effective pest monitoring is an essential tool for IPM, and pheromones have provided a tremendous boost to this field by attracting insects directly to traps. Efficient traps can support management decisions, to restrict the use of insecticides and reduce costs, non-target effects, and secondary pest outbreaks. The need for understanding the meaning a stink bug in a trap in IPM generally aligns with the biosecurity detection needs in countries like New Zealand, where high sensitivity at first detection will be essential for delimitation in a response (Vandervoet et al., 2019). The highly successful lures for BMSB have opened new opportunities for IPM as well as surveillance, but traps require more than simply attraction to be effective. Trap efficiency should remain relatively constant over time and, unfortunately, traps based on sticky panels are likely to eventually suffer from loss of efficacy through the accumulation of dust, leaves and detritus. Clear sticky panels are reported as suitable for BMSB monitoring and detection (Acebes-Doria et al., 2018), highlighting a lack of better alternatives.

At the forest–vineyard margin, the numbers of bugs sampled (alive and potentially killed or added to the laboratory culture) were substantially higher for the live traps than for the sticky traps. Neither trap caught many nymphs, which was in part because of the tendency of walking nymphs to avoid the sticky glue, although nymphs were observed to walk down the string to the live traps, and numbers on the outside of the traps apparently increased over time.

Catches of BMSB adults (and nymphs) typically build up over the first three days with the aggregation pheromone, independent of the trap type (Suckling et al., unpublished results). Catches in other trials (Suckling et al., unpublished data) have remained relatively steady thereafter, but here, a mid-week peak was twice followed by a >50% decline for the last three days, suggesting that an influence of adult removal could be having an effect on the local population, but this would need to be verified with absolute sampling techniques.

Our very short trial was resource-limited, but the results are sufficient to encourage further examination of this concept of live trapping, which avoids the need for a beating tray and other physical collection methods. This method of live collection has already added hundreds of insects to the colony at Fondazione Edmund Mach, and plans have been developed to test the approach further using commercially-available materials. There was a sex bias towards female BMSB in both traps, which is a useful finding because it is unlikely that both traps are biased and fail to represent the actual sex ratio. The live traps showed evidence of trap competition, as higher catches were generally made towards the ends of the transects, which is equivalent to the corner trap in an array, which faces less competition than central traps (Suckling et al., 2015). The sticky panels did not show this effect, suggesting that the lower catch efficiency could be masking the effect. While U.S. researchers have preferred to use 50 m trap spacings, their objectives were different, such as seeking calibration in absolute values of trap efficiency. Here, we sought to compare two trap types and to demonstrate the potential of live capture, rather than pursue the theoretical active space (Kirkpatrick et al., 2019). Leskey et al. (2012) and Rice et al. (2018) categorically state that “A killing agent is necessary for successful trapping of BMSB using pyramid traps with collection jars”, but perhaps the current work creates new options through design.

Live catches in this type of trap could be used for collection of insects aggregating pre-winter, as well as for irradiation and release of males for the sterile insect technique (SIT) (Welsh et al., 2017), and it may even be possible to generate overwintering behavior if suitable substrates are provided within the traps in late autumn. Males in particular are needed for SIT, so physical sorting by sex would still be necessary.

Such traps could also readily be converted into killing stations through the addition of long-life insecticide netting into the body of the trap, especially at the upwind panel where we observed the highest numbers of adults caught inside the traps (Figure 2.1). This type of technology could prove useful in an eradication of a delimited population. Sticky panels are currently preferred for surveillance in New Zealand (Vandervoet et al., 2019), but proactively established killing stations could complement this in high-risk sites. While our study of the new traps did not directly investigate the full potential for killing BMSB adults, we can conclude that this trap concept could be compatible with the needs of an eradication to reduce the numbers of a known and delimited population, while avoiding broadcast insecticide use. This type of mass trapping approach has the benefits of limiting broadcast insecticide usage and compatibility with biological control, but has material costs and ongoing labor costs (El-Sayed et al., 2006), although these costs could be sustained for the duration of an eradication (Brockerhoff et al., 2010). Identification of a suitable commercially-available low-cost fish trap or similar trap is proposed, now that the principle of this trap type has been demonstrated. Solid cylindrical walls, a mesh funnel opening downwind with a small non-return opening, a wind vane, and pivotal suspension are expected to be critical design features.

Reported observations of BMSB behavior around traps have focused on a range of attributes including walking (Leskey et al., 2015), but have apparently failed to consider the possibility that the plume can (hypothetically) be delivered from inside a cylinder, and apparently reach the population more successfully by extending the plume further than with lures that are fully exposed in the open air, for example, around a sticky trap. The mechanistic role of plume structure warrants further investigation, as the principle is general and may well enable improvement in catches to other lures and insects. Once in vogue for moth pheromones (Lewis & Macaulay 1976; Angerilli & McLean, 1984; Baker & Haynes, 1989), the literature on plume structure and insect behavior in and around pheromone-baited traps has reduced in recent decades, but perhaps the field needs review for other orders, as previous extensive research on BMSB trapping has apparently failed to discover how to greatly improve catches, as reported here.

The new trap concept may have considerable relevance to IPM, where its potential to help growers can now be evaluated within invaded jurisdictions. Recent approaches such as aggregating insects to sacrificial killing trees (Morrison et al., 2019) could potentially be replaced with more effective "lure and kill" traps to reduce environmental exposure from insecticides, if the initial promise of our new traps can be confirmed. An average improvement of ~7-fold and up to 15-fold over the recommended sticky panels appears to warrant further investigation for detection and surveillance, as well as for IPM, but needs to be placed in the context of overall insect population suppression.

2.5 Conclusions

The new traps were surprisingly effective at live trapping BMSB given extensive research on trapping this insect since the aggregation pheromone was identified. The new trap concept could represent a useful tool with a range of applications, although as yet there is limited understanding of why the trap design is working so well. More work on aspects of the trapping system (size, design, plume structure) would be useful to extract the essentials for development of even more effective traps in future. The immediately obvious applications of live capture include wild harvest for supporting rearing colonies, as well as supporting our objective of the sterile insect technique, because mass rearing is currently a limitation to that approach. Equally, the traps could provide the basis for a more sensitive surveillance system in biosecurity, given the much-improved catch rates.

Supplementary Materials: The following are available online at http://www.mdpi.com/2075-4450/10/11/376/s1

Table S1: Analysis of variance of log-transformed catch of *Halyomorpha halys* by trap type and replicate at the forest–vineyard margin in San Michele all'Adige (TN), Italy, from 22/8/19 to 5/9/2019, **Video S1**: Live Traps for Adult Brown Marmorated Stink Bugs.

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CHAPTER 3.

EFFECTS OF IRRADIATION ON BIOLOGY AND MATING BEHAVIOUR OF WILD MALES OF BROWN MARMORATED STINK BUG USING A 6 MV MEDICAL LINEAR ACCELERATOR

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Simple Summary

Controlling the brown marmorated stink bug without chemical insecticides is challenging. The sterile insect technique, based on use of irradiated sterile males to reduce fertility of wild females, is a valid method in area-wide pest management. This work complements previous investigations that were carried out by treating newly emerged males at lower irradiation doses. In this study, high irradiation doses (32 and 40 Gy) were applied, using a linear accelerator, to a colony of wild overwintering adults collected in large numbers in the field during the aggregation phase before the winter diapause. A sterility level of 95% was reached with a minimum 32 Gy X-ray irradiation dose and without significant impacts on other physiological parameters, such as fecundity and longevity.

Abstract

The brown marmorated stink bug, *Halyomorpha halys*, is a pentatomid bug of Eastern Asian origin that became an economically relevant pest in the Eurasian and American continents. Management of this species is limited to use of chemical insecticides: an inefficient method due to the strong adaptability of the target pest. The sterile insect technique (SIT) is potentially a valid tactic in the search for nontoxic alternatives. In this work, we investigated the suitability of masstrapped overwintering males, collected during the aggregation phase before the winter diapause, for their release as competitive sterile males in an SIT programme. Differently from previous studies, irradiation was applied with a linear accelerator device that produced high-energy photons. Following a similar scientific protocol with newly emerged irradiated males, the effects of X-ray irradiation on physiological parameters (longevity, fecundity and fertility) were assessed. In addition, behavioural bioassays were carried out in no-choice conditions to evaluate if irradiation interferes with mating processes. The results are very encouraging; the effects of the irradiation at 32 Gy did not differ from the controls in the longevity or fecundity of the exposed overwintering adults. The hatching rate of the eggs laid by the fertile females that had mated with the irradiated males was less than 5%. The results of behavioural bioassays showed that the irradiation did not cause a significant impact on the quality of the sterile males. More research is warranted to evaluate the mating competitiveness of sterile males in semi-field and field conditions.

Keywords: X-ray; *Halyomorpha halys*; integrated pest management; insect pest; pentatomids; diapause

3.1. Introduction

The brown marmorated stink bug (BMSB), *Halyomorpha halys* (Stål) (Hemiptera: Pentatomidae), is an invasive phytophagous species native to Eastern Asia (Lee et al., 2013). Due to its wide host-plant range, it is considered among the most harmful agricultural pests in Europe and the United States (Costi et al., 2017; Leskey & Nielsen, 2018). In addition, in the southern hemisphere, regions with moist and tropical, subtropical, Mediterranean and warm-temperate climates are at risk of establishment of BMSBs (Kriticos et al., 2017). Apples, peaches, nectarines, pears, grapes, sweet corn, soybeans and hazelnuts are among the most susceptible cultivated hosts (Leskey & Nielsen, 2018). In addition, this insect can cause nuisance in urban areas by invading buildings as shelters for overwintering and by emitting an unpleasant smell when disturbed (Inkley, 2012; Rice et al., 2014; Malek et al., 2018). Management of BMSB relies mainly on insecticide use, in particular, pyrethroids and neonicotinoids, in combination with other agronomic and biocontrol strategies (Kuhar et al., 2012; Leskey et al., 2014; Kuhar & Kamminga, 2017). There is a desire to limit use of broad-spectrum insecticides in favour of alternative, less-hazardous techniques in integrated pest management (IPM). Use of both native and exotic natural biocontrol agents is showing promising mid- and long-term results against BMSBs (Abram et al., 2017; Conti et al., 2021). Another potential control tactic is the Sterile Insect Technique (SIT). By definition, SIT is an environmentally-friendly pest control strategy based on mass rearing, sterilization, and inundative releases of predominantly male sterile insects. It is species-specific control method, and no documented off-target effects have been reported after decades of application (Enkerlin et al., 2017; Klassen & Curtis, 2021). To achieve sterility, mass-reared insects are typically irradiated with gamma rays or X-rays, making them unable to produce fertile offspring. Populations reduce when enough sterile males are mated with wild females (Lance & McInnis, 2021). The Sterile Insect Technique has been successfully used to control insect pests in many ecosystems. For instance, SIT has protected the horticultural industries from *Ceratitis capitata* (Wiedemann) (Diptera: Tephritidae) in many countries such as Guatemala, Belize, Mexico, and the USA (Enkerlin et al., 2017); SIT applied against the tsetse fly, *Glossina austeni* (Wiedemann) (Diptera: Glossinidae), made its eradication possible from Unguja Island, Zanzibar where it represented a severe problem for livestock (Vreysen et al., 2000). Finally, SIT is used in various programs to control different species of mosquitoes, which are vectors of pathogens causing serious human diseases such as malaria and dengue (Lees et al., 2021) To guarantee a successful outcome of SIT, many assumptions must be met, such as the absence of parthenogenesis, the possibility of mass rearing of the insects, a good knowledge of the insect mating behaviour and reproductive biology, size, and dispersal of natural populations, little to no adult crop damage, and no risks for human health and animals (for instance by transmitting pathogens). In the case of invasive hemipterans, the main obstacle to SIT application as a control method is the unwanted damage to host crops that could be caused by the release of adult pests, despite them being sterile (Klassen & Curtis 2021). Nonetheless, the application of SIT is particularly capable of giving successful pest suppression and even eradication, regardless of the challenges, when applied in specific geographic and infrastructural conditions with a closed population such as islands, greenhouses and siloes with particular respect to several conditions (Lance & McInnis. 2021).

To prepare for a possible application of the SIT against BMSBs, previous studies investigated the effects of irradiation on different phenological stages (Welsh et al., 2017; D. Suckling et al., 2019; Nguyen et al., 2021). To date, however, the SIT has not yet been included in any control strategy against BMSB despite the great potential it offers for some situations. More studies are needed to develop a comprehensive and affordable technique before deployment. A critical problem facing SIT implementation on brown marmorated stink bugs is the impracticability of sustainable mass rearing.

It has been proposed that this challenge could be mitigated by rearing, sterilising and releasing populations of previously wild-harvested aggregated overwintering males (Suckling et al., 2019a).

Overwintering is an important phase for both male and female adults of BMSB; the process is extremely selective since it is characterised by extremely high mortality. For example, Costi et al. (2017) observed that in Emilia Romagna (Italy), in outdoor conditions, only 28% of BMSB adults survived overwintering, and only half of them (14%) were capable of reproducing. Moreover, it has been demonstrated that BMSB females overwinter unmated and sexually immature (Nielsen et al., 2017), and to reach sexual maturity, they have to go through a period of cold storage followed by a postdiapause period under specific temperature (Taylor et al., 2017) and photoperiod (Nielsen et al., 2017) conditions. Our preliminary observations on about 11,000 overwintering adults, collected during autumn of 2018, showed that BMSBs need seven weeks of winter diapause in outdoor conditions, followed by a three-week postdiapause period in controlled conditions before oviposition. While the postdiapause period is essential for females to reach sexual maturity, nothing is known yet about males (Nielsen et al., 2017; Taylor et al., 2017; Koutsogeorgiou et al., 2022). However, we hypothesise that a postdiapause period may also be necessary for males to reach sexual maturity, as irradiation during diapause could have stronger effects on sterility than irradiation after this period, which we know lasts three weeks in females.

Here, we report the first study testing the use of wild-harvested overwintering males of BMSB as an alternative option to mass rearing for the SIT. We evaluated the effects of irradiation on the fertility, longevity and mating behaviour of overwintering males of BMSB.

3.2 Materials and Methods

3.2.1. Insects and Rearing

During autumn of 2019 and 2020 (from the end of September until the end of November), 2500 and 3600 BMSB adults were, respectively, harvested in the field at Fondazione Edmund Mach (46°11’43” N, 11°8’5” E), San Michele all’Adige (Trento), Italy, using live traps (Suckling wt al., 2019 b). The captured adults were reared in cloth cages (30 x 30 x 30 cm; BugDorm®, Taichung, Taiwan). Insects were provided with a diet consisting of tomatoes, green beans, carrots and apples ad libitum and wet cotton for a water supply. They were kept in a greenhouse with a natural photoperiod, where T = 18–15 °C and RH = 70%, as a form of preparation for overwintering until the end of November, when they were then transferred outside under a wooden shed. Cloth cages were set up with cardboard rolls as shelter, and a data logger (EL-USB-2, Lascar Electronics, Whiteparish, UK) was inserted into the wooden shed.

Insects were maintained in overwintering conditions for about two months, in accordance with the recommendations by Taylor et al. (2017) that suggest a minimum of seven weeks before breaking diapause; the insects were, then, moved to a climatic chamber with standard conditions (L:D = 16:8, T = 25 °C, RH = 70%). However, to reduce possible negative effects on longevity due to thermal shock, BMSB adults were kept for three days at 18 °C before being moved to 25 °C. Afterwards, the cardboard rolls were removed, and the males and females were placed in separate cages to prevent them from mating before these experiments began. Then, we provided insects with the same diet mentioned above.

Considering the high mortality of individuals during overwintering, we chose to irradiate the insects at the end of the diapause.

3.2.2. Irradiation

Irradiation took place at the Radiation Oncology Department of Santa Chiara Hospital (Trento, Italy), using an alternative technique that involves the use of the Elekta Precise Sli linear accelerator (LINAC), from Elekta AB (Stockholm, Sweden), with high-energy photons (energy used: 6 MV) instead of the conventional method, based on Co-60, used in previous studies on BMSBs (Welsh et al., 2017; Suckling et al., 2019a; Nguyen et al., 2021) and other hemipteran pests (Horrocks et al., 2020; Cristofaro et al., 2022). The decision to use a hospital LINAC (standard for treatment of oncological diseases) was dictated by the need to have an irradiator near the insect-collection site to reduce the time required to irradiate and maintain insect quality by limiting the stress associated with transport to an irradiator. For accurate dose delivery, photons must pass through a material with a homogeneous density. To obtain this condition, we used water-equivalent materials (see irradiation details below). At the same time, we kept the insects in Petri dishes (diameter = 90 mm) containing wet, absorbent paper that reduced the presence of air inside; to achieve this, a phase of cold-induced torpor was deemed necessary. To chill the insects, they were placed in a plastic container (Ø = 133 mm; V= of 1180 mL) equipped with a cap with fine organza mesh and kept in a freezer at - 20 ◦C for two minutes; then, the insects were transferred in a series of Petri dishes secured with parafilm. Each Petri dish contained an average of 15 individuals. The number of males irradiated for each treatment was in a range of 30–35 in January and February of 2020 and 60–62 in February of 2021.

The Petri dishes containing the insects arranged for the irradiation were transported to the laboratories of the Santa Chiara Hospital in a polystyrene box. The temperature inside the box was regulated with an ice tablet and registered with a data logger; the values ranged between 5 °C and 10 °C. Once the insects were brought to the linear accelerator, four Petri dishes at a time were inserted in a plexiglass panel, measuring 30 x 30 x 2 cm, positioned between two layers, each consisting of five

30 x 30 x 1 cm sheets of a water-equivalent plastic material, PWW RW3 Slab Phantom® (Freiburg, Germany), which simulates the density of water. The 4 mm space between the Petri dishes and the plexiglass was filled with four 1 mm-thick plexiglass disks. The entire structure was then placed under the LINAC. The irradiation was performed with two fields in the isocentric technique, one anterior and one posterior, with dimensions of 30 x 30 cm, so as to uniformly irradiate the entire system; the dose rate used was 250 cGy/min. Meanwhile, for a duration equal to the mean irradiation time (about five minutes), the Petri dishes containing the control insects with no irradiation (0 Gy), were kept outside the polystyrene box, in a room next to the irradiator, with the same room-temperature parameters. After irradiation, the insects were placed in cloth cages (24.5 x 24.5 x 24.5 cm; BugDorm®, Taichung, Taiwan), transported to the climatic chamber and fed with the same diet mentioned above until the formation of crosses for these experiments. The entire transport and irradiation procedure was performed within one hour.

3.2.3 Male Dose Response, Year 1

The first dose-response experiment was conducted in 2020, irradiating BMSB overwintering males at 16 and 24 Gy during two different physiological conditions: immediately after induced interruption of diapause (Group 1) and three weeks later (Group 2).

In particular, the data collected in terms of sterility were compared between Group 1 and Group 2 to understand if one of the two conditions were more suitable to induce stable sterility with irradiation.

The males of Group 1 were irradiated during their dormancy (23 January 2020) and then fed for about three weeks in standard postdiapause conditions (L:D = 16:8, T = 25 °C, RH = 70%) until the females were ready for mating.

Before irradiation, the males of Group 2 were transferred into postdiapause conditions (23 January 2020), fed for three weeks, and then irradiated (13 February 2020) and crossed with sexually reproductive females (14 February 2020).

A total of two treatments were tested for each group, plus the control. All crosses were carried out using overwintering males and females, as below.

Group 1:

(a) 0 Gy male x 0 Gy female (zero-dose control);

(b) 16 Gy male x 0 Gy female;

(c) 24 Gy male x 0 Gy female.

Group 2:

(a) 0 Gy male x 0 Gy female (zero-dose control);

(b) 16 Gy male x 0 Gy female;

(c) 24 Gy male x 0 Gy female.

A minimum of twenty replicates were performed for each treatment. Crosses were accomplished by confining each pair in a transparent plastic container (Ø = 133 mm and V = 1180 mL) equipped with a cap with fine organza mesh and a paper-towel sheet folded into four as oviposition substrates.

Each pair was fed with two green beans and one cherry tomato; a 3 cm diameter plastic cup, in which was inserted a wet cotton, was utilised as a water source ad libitum. The diet and substrate were refreshed twice a week.

BMSB pairs were checked twice a week to record the survival rate and oviposition. The collected egg masses were transferred into 90 mm-diameter Petri dishes and kept under the same climatic conditions to evaluate the hatching rate. Eggs that did not hatch were still checked for several weeks until they collapsed or blackened.

The oviposition experiment was concluded after three weeks (6 March 2020).

3.2.4. Male Dose Response, Year 2

During 2021, a second dose-response experiment was carried out using the same approach as for Group 2. BMSB males were transferred (12 January 2021) for 3 weeks in postdiapause conditions before being irradiated (2 February 2021) with X-rays at 16, 24, 32 and 40 Gy. The last two dosages were added with the aim to reach complete sterility.

Since it was the first time that we irradiated insects with this technique and several manipulations were required in preparation for irradiation, including transport to the irradiator, we decided to evaluate the effects of such variables on insects' biology (longevity, fertility, fecundity) so that we could separate these effects from those due to the irradiation. Therefore, we introduced an additional control (0 Gy Cell) that was not prepared for irradiation and not transported to the irradiator but remained in the climatic chamber under the standard conditions used for all postdiapause insects (L:D = 16:8, T = 25 ◦C, RH = 70%). As in Experiment 1, the insects were mated after irradiation. Six treatments were carried out including the two controls:

(a) 0 Gy male x 0 Gy female (zero-dose Cell control);

(b) 0 Gy male x 0 Gy female (zero-dose control);

(c) 16 Gy male x 0 Gy female;

(d) 24 Gy male x 0 Gy female;

(e) 32 Gy male x 0 Gy female;

(f) 40 Gy male x 0 Gy female.

In the second year, the number of replicates (n = 40) per treatment was doubled from the previous year to obtain more-robust data. In addition, before starting of this experiment, twenty overwintering BMSB females were dissected to record the physiological statuses of the spermathecae according to Nielsen et al. (2017), which were then compared with the data of fifteen one-week-old mated females with the expectation that spermathecal width (proximal part) would be significantly greater in mated than in overwintering females, confirming the unmated status of the latter.

Parameters such as fertility of males, fecundity (expressed as the number of eggs laid by each ovipositing female) and longevity of both males and females were compared. The oviposition experiment was concluded with the death of the females. Likewise, their longevity was recorded until the death of all insects.

3.2.5. Cumulative Mortality in F1

In order to detect the potential presence of inherited sterility, the survival of the F1 generation derived from the second experiment (male dose response, year 2) was also recorded at various development stages, from nymphs to adults, for 0 Gy, 32 Gy and 40 Gy to evaluate possible cumulative mortality effects due to irradiation.

For each treatment, twenty-five first instars of BMSB were individually isolated in a transparent plastic container (Ø = 133 mm and V = 1180 mL) equipped with a cap with fine organza mesh.

The insects were fed with a green bean, a cherry tomato and a shelled peanut, while a wet cotton ball (Ø x 10 mm) was provided as a water source. The diet was refreshed twice a week. Insects were checked to record the survival rate and the stage (first to fifth nymphal instars or adult stage).

3.2.6. Mating-Behaviour Bioassays

Mating-behaviour bioassays were also performed to assess the mating performance of irradiated versus nonirradiated males in no-choice test conditions. The purpose was to evaluate possible differences in the durations and numbers of matings between irradiated males at the highest doses and nonirradiated insects.

A six-hour trial was carried out comparing irradiated overwintering adult males at 32 Gy and 40 Gy, with untreated (0 Gy) individuals.

Crosses between different treatments were formed by confining one couple/treatment in a transparent plastic container (Ø = 133 mm and V = 1180 mL) equipped with a transparent cap. No food was provided during the test, except for water provided ad libitum with wet cotton in a small plastic cup. Bioassays were carried out at 25 °C, RH = 60% and an illuminance of ≈ 800 lx.

Three treatments were evaluated, with 12 replicates each:

(a) 0 Gy male x 0 Gy female (zero-dose control);

(b) 32 Gy male x 0 Gy female;

(c) 40 Gy male x 0 Gy female;

The number of matings and the duration of each mating were recorded based on direct observation.

3.2.7. Statistical Methods

During the first year, differences in terms of fertility, measured in the egg-hatching percentage as a function of male irradiation dose, were evaluated among treatments (0, 16 and 24 Gy) and groups (1 and 2) using a generalised linear model with "quasibinomial" distribution.

In the second year, other parameters were evaluated. A Shapiro–Wilk test was performed to assess normality, and Levene's test for homogeneity of variance was also performed to assess homoscedasticity. Because the parametric assumptions failed for all datasets, we adopted a nonparametric approach for data analysis:

(a) The mated status was ascertained by comparing the spermathecal widths of overwintering versus mated one-week-old females. A one-tailed Welsh t-test (Welch, 1947), which considered the two means equal, was performed to test the null hypothesis.

(b) The fecundity of the females was compared using a two-sample Brown–Mood median test (Brown & Mood, n.d.) followed by a contingency table (2 × 6) $\chi2$. The number of egg masses produced per female and the number of eggs per egg mass were compared with the Kruskal–Wallis test.

(c) The fertility of males and the longevities of both males and females for the irradiated (16, 24, 32 and 40 Gy) and two unirradiated control groups (0 Gy Cell and 0 Gy) were compared through a Kruskal–Wallis test. In cases of significance, a Mann–Whitney pairwise test with the Bonferroni correction was then performed as a post hoc test.

(d) To evaluate possible cumulative mortality effects in the F1 generation among the two highest dosages of male irradiation (32 and 40 Gy) and a 0 Gy control, a $\chi 2$ test in a contingency table (2 x 3) was conducted for each stage (nymph from second to fifth instar and adult). Ryan's test for multiple comparisons of proportions (Ryan, 1960) was performed as a post hoc test.

(e) The numbers of matings, the mating durations and the total mating times were compared across treatments with the Kruskal–Wallis test, which was followed by the Mann–Whitney pairwise test with the Bonferroni correction as a post hoc test in cases of significance.

The differences between Group 1 and Group 2 in the first-year dose-response experiment were obtained in R (R Core Team, 2023) through the application of a generalised linear model (glm) with a quasibinomial distribution with the formula "H1 ~ Group * Radiation", using the "lme4" package (Boeck et al., 2011), where H1 is the egg-hatching percentage.

The following tests were performed to analyse the second-year data using Past (Hammer et al., 2001) (version: 4.12b): the Shapiro–Wilk test, Levene's test, the χ^2 test, the Kruskal–Wallis test and the Mann–Whitney pairwise test.

3.3 Results

3.3.1 Male Dose Response, Year 1

The fertility (median percentage of hatching rate) was higher in the controls (96.49% for Group 1 and 94.64% for Group 2), decreasing when males were irradiated at 16 (42.86% for Group 1 and 35.71% for Group 2) and declining up to 19.25% and 21.42% (for Group 1 and Group 2) at 24 Gy (Figure 3.1). The model performed did not display a significant difference in the effects of irradiation between the two groups nor the interactions between groups and the male radiation dose in terms of hatching rate. On the contrary, significant differences in terms of fertility were found between the irradiated (16 and 24 Gy) and nonirradiated males (0 Gy) (Table 3.1).

The Group 2 protocol was selected as the most suitable, considering the high postdiapause mortality and the need to know precisely how many insects were available experimental

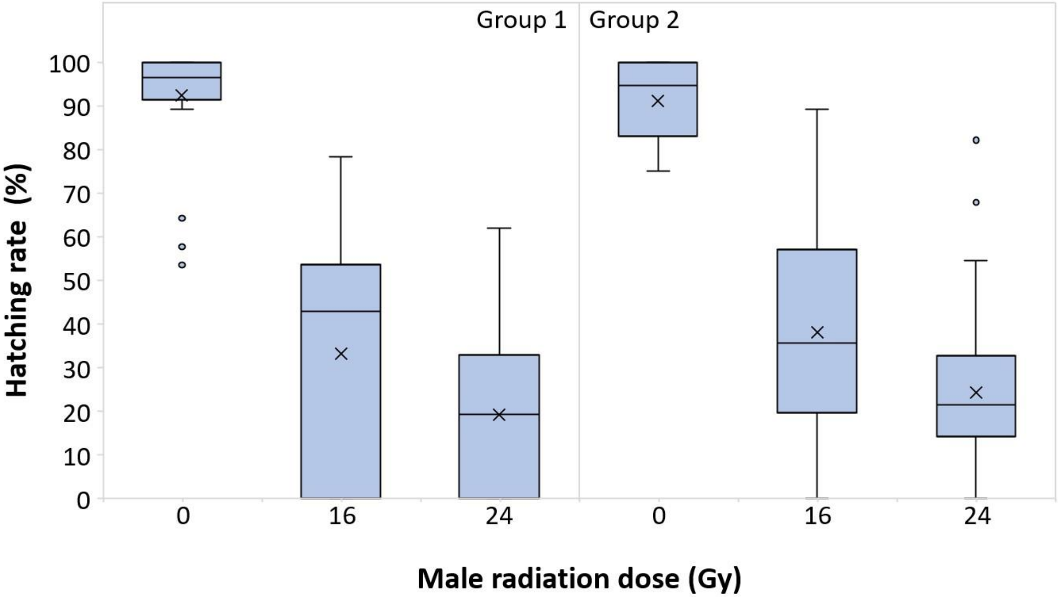

Figure 3.1. Hatching rates of all batches of eggs from crosses of irradiated (16 and 24 Gy) or untreated males (0 Gy), mated with virgin females of *Halyomorpha halys*, for Group 1 (left) and Group 2 (right). The upper whisker extends to the highest data value within the upper limit (Q3 + 1.5 (Q3 − Q1)); the boxplots represent the interquartile range, with a

horizontal bar as the median, and the lower whisker extends to the lowest value within the lower limit (Q1 − 1.5 (Q3 − Q1)). The "×" symbol represents the mean value; the dots represent outliers.

Table 3.1. Results of the generalised linear model for hatching rates, showing the independent variables of the chosen model.

Coefficient	**Estimate**	**Std. Error**	**t-Value**	**Pr(>|t|)**
Intercept	−0.07863	0.02680	−2.934	0.00377 ** [1]
Group 2	−0.01489	0.05438	−0.274	0.78447
Radiation, 16 Gy	−1.02482	0.12472	−8.217	3.39×10^{-14} *** [1]
Radiation, 24 Gy	−1.57294	0.18732	−8.397	1.12×10^{-14} *** [1]
Group 2; Radiation, 16 Gy	0.15456	0.15784	0.979	0.32874
Group 2; Radiation, 24 Gy	0.25251	0.22973	1.099	0.27311

[1] ** $p < 0.01$, *** $p < 0.001$.

3.3.2 Male Dose Response, Year 2

The mortality of the overwintering adults recorded in about two months of diapause was 59% for both males and females. The mortality of the remaining survivors in the following three weeks was 15% for males and 20% for females. As a result, 560 males and 600 females were available for experiments. The mated status of the overwintering females, verified with spermathecal width, was significantly different if compared with that of one-week-old mated females ($t = -10.3$; df = 17; $p = 5.05 \times 10^{-9}$), confirming the unmated status at the end of the diapause and consequently the suitability of overwintering females for these experiments (Figure 3.2).

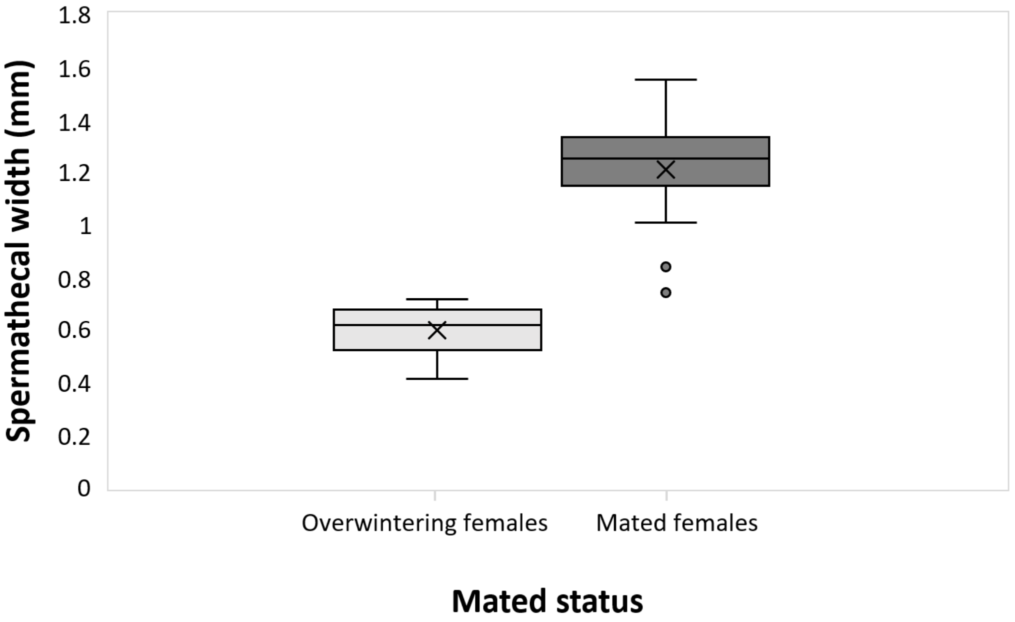

Figure 3.2 The mated status was verified as a function of spermathecal width by comparing the data between overwintering females and mated one-week-old females. The upper whisker extends to the highest data value within the upper limit (Q3 + 1.5 (Q3 − Q1)); the boxplots represent the interquartile range, with a horizontal bar as the median, and the lower whisker extends to the lowest value within the lower limit (Q1 − 1.5 (Q3 − Q1)). The "×" symbol represents the mean value; the dots represent outliers

No significant differences were recorded between the groups in fecundity (eggs produced per female), number of egg masses per female or number of eggs per egg mass (Table 3.2).

Table 3.2 The percentage of egg-laying females was calculated on n1 (females alive after three days); in recording the first egg mass four days after the formation of the crosses, it was assumed that the females that died before the fourth day did not have enough time to lay eggs.

Male Dose (Gy)	***n***	***n1***	**No. of Egg Masses**	**Total Eggs Collected**	**% Egg-Laying Females**	**Eggs/Egg Mass**	**Egg Masses/Egg-Laying Females**	**Eggs/Fem-ale**
0 Cell [2]	40	35	78	2052	60	26.31	3.75	56
0	40	36	79	2018	80.56	25.54	2.72	50
16	40	35	75	1954	57.14	26.05	3.45	41.5
24	40	32	46	1185	43.75	25.76	3.29	27.5
32	40	35	98	2513	62.86	25.64	4.45	95.5
40	40	33	71	1827	48.48	25.73	4.60	96
p						0.9 [1]	0.09 [1]	0.16 [1]

[1] The p-values were calculated with a 95% confidence interval. 2 The 0 Cell control is the negative control: insects that were not irradiated or transported to the irradiator.

In terms of fertility (median), there was a significant difference among the treatments (Kruskal–Wallis test: H = 264.4; df = 447; $p = 1.42 \times 10^{-55}$). The results showed a declining trend from the two controls, 0 Gy Cell and 0 Gy, which had the identical value of 92.86%, through the irradiation dosages. The hatching rates for the doses of 16 and 24 Gy were, respectively, 37.93% and 16.39%, while they were 4.76% and 3.57%, respectively, for 32 and 40 Gy, which showed sterility of more than 95% (Figure 3.3).

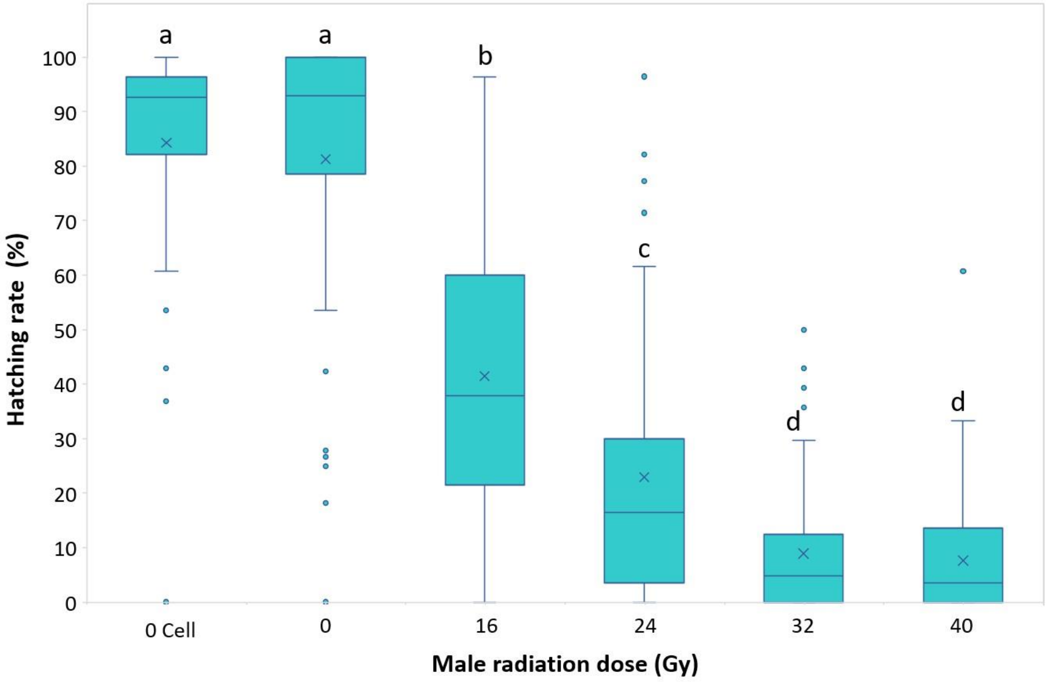

Figure 3.3. Hatching rates of all batches of eggs from crosses of irradiated (16, 24, 32 or 40 Gy) or untreated males (0 Gy Cell and 0 Gy) mated with virgin females of *Halyomorpha halys*. Letters (a–d) indicate differences among groups. The upper whisker extends to the highest data value within the upper limit (Q3 + 1.5 (Q3 − Q1)); the boxplots represent the interquartile range, with a horizontal bar as the median, and the lower whisker extends to the lowest value within the lower limit (Q1 − 1.5 (Q3 − Q1)). The “×” symbol represents the mean value; the dots represent outliers.

In terms of longevity (Figure 3.4), no statistical difference was observed for females paired with irradiated (16, 24, 32 and 40 Gy) and unirradiated (0 Gy Cell and 0 Gy) males (Kruskal–Wallis test: H = 5. 234; df = 7; $p = 0.28$). Moreover, the mortality among the irradiated males was similar to that of both of the controls, except for the strongest dose (40 Gy), which was significantly different from the 0 Gy Cell group (Kruskal–Wallis test: H = 13.39; df = 239; $p = 0.01$).

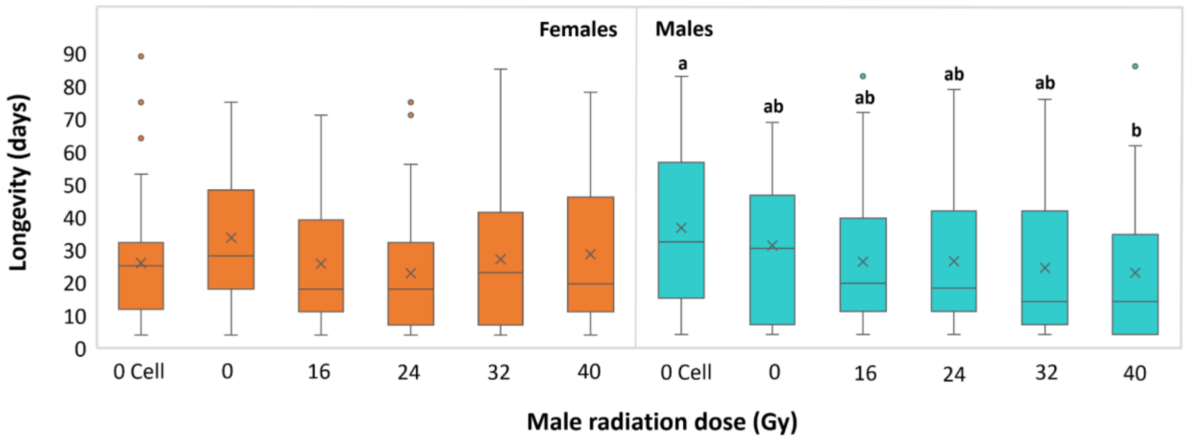

Figure 3.4. Longevities of untreated *Halyomorpha halys* females paired with irradiated males (left) and irradiated male adults (right), as a function of male irradiation dose. Letters (a, ab, b) indicate differences among groups. The upper whisker extends to the highest data value within the upper limit (Q3 + 1.5 (Q3 − Q1)); the boxplots represent the interquartile range, with a horizontal bar as the median, and the lower whisker extends to the lowest value within the lower limit (Q1 − 1.5 (Q3 − Q1)). The “×” symbol represents the mean value; the dots represent outliers.

3.3.3 Cumulative Mortality in F1

A clear difference in the F1-generation survival rate was recorded between the 0 Gy control and the two irradiation doses of 32 Gy and 40 Gy (Figure 3.5).

The difference between the control of 0 Gy and the two radiation doses was significant starting from the second instar ($\chi^2 = 7.91$; df = 2; $p = 0.019$). As for the third instar ($\chi^2 = 24$; df = 2; $p = 6.16\times 10^{-6}$), all treatments differed from each other, whereas the fourth ($\chi^2 = 21{,}7$; df = 2; $p = 1.95\times 10^{-5}$) and fifth ($\chi^2 = 24.74$; df = 2; $p = 4.26\times 10^{-6}$) instars and also the adults ($\chi^2 = 26.39$; df = 2; $p = 1.86\times 10^{-6}$) showed significant differences only between the control of 0 Gy and the two irradiation doses.

To summarise, the percentages of F1 generation insects that reached the adult stage were 76% for 0 Gy and 20% and 12% for 32 Gy and 40 Gy, respectively.

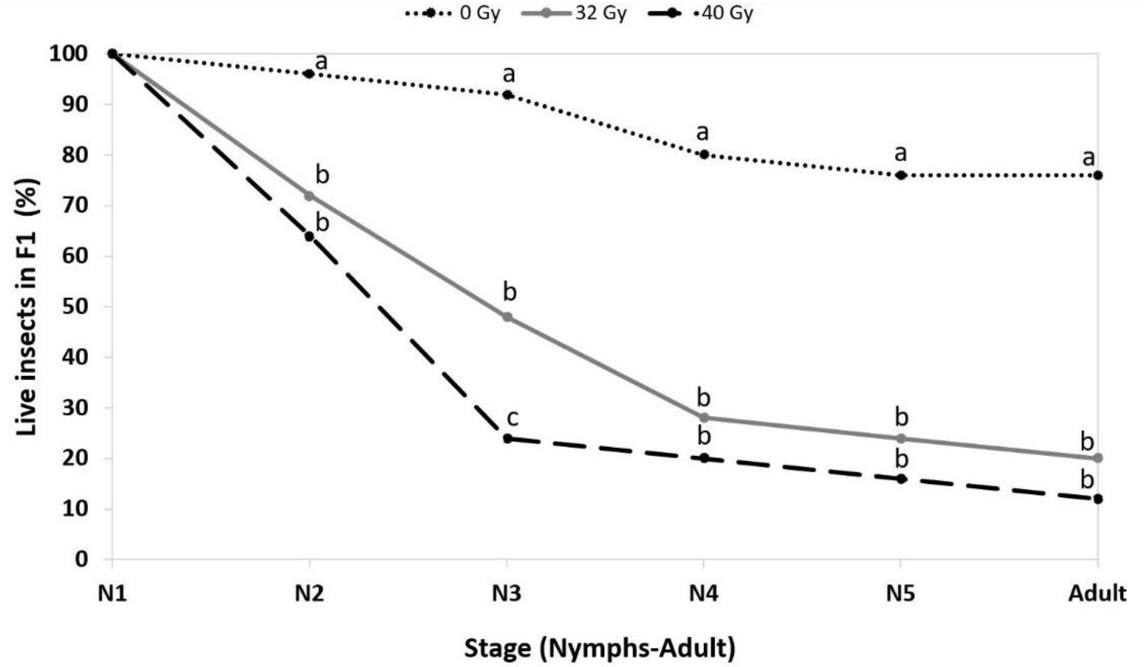

Figure 3.5. Percentage of insects alive at each stage in the F1 generation, considering the starting point (100% alive) of 25 first-instar insects. The different lines represent the treatments, and the letters (a–c) indicate the differences between the three treatments at each stage.

3.3.4 Mating Behavioural Bioassays

In the six-hour mating trial, no statistically significant differences were found for the number of matings (Kruskal–Wallis test: H = 3.648; df = 34; p = 0.146) or the total mating time (Kruskal–Wallis test: H = 1.19; df = 34; p = 0.55). An increasing trend was observed in mating duration (mating time), with statistically significant differences between 40 Gy compared to 32 Gy and 0 Gy (Kruskal–Wallis test: H = 22.6; df = 162; $p = 6 \times 10^{-11}$) (Table 3.3).

Table 3.3. Numbers of matings, mating times and total mating times are shown in the table as medians. For the mating times, the letters "a" and "b" indicate the differences among the groups.

Male Dose (Gy)	***n***	**No. of Matings**	**Mating Time (min)**	**Total Mating Time (min)**
0	12	6	12 a	72
32	11	5	13 a	64
40	12	5	13.5 b	66
p		0.15 [1]	5.997×10^{-6} [1]	0.55 [1]

[1] The *p*-values were calculated with a 95% confidence interval.

3.4 Discussion

The success of an area-wide pest management programme that has the SIT as its strategic core relies on setting up a system to release an adequate number of sterile males able to compete with wild males for mating opportunities. Therefore, this work was focused on the selection of the correct irradiation dose, able to induce sperm sterility in irradiated males while at the same time maintaining the mating competitiveness of the sterile BMSB males with respect to the wild insects (Suckling et al., 2019a). Given the difficulty in mass rearing of BMSBs, the possibility of using post diapause overwintering males following large autumn mass trapping has been considered (Suckling et al., 2019a). An irradiation technology that does not involve radioactive isotopes was also used, with the purpose of promoting the development of the SIT for future applications. In order to set up a suitable protocol to release competitive sterile males, it was crucial to consider the impact of diapause and postdiapause mortality of BMSBs. According to the data presented by Costi et al. (2017), it seemed pointless to irradiate BMSB adults before their winter diapause. In fact, those authors reported that only 28% of adults were able to survive to the overwintering diapause when kept in outdoor conditions until spring, and only 50% of the surviving adults then reached reproduction. We thus focused our efforts to evaluate the performance of males that were irradiated to achieve sterility after surviving the postdiapause period. The results showed differences in hatching rates within the groups between the negative control, 0 Gy, and the two irradiation doses, 16 Gy and 24 Gy, but no differences between the groups. In the second dose-response experiment, performed during 2021, we chose the Group 2 protocol, considering it the most suitable, and applied irradiation only on the surviving males at the postdiapause mortality phase.

The effects of irradiation on reproduction were different from the data presented by other authors who used gamma rays (Welsh et al., 2017; D. Suckling et al., 2019a; Nguyen et al., 2021). The rapid decline in fecundity observed by Welsh et al., 2017 when a female mated with a male irradiated at 32 Gy or a higher dose was not recorded in our study. Furthermore, unlike what was reported by Welsh et al. (2017), our hatching-rate data were higher, while they were lower than those reported by Suckling et al. (2019a) and more in line with those of Nguyen et al. (2021). Considering the same radiation doses, the observed differences could have depended on the different ages of the irradiated males (Suckling et al., 2019a).

Regarding effects on the longevity of irradiated males, our results are also different from the data already reported in the literature (Welsh et al., 2017; D. Suckling et al., 2019a; Nguyen et al., 2021), where constant declines in longevity with increasing radiation doses were observed. Indeed, in the present study, only the males irradiated at 40 Gy showed significantly lower longevity than the control of 0 Gy Cell. Differently from what was reported by Welsh et al., 2017, the longevity of the females was constant, independently from the irradiation dose of the male with whom a female was mating. The number of eggs laid per female was variable regardless of the male irradiation dose, as in the research of Nguyen et al., 2021, unlike what has been observed in other works (Welsh et al., 2017; D. Suckling et al., 2019), which have reported declines related to higher radiation doses.

At the same time, while increasing the irradiation doses on males, we observed a negative impact on reproduction, with high levels of sterility at the highest dose. Moreover, the results of the cumulative mortality experiment showed that even if sperm sterility were not complete, the longevity and development of offspring were severely compromised (Figure 3.5).

Even though most SIT programmes have been applied to target pests from Diptera and Lepidoptera (Vreysen et al., 2006), recent studies have shown the feasibility of including the SIT in area-wide control programmes against invasive alien pentatomids and even considering eradication strategies (Suckling et al., 2019a; Horrocks et al., 2020; Cristofaro et al., 2022). Concern about the suitability of implementing mass rearing facilities for the species belonging to pentatomids has led to new ideas and approaches based on mass harvesting, irradiation and release of large numbers of sterile males of the target pest species instead of multiplying them in laboratory (Suckling et al., 2019a; Cristofaro et al., 2022). For this reason, recent studies have been addressed to develop a new live mass-trapping tool baited with the specific aggregation pheromone that enables collection of large numbers of BMSB adults during autumn (Suckling et al., 2019a). The success of a SIT programme in AW-IPM also depends on the quality of the insects to be released. In this context, it is essential to conduct studies on mating behaviour (Hendrichs et al., 2002). Often, poor performance of sterile males in terms of mating competitiveness has been wrongly attributed to side effects of irradiation (De Beer et al., 2020; Ilboudo et al., 2022); on the contrary, the bottleneck is often due to the fact that prolonged captivity can induce drastic shifts, resulting in a clear increase in genotypes that are better adapted to indoor conditions rather than natural environments (Cristofaro et al., 2022). In the present study, the insects were wild-harvested, and thus their mating competitiveness would mainly have been influenced by irradiation. To assess possible side effects of irradiation on mating behaviour, mating times were recorded for the highest doses (32 and 40 Gy). The results showed that sterile wild-type

BMSB males did not lose mating competitiveness when irradiated with the tested dose range. However, at 40 Gy, the mating time was significantly longer. To clarify these data, further studies should also consider sperm transmission and competition. Indeed, as modelled in a polyandrous species, in which it is uncertain whether a female would use the sperm of a single male, the effect of multiple matings can be assumed to depend on competition between sperm and between males (Berryman, 1967; Zouros, 1969). Consequently, it will be crucial to understand whether the observed difference is related to sperm transmission and how females store sperm in the spermatheca.

The mating times recorded in our study are higher (12, 13 and 13.5 min for 0, 32 and 40 Gy, respectively) if compared to a previous study (10 min) (Kawada & Kitamura, 1983) that described the mating behaviour of BMSBs. However, the experimental protocols were different: in our case, the insects were kept in separate pairs and were observed continuously, while in the previous study, the insects were all in the same container and data were collected every 10 min.

Moreover, in the same work, it was observed that males required a 50–60 interval between successive coupling and that the average number of matings was 5.28 ± 0.15 in 24 h for both males and females (Kawada & Kitamura, 1983). In our study, the interval between matings was similar and the number of matings almost the same in comparing different treatments (6 for 0 Gy and 5 for 32 and 40 Gy), but the duration of the experiment was four times shorter (6 h versus 24 h). However, Kawada & Kitamura (1983) observed that both males and females of BMSB can mate more than five times in a day, and these results are very relevant because those tests were performed immediately after sexual maturation.

Our team conceived mass field collection and exploitation of overwintering BMSB males (Suckling et al., 2019a) whose preliminary parameters for a possible practical application have been further defined in the present work. Indeed, it was necessary to test the feasibility of a programme involving efficient interruption of diapause that would not compromise the health or mating competitiveness of the insects, also considering the eventuality of transporting sterilised insects to another location (e.g., from one jurisdiction to another) and releasing them. In our case, the mortality recorded in about two months of diapause (59% for both males and females) and in the following three weeks (15% for males and 20% for females) was much lower than that recorded by Costi et al. (2017), as the diapause was stopped long before spring. Furthermore, using suitable climatic chambers is possible to keep insects in optimal standard diapause conditions (at 9 °C) and optimise the survival of the insects until spring or, if necessary, successfully interrupt the diapause after only 7 weeks (Taylor et al., 2017).

The 95% sterility rate achieved in this study at 32 Gy and 40 Gy is likely to be sufficient for suppression (if $\lambda \leq 20$) according to the model constructed by Klassen (1971): $q < 1/\lambda$ is applied when sterility is incomplete (where q is considered as a fraction of males that has remained fertile and λ is the population's intrinsic rate of increase). Furthermore, to have effective suppression, the target pest species must be present in low numbers (Vreysen et al., 2006) and the ratio of "sterile males: fertile males" for BMSBs should be at least 5:1 (Suckling et al., 2019a).

Incomplete sterility is probably insufficient for eradication; nonetheless, if the SIT is used as a component of an IPM strategy, the possibility of having effective pest control in a limited area will increase. Indeed, use of the SIT as a component of area-wide pest management strategies, as demonstrated for Lepidoptera and Diptera, may effectively achieve both suppression and eradication of insect pests in many cases (Vreysen et al., 2006). To incorporate BMSBs into an IPM programme, it is a primary factor to know the number of generations per year and the peak filial adult population, as that is the most damaging stage (Nielsen et al., 2008). In invasion areas, both where BMSBs are univoltine, as in Switzerland (Haye et al., 2014), and where they are multivoltine, as in Southern

Europe (Costi et al., 2017) and warmer United States areas (Nielsen et al., 2008), the only potential window of release for sterile, overwintering male populations is at the beginning of spring, when only the overwintering generation is present.

For BMSBs, the aspect concerning the damage caused by adults to crops remains critical; however, in an eradication programme, capture (Suckling et al., 2019a) and release of sterile BMSB males and any short-term and localised crop damage would not be considered as important as achieving removal long-term (risk–benefit analysis). Furthermore, it has been demonstrated on *Pistacia vera* (L.) (Sapindales: Anacardiaceae) that feeding damage caused by males is significantly lower than that caused by females (Lara et al., 2017).

Another important aspect in support of the concept of an AW-IPM or eradication programme for management of BMSBs in its invasive range, with the involvement of the SIT, is the result of its synergism with classical biological control approaches (Knipling, 1979; Liebhold et al., 2016).

Safety concerns related to implementation of radioactive sources for insect sterilization (Kutsaev et al., 2021) can limit development of SIT programmes. Use of linear accelerators, which has already been explored in Diptera to induce sterility (Wang et al., 2023; Balestrino et al., 2016), can reduce irradiation costs and avoid the need for radioactive sources, thus eliminating the problems associated with safety, transport, handling and possible misuse of these materials (Kutsaev et al., 2021; Wang et al., 2023; Balestrino et al., 2016).

High-energy photons produced by linear accelerators seem to be a valid alternative to other radiation sources and are suitable for some SIT programmes. In our study involving a 6 MV LINAC, the developed protocol allowed the achievement of a high level of sterility of overwintering males (over 95%) at only 32 Gy without compromising male mating behaviour (in no-choice conditions).

Males’ longevity and fertility did not show significant differences between the two types of control, indicating that the abiotic stress related to the irradiation protocol was negligible up to 32 Gy. In addition to offering sterilisation of field-collected males as an alternative to expensive (and probably impossible) BMSB mass rearing, our results support use of medical linear accelerators that are present in hospital facilities, since these are more widely available compared to gamma sources, to promote SIT programmes.

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CHAPTER 4.

COMBINING IRRADIATION AND BIOLOGICAL CONTROL AGAINST BROWN MARMORATED STINK BUG: ARE STERILE EGGS A SUITABLE SUBSTRATE FOR THE EGG PARASITOID *TRISSOLCUS JAPONICUS*?

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Simple Summary

Halyomorpha halys is an alien stink bug species native to south-eastern Asia, and is now widely distributed and very invasive worldwide. Management of this pest by chemical insecticides is not very effective because of the high mobility of the pest species and its innate ability to develop resistance to most of the synthetic insecticides applied. For this reason, classical biological control has been considered, with attention focused on co-evolved egg parasitoids, which oviposit and complete their larval development within a single egg of the host. The detection and collection of the egg parasitoid species is often conducted by exposing for few days newly oviposited egg-masses of the stink bug pest in the field for a few days as sentinels, which can then be parasitized by the egg parasitoid. In newly invaded areas, limiting factors in the use of sentinel eggs include the short amount of time that they are a suitable substrate for the oviposition of the egg parasitoid and the risk of the unintentional releases of additional pests from unparasitized eggs. A valid potential alternative is to use sterile sentinel eggs of the pest species. This study is focused on the evaluation of the performance of three types of sterile sentinel eggs for the oviposition and larval development of the egg parasitoid *Trissolcus japonicus*.

Abstract

The brown marmorated stink bug (BMSB), *Halyomorpha halys*, is a phytophagous alien pest native to South-Eastern Asia and now distributed worldwide. This species is considered among the most dangerous insect pests in North America and in Europe. In agriculture, the predominant approach to managing BMSB is based on the use of insecticides, specifically pyrethroids and neonicotinoids. Unfortunately, the biology of the species and the facility to build up mechanisms of resistance to most of them, induced farmers and scientist to develop different least toxic and more effective strategies of control. In a territorial area-wide approach, the use of classical biological control program in combination with other least-toxic strategies has been taken under consideration. Subsequently to some explorations in the native range, the attention was focused on *Trissolcus japonicus*, a small egg parasitoid scelionid wasp, able to oviposit and complete the larval development in a single egg of *H. halys*. Egg parasitoids are recorded in the native area releasing egg masses, so called sentinel eggs, of the pest in the environment for a short period, in order to get back in the laboratory some of them parasitized. Outside of the area of origin, the use of fertile sentinel eggs of the alien species may lead to the accidental release of other individuals of the pest species; the alternative is to use sterile sentinel eggs to record the presence of new indigenous egg parasitoids or to detect the dispersal of the alien ones (in this case *T. japonicus*) released in a new environment to control the target insect pest species. This study is focussed on the evaluation of the performance of 3 types of sterile sentinel eggs as suitable substrate for the oviposition and larval development of the egg parasitoid *T. japonicus*, in a context of combining classical biological control with the Sterile Insect Technique (SIT).

Keywords: SIT; CBC; *Samurai wasp*; *Halyomorpha halys*; IPM; Sentinel eggs

4.1 Introduction

The brown marmorated stink bug (BMSB) *Halyomorpha halys* (Stål) (Hemiptera: Pentatomidae) is a phytophagous insect native to Eastern Asian (Lee et al., 2013). It has successfully invaded various regions, including Europe, the United States (Costi et al., 2017; Leskey & Nielsen, 2018), and the Caucasus (Musolin et al., 2018). Its potential for further expansion is substantial, particularly in the southern hemisphere (Kritickos et al., 2017; Faúndez et al., 2017. Due to its wide host range, BMSB is considered an important pest species in the invaded areas, causing significant crop losses (Bergmann et al., 2016; Leskey & Nielsen, 2018). The predominant approach to managing BMSB involves the use of insecticides, specifically pyrethroids and neonicotinoids, along with other agronomic and biocontrol strategies (Kuhar et al., 2012; Leskey et al., 2014; Kuhar & Kamminga, 2017). However, broad-spectrum insecticides are a short-term solution, and their frequent use can induce insecticide resistance and sometimes lead to secondary pest outbreaks (Leskey et al., 2012a; Leskey 2012b). On the other hand, the implementation of both indigenous and exotic natural biocontrol agents has evinced promising medium- and long-term outcomes in controlling BMSB (Abram et al., 2017; Leskey & Nielsen, 2018; Conti et al., 2021; Falagiarda et al., 2023). In particular, the exotic egg parasitoid *Trissolcus japonicus* (Ashmead) (Hymenoptera: Scelionidae) has been identified as the most promising candidate for classical biological control of BMSB (Zhang et al., 2018; Conti et al., 2021) due to its host-specificity and the high parasitization rate (Hedstrom et al., 2017; Haye et al., 2020). The presence of adventive populations of *T. japonicus* was documented in the United States since 2014 (Talamas et al., 2015) and subsequently observed in Europe since 2017 (Sabbatini et al., 2018; Stahl et al., 2019). In the United States, initiatives have been taken to facilitate the dispersal of the adventive populations of *T. japonicus* (Jentsch et al., 2017; Lowenstein et al., 2019). In Europe, Italy adopted a different approach, implementing a conventional biological control program, inoculating individuals to establish self-sustaining populations that would subsequently proliferate in response to BMSB presence (Falagiarda et al., 2023).

In the context of an IPM strategy, combining CBC and SIT may represent a synergistic and effective approach. The Sterile Insect Technique is considered a species-specific method that relies on the mass production, sterilization, and subsequent inundative releases of predominantly sterile male insects (Klassen & Curtis, 2021). The synergy between SIT and classical biological control approaches (Knipling, 1979, Liebhold et al., 2016) further strengthens the opportunity to implement IPM or area-wide eradication programs for managing BMSB within its invasive range. In recent years, there has been a notable surge in the adoption of integrating biological control and SIT specifically (Hendrichs et al., 2009; Gurr & Kvedaras., 2010).

A pest management program that involves the use of BCAs) requires to have a good field monitoring system for their detection which for BMSB is based on sentinel eggs for egg parasitoids (Konopka et al., 2019; Abram et al., 2020; Tillman et al., 2020; Cornelius et al., 2021). To minimize the risk of inadvertently introducing the pest in the environment by using fertile sentinel eggs, some alternatives were evaluated. Frozen BMSB egg masses at – 80 °C are commonly used (Roversi et al., 2016, Abram et al., 2017, McIntosh., 2019), although lower *T. japonicus* emergence was observed from frozen BMSB eggs compared fresh eggs (McIntosh et al., 2019). Unfertilized and refrigerated eggs were then evaluated in substitution of frozen eggs, showing better performance in the emergence and development of *T. japonicus* (Yang et al., 2018; Wong et al., 2021).

Cristofaro et al. (2022), proposed for *Bagrada hilaris* (Burmeister), another pentatomid species, a new concept of sterile sentinel eggs based on sterile eggs derived by SIT. Consequently, in the present study, besides the classical SIT approach, based on the release of sterile irradiated males, we are comparing the performance of 2 different types of sterile eggs -derived by SIT and by irradiation of fresh eggs- to be used as sentinel eggs for *T. japonicus*. The work was divided into three parts. The first two parts aimed to identify the correct dose of radiation needed to produce sterile eggs and sterile males of the BMSB species, while the third experiment evaluated the (Wong et al., 2021).

4.2 Materials and Methods

4.2.1 Insects and Rearing

Halyomorpha halys colony originated from adults collected using traps in May 2018 from infested field sites in Rome (42° 2' 36.96" N, 12° 17' 53.66" E). Laboratory colonies of BMSB were maintained in a temperature-controlled climatic chamber (27 ± 1 °C, photo-period L.D. = 16:8, RH = 70 ± 10%) in the laboratories of entomology, at the Italian National Agency for New Technologies, Energy and Sustainable Economic Development (ENEA), Rome (Italy). Adults were reared in cages (60 x 30 x 30 cm BugDorm®, Taichung, Taiwan) at the density of about 50-70 adults per cage with approximately a 1:1 sex ratio. Insects had access ad libitum to water in a glass vial with a cotton plug and were fed with potted plants of *Phaseolus vulgaris* (L.) and *Vicia faba* (L.), and other fresh vegetables and fruits: green beans, carrots, apples and kiwi. Senesced and dead host plants were removed and replaced with new plants weekly or as needed. Each week, the bottoms of all rearing cages were cleared of dead insects and debris (exuviae, loose soil, fallen leaves, and supplemental foods) and scrubbed clean. Food was replenished three times per week. Females in the cages laid clusters of ~28 eggs on host plant leaves and on the top of the cages. BMSB egg masses were collected daily (at 9:00 am), and 1 day-old or 2 days-old egg masses were used in the trials. The colony of BMSB was regularly renewed or supplemented with adults of the rearing and sometimes by field-collected adults. Plants within the cages were watered two to three times per week. The same treatment was performed to rear the juvenile forms.

A second BMSB colony was reared at the entomology laboratories of the Fondazione Edmund Mach (FEM), Trento (Italy), following the same protocol mentioned above. The colony originated from a mass trapping collection realized during autumn 2021 in fields near FEM (46°11’43’’ N, 11°8’5’’ E), using live traps (Suckling et al., 2019a).

Trissolcus japonicus colonies were supplied by CREA-DC (Italy) and originated from the same population on which the risk assessment study was conducted (CREA, 2020). They were reared at FEM facilities using BMSB egg masses obtained from specifically maintained colonies. Following the same national protocol adopted for *T. japonicus* release (Falagiarda et al., 2023), the individuals were placed in plastic tubes (VWR 50-mL centrifuge tubes, 525–0611) containing honey and maintained in climatic chambers at a temperature of 24°C ± 1 °C, photoperiod L.D. = 16:8, and relative humidity (RH) of 60 ± 5%.

4.2.2 Egg Sterilization

Sterile eggs were obtained in three different ways:

a) Irradiation of fresh eggs with gamma rays (irradiated eggs);

b) Eggs obtained by mating BMSB fertile females with irradiated males (SIT eggs);

c) Refrigeration of fresh eggs of BMSB at 8 °C for a minimum of 14 days (as control).

4.2.3 Irradiation

Egg masses of 24 and 48 hours after oviposition were exposed to γ radiation using cardboard tubes with a diameter of 9 cm. The radiation doses used were 0, 16, 24, 32, and 40 Gy, which were delivered using the Co-60 gamma facility at ENEA (Baccaro et al., 2019), with a dose rate of 175.03 Gy/h (2.92 Gy/min).

Male adult bugs were irradiated ~ 24 hours after emergence. Insects were exposed to radiation using the same protocol adopted for the egg masses; the radiation doses used were 24 and 50 Gy.

4.2.4 Egg Refrigeration

Refrigerated eggs, demonstrated excellent quality as a substrate for oviposition and development of the offspring of *T. japonicus*, showing higher parasitoid emergence than frozen eggs, and with minimal or no sublethal effects on the progeny (Wong et al., 2021). Therefore, in the present study, we adopted refrigerated eggs as a positive control.

Refrigerated sterile eggs were obtained by keeping fresh eggs (<24 h since being laid) at 8 ° C for 14 days according to Wong et al. (2021).

4.2.5 Egg Dose Response

An initial dose-response experiment for BMSB eggs was conducted in order to identify the dose of irradiation needed to induce complete egg sterility. In autumn 2021, fresh eggs from the rearing colony at the ENEA facilities in Rome were irradiated at doses of 16, 24, 32, and 40 Gy. The aging factor was also considered. Consequently, egg masses were irradiated 24 and 48 h after oviposition. Unirradiated eggs (0 Gy dose) of 24 and 48 h were used as the control. The number of replicates was approximately 10, with at least 6 replicates per treatment. After irradiation, eggs were placed in 9 cm diameter Petri dishes and were moved to a climatic chamber (27 ± 1 °C, photoperiod L.D. = 16:8, RH = 70 ± 10%) for 8 days, which is when all of the eggs should be virtually hatched, according to Nielsen et al. [42]. The number of hatched eggs per egg mass, the hatching rate (%) of eggs per egg mass, the number of immature embryos per egg mass, and their percentage were then all recorded with a Zeiss Stemi 508 KMAT stereomicroscope (10× magnification) (Zeiss, Oberkochen, Germany). Immature embryos were recorded regardless of subsequent egg hatching.

4.2.6 Male Dose Response

A second experiment was conducted aiming to reach complete sterility of BMSB adult males in order to obtain sterile eggs by conventional SIT approach. Male adult BMSB from rearing colony of ENEA were irradiated ~ 24 hours after emergence at the doses of 24 and 50 Gy. Unirradiated males (0 Gy dose) were used as control. After irradiation, males were moved in a climatic chamber (27 ± 1 °C, photoperiod L.D. = 16:8, RH = 70 ± 10%) and reared with newly emerged fertile females in cages (60 x 30 x 30 cm BugDorm®, Taichung, Taiwan) at the density of about 50-70 adults per cage with a 1:1 sex ratio. The eggs laid were collected daily and kept in 9 cm diameter Petri dishes for approximately 8 days, until hatching. The same parameters recorded for irradiated eggs were then recorded: the hatching rate (%) of eggs and the number of immature embryos in the eggs.

4.2.7 Evaluation of Sterile Eggs as an Oviposition Substrate

From March to June 2022, BMSB irradiated and SIT eggs ≤ 24 hours old were sent from ENEA to FEM using a courier with delivery in 24 hours. The egg masses were stored in Petri dishes fixed in polystyrene boxes for transportation. The temperature inside the box was registered with a data logger (EL-USB-2, Lascar Electronics, Whiteparish, UK), and the values ranged between 18 °C and 23 °C. After delivering, egg masses (number of eggs per egg mass = 25-28) were glued with a drop of odourless polyvinyl acetate-based glue Vinavil® (Vinavil, Milan, Italy) on a small cardboard (2 x 8 cm) and then placed into a plastic tube (VWR 50-mL centrifuge tubes, 525–0611) containing honey (some droplets on the inside part of the screw cup). Each egg mass had been exposed to one 2-

week-old *T. japonicus* mated female originating from the FEM rearing colony. The age of the females was chosen to maximize the ovary egg load without compromising their lifespan (Sabbatini et al, 2020). Moreover, these females were isolated in the plastic tube containing honey provided ad libitum as a food source for 24 hours before being offered to BMSB egg masses. Egg masses were maintained in climatic chamber at a temperature of 24 °C ± 1 °C, photoperiod L.D. = 16:8, and relative humidity (RH) of 60 ± 5% until the emergence of *T. japonicus* (generally after 15 ± 2 days). To evaluate the suitability of BMSB eggs over time and their potential use in male sterile release programs and field monitoring of egg parasitoids, we kept the eggs at standard climatic conditions, before to be exposed to the egg parasitoid before parasitism (L:D = 16:8, T = 20 °C, RH = 60%). The eggs were placed in a climatic chamber with the above-mentioned parameters for different periods: 4, 7, 10, 15, and 20 days before the exposure to a *T. japonicus* female. As a positive control, refrigerated eggs were used, following the protocol of Wong et al. (2021). Refrigerated eggs were placed in a climatic chamber, like the other two types of sterile eggs, for different times: 1, 4, 7, 10, 15, and 20 days before their exposition to a *T. japonicus* ovipositing female. From here on, when we refer to the age of the eggs, we mean the elapse of time spent in the climatic chamber (L:D = 16:8, T = 20 °C, RH = 60%) before to be exposed to the parasitoid.

The number of replicates was approximately 30 per treatment (irradiated, SIT, and refrigerated) at different ages.

After 24 hours, the female was removed from the vial with the egg mass, and the screw cup with honey was substituted with another without honey to avoid feeding the emerged individuals before recording Key parameters (i.e., the dry weight).

To evaluate eggs acceptance and possible non-lethal impacts in the larval development of *T. japonicus* caused by sterile eggs either obtained by direct irradiation or by mating with sterile male, we measured the following parameters:

(a) The percentage of parasitoids that emerged on the total number of eggs;

(b) The sex ratio of the emerged parasitoids;

(c) The longevity of the emerged parasitoid females;

(d) The fecundity of the parasitoid females at 0 and 12 days after emergence;

(e) The female dry weight (in the F1 generation).

To estimate the longevity, one emerged *T. japonicus* female per egg mass was randomly selected and then isolated in a plastic tube (VWR 50-mL centrifuge tubes, 525–0611) containing honey and folded blotting paper stapled to a small card as a shelter. The mortality was checked weekly, and the honey was refreshed when necessary (at least once a week).

To measure fecundity, we dissected two *T. japonicus* female specimens per egg mass at two intervals following their emergence (0 days and 12 days), to record the initial egg load (0 days, < 24 hours) and the maximum egg load (12 days post-emergence), according to Wong et al. (2020). To determine the egg count immediately after emergence (within 24 hours), we employed a method involving rapid freezing (-20 °C) of each female specimen for 30 minutes. Subsequently, the female was preserved in a 1.7 ml microcentrifuge tube containing a solution of NaCl 0,9 %. To record fecundity after 12 days, each female was isolated for 12 days in a plastic tube (VWR 50-mL centrifuge tubes, 525–0611) containing honey, then processed as the 0-day fertility samples. Subsequently, by dissecting the abdomen of a female, with two entomological pins under a stereo-microscope, it was possible to count the number of mature ovarioles.

Female dry body weight, serving as an indicator of body size, was assessed following the methodology outlined in Wong et al. (2020). Three females of *T. japonicus* per egg mass were randomly selected and then placed in a 1.7 ml microcentrifuge tube containing 75% ethanol. Subsequently, they were transferred to a glass chamber equipped with desiccant (silica gel) for a period of 48 hours to facilitate drying. The desiccated parasitoids were then extracted from the chamber and weighed with a Sartorius CP2P microbalance (Sartorius, Göttingen, Germany) to the nearest microgram (µg). The weight of three females from each egg mass was measured simultaneously, and the total weight was divided by three to determine the average dry weight for each specific egg mass.

All unhatched eggs were dissected two weeks after the eggs had hatched, to check for the presence of an undeveloped or unemerged larva.

4.2.6. Statistical Methods

Eggs dose-response and male dose-response: Data analyses were performed using SPSS inc. PASW Statistics 17.0 (Kinnear et al., 2010).

Egg Dose Response: Effects of gamma irradiation on the examined response variables (number of neanids, the percentage of eggs hatching and the number of immature embryos) were analyzed by a Generalized Linear Model (GLM) (Nelder and Wedderburn, 1972; Madsen & Thyregod, 2011). Due to the strong overdispersion in the dataset, a negative binomial distribution was applied. Two factors were considered as explanatory variables in the analysis: the applied dose (0, 16, 24, 32 and 40 Gy) and the time elapsed from the oviposition up to the irradiation treatment (24 and 48 hours). A model with a full factorial design was chosen. The dose Gy wass treated as a covariate in the model. A minimum of six repetitions were performed. Dunn’s test with Bonferroni correction was used as a post hoc test.

Male Dose Response: In the case of irradiated adults the same response variables were analyzed, and a simple GLM model with a negative binomial distribution and one factor as an explanatory variable (the dose Gy at 0, 24 and 50 Gy) was applied. In the case of the tests with irradiated adults, 10 repetitions for both 0 and 24 Gy doses were carried out, whereas 100 repetitions were made for the 50 Gy dose, to more precisely estimate the frequency of eggs eclosion and the survival of the juvenile forms when these values approached zero. Dunn’s test with Bonferroni correction was used as a post hoc test.

Evaluation of sterile eggs as an oviposition substrate: data analysis and plotting were performed with R version 4.2.2 (R Core Team, 2022) and ggplot2 (Wickam, 2016). Kruskal-Wallis test and Dunn post hoc test with Bonferroni correction were used to compare the proportion of emergence, sex ratio, female dry weight, the longevity of emerged females and fecundity (at 0 and 12 days) for the three treatments (I, R, S), according to the age of the exposed egg masses (1, 4, 7, 10, 15 or 20 days).

To determine if the interaction between treatment and egg mass age was significant, we used generalized additive models (GAMs), considering the non-linearity of the data (Wood, 2011). For proportion of emergence and sex ratio, a binomial error distribution was applied, while the remaining parameters were analysed with a Poisson error distribution. The Treatment (I, R or S) was modelled as a factor (nominal variable), while a smoothing function was used for the egg mass age.

4.3 Results

4.3.1 Egg Dose Response

According to the GLM (Table 4.1), a strong negative effect of gamma irradiation was observed both on the development of the BMSB embryo and on the number of eggs hatching. In fact, only a few eggs hatched already starting from the lowest dose of irradiation of 16 Gy (Table 4.2). As regards the egg aging factor (24 or 48 hours from oviposition), a statistically significant interaction was observed (Table 4.2), suggesting a differential effect of the irradiation on the eggs hatching depending on the timing of treatment. No egg hatch was observed when the irradiation was performed within the first 24 h after oviposition, while sporadic cases of eggs hatching were observed at 48 hours up to 32 Gy (Table 4.2).

Table 4.1 Effects of gamma irradiation on the *Halyomorpha halys* egg masses at 24 and 48 hours after egg collection.

	n. of Visible Embryos		**n. of Hatched Eggs**	
	χ^2	p-value	χ^2	p-value
Intercept	46.186	1.1×10^{-11}	67.274	2.4×10^{-16}
Gy	28.514	9.3×10^{-8}	44.762	2.2×10^{-11}
hours	0.641	0.423	0.091	0.763
Gy x hours	0.480	0.488	7.662	$5.6\ ^{\mathrm{X}}\ 10^{-3}$

Table 4.2. Effects of gamma irradiation on *Halyomorpha halys* egg masses irradiated at 24 h and 48 h after egg collection. The data refer to a period of 6–8 days after oviposition, when virtually all eggs were hatched, and only the first instar nymphs that were still alive were considered in the eggs hatched.

Dose (Gy)	**Egg Age (hours)**	***n***	**Immature Embryos**		**Eggs Hatched**	
			(n.) [1]	**(%) [1]**	**(n.) [1]**	**(%) [1]**
0	24	10	25.67 ± 1.48 **a**	91.67 ± 5.29 **a**	21.80 ± 2.74 **a**	77.86 ± 9.80 **a**
0	48	6	26.00 ± 1.53 **a**	92.86 ± 5.46 **a**	24.67 ± 1.86 **a**	88.10 ± 6.63 **a**
16	24	12	9.77 ± 2.58 **b**	30.95 ± 5.83 **b**	0.00 **b**	0.00 b
16	48	10	7.00 ± 3.69 **bc**	25.00 ± 13.17 **bc**	0.60 ± 0.43 **b**	2.14 ± 1.52 **b**
24	24	14	8.50 ± 3.11 **b**	30.36 ± 11.10 **b**	0.00 b	0.00 b
24	48	10	0.00 **c**	0.00 **c**	1.50 ± 1.50 **b**	5.36 ± 5.36 **b**
32	24	12	0.00 **c**	0.00 **c**	0.00 **b**	0.00 b
32	48	24	2.79 ± 1.56 **c**	9.97 ± 5.56 **c**	2.04 ± 1.39 **b**	9.07 ± 5.20 **b**
40	24	10	0.00 **c**	0.00 **c**	0.00 **b**	0.00 **b**
40	48	10	0.00 **c**	0.00 **c**	0.00 **b**	0.00 **b**

[1] Mean ± se; different letters indicate significant differences ($p < 0.05$) in the same column, according to a Dunn's test with Bonferroni correction.

4.3.2. Male Dose Response

The effect of irradiation on the adult males and their progeny was analysed at 24 and 50 Gy in comparison to the untreated control. The effect of irradiation was strong on eggs hatching according to the GLM ($\chi2$ = 412.3, df = 1, p < 0.001) as well as on immature embryos formation ($\chi2$ = 421.2, df = 1 p < 0.001) and very low values of eggs hatching were detected at 50 Gy (0.81% ± 0.21 in comparison to the 92.1% ± 4.39 of the untreated control) (Table 4.3), while at the 24 Gy dose percentage of eggs hatched was intermediate (23.47 ± 6.1) between the untreated control and 50 Gy, and significantly differing from the 0 and 50 Gy doses according to the Dunn's test.

Table 4.3 Immature embryos (visible embryos after oviposition) and eggs hatched after 6-8 days from the oviposition of SIT eggs.

Dose (Gy)	***n***	**Immature Embryos**		**Eggs Hatched**	
		n. [1]	**%** [1]	**n.** [1]	**%** [1]
0	10	26.50 ± 0.582 **a**	95.96 ± 1.63 **a**	25.50 ± 1.327 **a**	92.14 ± 4.39 **a**
24	10	8.83 ± 1.662 **b**	31.55 ± 5.93 **b**	6.57 ± 1.702 **b**	23.47 ± 6.08 **b**
50	100	1.08 ± 0.176 **c**	3.86 ± 0.63 **c**	0.23 ± 0.060 **c**	0.81 ± 0.21 **c**

[1] Mean ± se; different letters indicate significant differences ($p < 0.05$) in the same column according to Dunn's test with Bonferroni correction.

4.3.3. Evaluation of Sterile Eggs as an Oviposition Substrate

The proportion of emergence (%) of parasitoids had quite different trends for the three egg types (Figure 4.1; Table 4.4). One-day-old SIT eggs showed values above 90% and continued with similar values at 4, 7 and 10 days of eggs age; only at 15 and 20 days, the proportion of emerged insects dropped to 83.12% and 75.56%, respectively. In contrast, irradiated and refrigerated eggs had lower values for 1-day-old eggs (84.28% and 77.85%), while values raised at 4 days (91.39% and 90.66%) and remained at similar values even at 7 days (88.86% and 88.58%), and did not differ statistically significantly from SIT eggs of 4 and 7 days. From 10 to 20 days of eggs age, the proportion of emergence of irradiated and refrigerated eggs dropped significantly compared to SIT eggs.

The sex ratio, expressed as the percentage of emerged females on the total number of emerged parasitoids (Table 4), was significantly higher in 1-day-old SIT eggs (88.34%) compared to irradiated (84.28%) and refrigerated eggs (78.48). For 4- and 7-days aged eggs, the parasitoid emergence rate was similar for all three types of eggs (values variable between 81.45% and 89.25%), while dropped significantly in 10-day-old eggs for irradiated (61.79%) and refrigerated eggs (73.82%), with respect to SIT eggs (91.95%). With the increase of the age of BMSB eggs, the decline in the percentage of emerging parasitoid females was stronger for irradiated and refrigerated eggs than for SIT eggs. Indeed for 20-day-old eggs, the emergence rate of *T. japonicus* females was 40.55% for irradiated, 39.67% for refrigerated, and 73.45% for SIT eggs (Table 4.4)

Possible sub-lethal effects on the progeny of *T. japonicus* due to the larval development in sterile eggs were evaluated by measuring fecundity, dry weight, and longevity in the emerged parasitoids.

The fecundity at initial egg load (Fo) did not differ among the three typologies of eggs 1, 4, and 10 days old, whereas at 7, 15, and 20 days was significantly lower for irradiated eggs than refrigerated and SIT (Table 4.4). On the other hand, the fecundity at maximum egg load (F12) was constant for SIT eggs which showed in the complex the highest values considering all the ages of the eggs, while were quite variable for the other two types of eggs (Table 4.4).

The dry weight was even constant for SIT eggs which showed the highest values for each age of the eggs. The other two types of eggs showed highly variable values among the different ages, sometimes similar to SIT eggs and sometimes significant different (Table 4.4).

Regarding longevity (expressed in days), the only statistically significant differences were observed in the progeny from eggs of 7 and 20 days old. In both cases, the highest values were observed for irradiated eggs (Table 4.4).

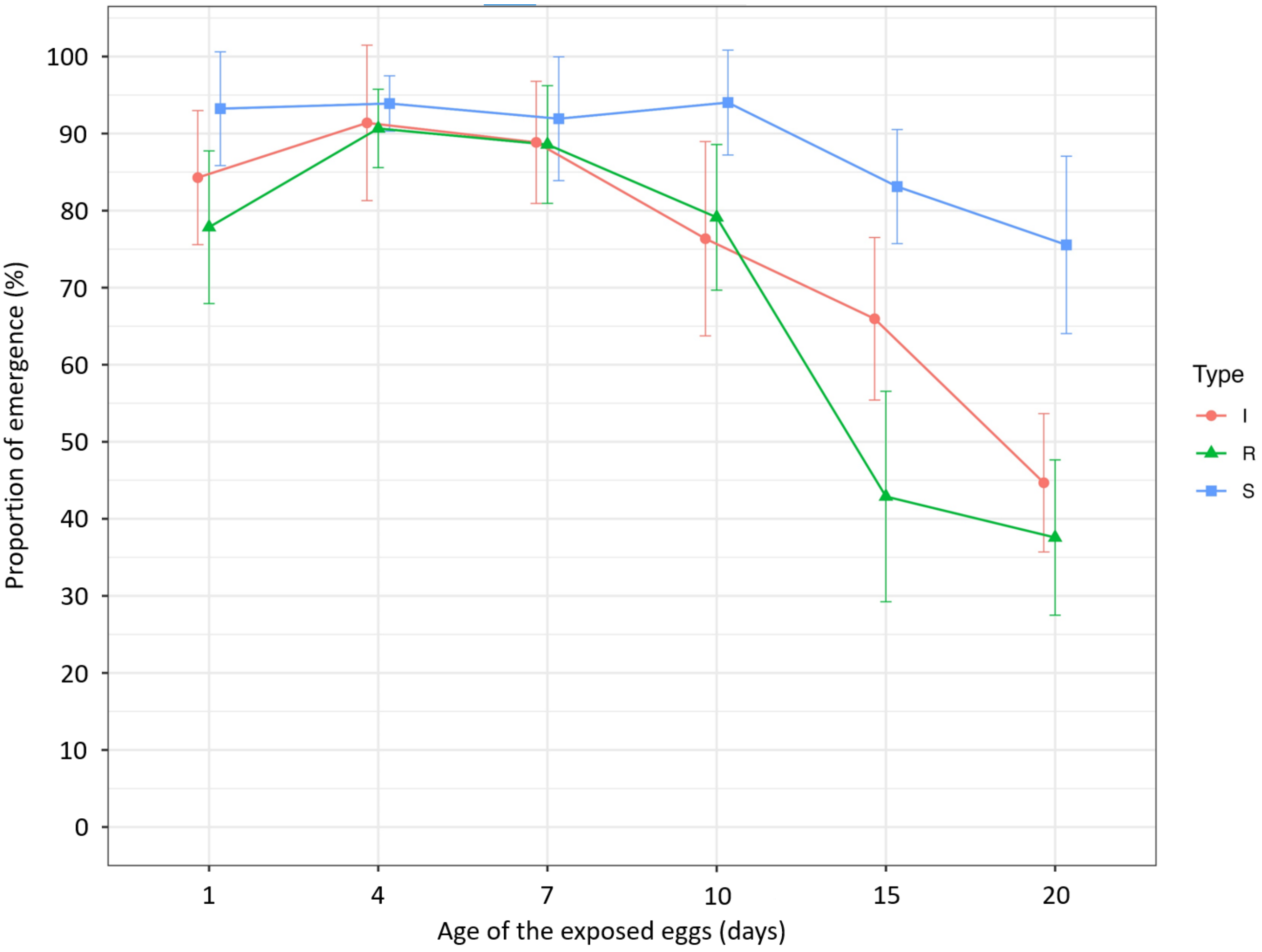

Figure 4.1. Average (± 95% confidence interval) proportion of emergence (%) for the egg mass type (I, Irradiated; R, Refrigerated; S, SIT) at different egg age.

Table 4.4. Mean and standard deviation for the different parameters, according to the age of the exposed egg masses. Significant differences verified with Kruskal–Wallis test (H) and post hoc Dunn's test with Bonferroni correction, where letters indicate significant differences ($p < 0.05$).

Parameter	**Age (days)**	**H**	**df**	**P-value**	**Irradiated**	**Refrigerated**	**SIT**
					Mean ± sd	**Mean ± sd**	**Mean ± sd**
Proportion of emergence	1	17.591	2	<0.001	84.28 ± 27.9 **b**	77.85 ± 27 **c**	93.23 ± 20.12 **a**
	4	4.464	2	0.107	91.39 ± 25.49	90.66 ± 14.58	93.90 ± 9.45
	7	5.777	2	0.056	88.86 ± 21.23	88.58 ± 20.84	91.93 ± 23.4
	10	15.244	2	<0.001	76.36 ± 34.38 **b**	79.13 ± 27.51 **b**	94.03 ± 18.23 **a**
	15	19.226	2	<0.001	65.96 ± 32.1 **b**	42.9 ± 35.9 **c**	83.12 ± 19.82 **a**
	20	25.284	2	<0.001	44.68 ± 24.9 **b**	37.57 ± 27.49 **b**	75.56 ± 30.25 **a**
Sex ratio	1	13.791	2	0.001	81.66 ± 24.06 **b**	78.48 ± 18.15 **c**	88.34 ± 19.95 **a**
	4	2.460	2	0.292	84.20 ± 25.95	81.45 ± 25.15	89.25 ± 10.45
	7	7.664	2	0.022	84.11 ± 20.44	84.15 ± 17.19	87.61 ± 22.79
	10	19.095	2	<0.001	61.79 ± 38.59 **c**	73.82 ± 26.47 **b**	91.95 ± 5.28 **a**
	15	4.210	2	0.122	60.37 ± 31.42 **b**	53.85 ± 28.8 **b**	68.56 ± 32.45 **a**
	20	24.839	2	<0.001	40.55 ± 21.95 **b**	39.67 ± 23.26 **b**	73.45 ± 26.61 **a**
Fecundity - 0 days	1	3.748	2	0.154	28.82 ± 4.19	26.14 ± 1.66	26.58 ± 3.36
	4	1.851	2	0.396	27.7 ± 2.99	26.31 ± 2.92	27.12 ± 3.60
	7	7.726	2	0.021	23.5 ± 5.43 **b**	28.95 ± 3.33 **a**	28.15 ± 3.52 **a**
	10	3.520	2	0.172	28.5 ± 3.03	26.48 ± 3.65	28.78 ± 2.97
	15	12.207	2	0.002	23.59 ± 4.58 **b**	26.36 ± 1.96 **ab**	28.35 ± 2.31 **a**
	20	24.363	2	<0.001	22.43 ± 1.79 **b**	27.47 ± 2.07 **a**	26.75 ± 2.63 **a**
Fecundity - 12 days	1	2.283	2	0.319	53.78 ± 3.38	55.78 ± 3.28	55.21 ± 4.24
	4	2.556	2	0.279	53.89 ± 4.19 **b**	56.33 ± 3.2 **a**	55.96 ± 4.28 **ab**
	7	16.595	2	<0.001	49.5 ± 3.67 **b**	53.63 ± 3.52 **b**	56.29 ± 3.11 **a**
	10	2.492	2	0.288	53.06 ± 3.25	52.78 ± 3.08	55 ± 4.22
	15	17.751	2	<0.001	49 ± 3.14 **b**	53.75 ± 3.72 **a**	56.63 ± 3.89 **a**
	20	5.646	2	0.059	50.83 ± 1.8 **ab**	46.53 ± 8.64 **b**	53.25 ± 3.05 **a**
Dry weight	1	7.499	2	0.024	116.57 ± 11.98 **a**	108 ± 5.87 **b**	115.98 ± 8.04 **a**
	4	10.717	2	0.005	107.59 ± 5.65 **b**	113.41 ± 6.22 **a**	112.65 ± 8.96 **a**
	7	14.239	2	0.001	107.87 ± 7.57 **b**	111.65 ± 4.51 **ab**	115.68 ± 5.89 **a**
	10	7.715	2	0.021	108.54 ± 7.56 **ab**	107.26 ± 9.6 **b**	113.6 ± 8.12 **a**
	15	21.866	2	<0.001	101.57 ± 7.77 **b**	115.25 ± 13.13 **a**	112.12 ± 7.85 **a**
	20	14.879	2	0.001	104.57 ± 8.65 **b**	106.51 ± 4.83 **b**	112.64 ± 5.23 **a**
Longevity	1	2.898	2	0.235	115.75 ± 44.54	93.41 ± 55.16	106.2 ± 44.26
	4	1.504	2	0.471	109 ± 48.67	97.84 ± 39.43	96.21 ± 43.19
	7	6.399	2	0.041	133.64 ± 36.1 **a**	121.67 ± 39.51 **ab**	105.5 ± 49.97 **b**
	10	1.539	2	0.463	107.67 ± 53.8	107.35 ± 34.85	116.24 ± 40.19
	15	4.877	2	0.087	104.9 ± 39.58	74.58 ± 50.52	101.65 ± 39.3
	20	11.127	2	0.004	120.46 ± 41.53 **a**	78.9 ± 45.54 **b**	103.22 ± 43.8 **ab**

The estimated smoothing curves for the GAMs and factor variables (Figure 4.2) supported a non-linear relationship between the recorded parameters and the age of the egg mass, which varied with the treatment. A backwards selection using the Akaike Information Criteria indicated that no terms should be dropped from the models. According to egg mass type, the analysis confirmed a significant influence of the age of the eggs for the parameters considered (Table 4.5).

Regarding the proportion of emergence (%) of parasitoids, SIT eggs showed an initial plateau, and no significant decrease was observed for up to 10 days. On the other hand, for irradiated and refrigerated eggs, the proportion of emergence was inversely proportional to the percentage of hatching immediately and increased on the fifth day when the highest proportion of emergence was recorded. After that, it decreased significantly (Figure 4.2 a; Table 4.5).

The sex ratio (%) was also influenced by the age of the eggs (Table 4.5). The decrease of the proportion of females was more influenced by the egg age for irradiated and SIT eggs than for refrigerated eggs (Figure 4.2 b). Specifically, a significant decline in sex ratio was observed after 5 days for irradiated eggs and after 10 days for SIT eggs, while for refrigerated eggs the influence of the egg age was less significant compared to the other two types of eggs (Table 4.5). The pattern observed in refrigerated eggs exhibited considerable variability, with a decline at 5 days followed by a subsequent increase.

The fecundity F0 was strongly influenced by the age of the eggs for irradiated eggs. The same influence was slightly significant for SIT eggs, while was not significant for refrigerated (Figure 4.2 c; Table 4.5).

The fecundity F12 was significantly influenced by the egg age for irradiated and refrigerated eggs, declining as eggs were older. The SIT eggs were not influenced by egg age for this parameter

(Figure 4.2 d; Table 4.5); indeed, the load of eggs was constant independently of the age of the eggs (Table 4.4).

The dry weight declined significantly for irradiated and refrigerated eggs influenced by egg age. The strongest decline was observed for irradiated eggs already at 5 days, while SIT eggs were not influenced by egg age and showed a linear trend (Figure 4.2 e; Table 4.5).

Longevity was not affected by the egg age for irradiated and SIT eggs, which both showed a linear trend, while it decreases significantly for the refrigerated eggs (Figure 4.2 f; Table 4.5).

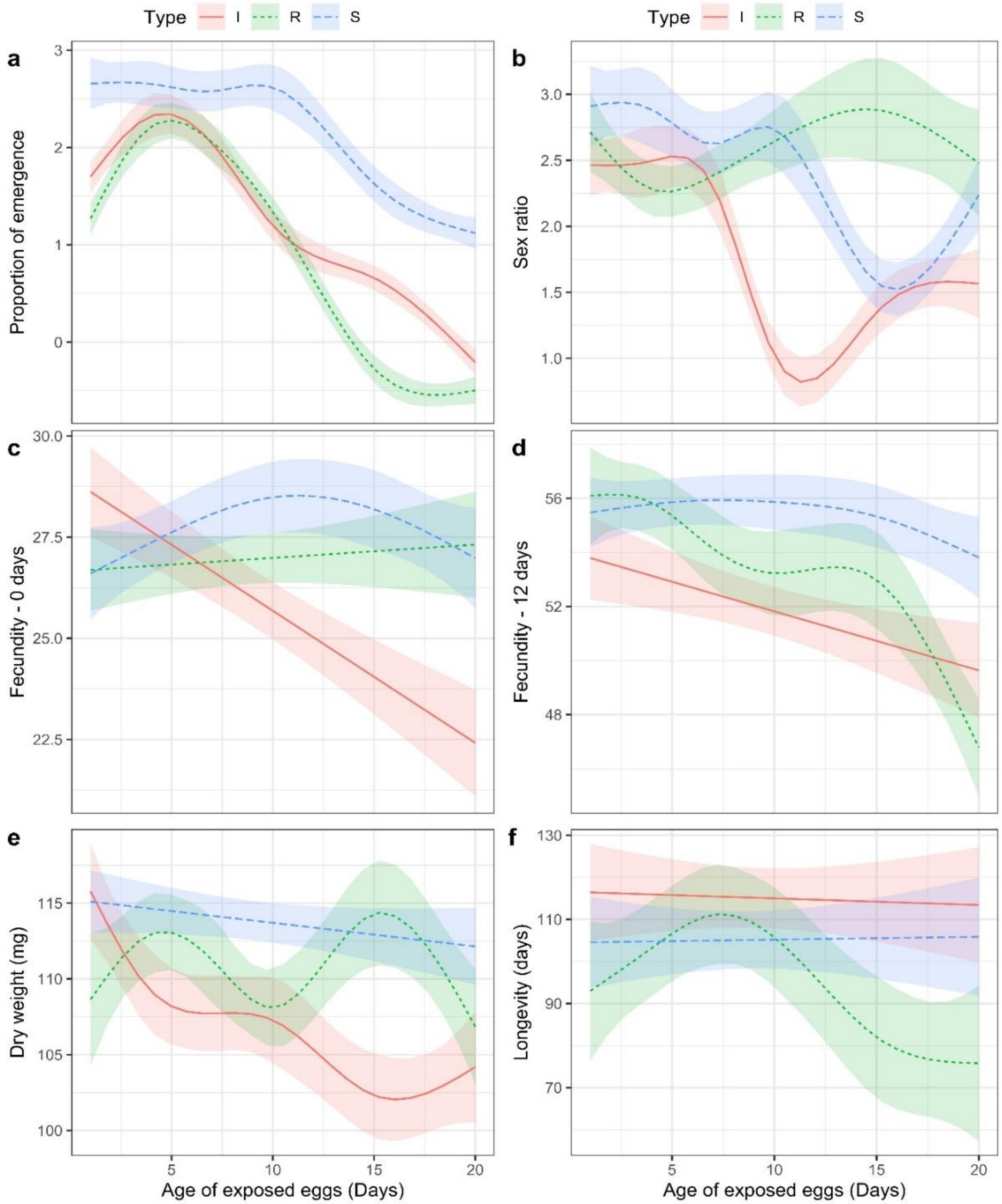

Figure 4.2. Figure Estimated smooth functions for the generalized additive models (GAMs), according to egg mass type (I, Irradiated; R, Refrigerated; S, SIT). Smoothing parameters estimated using restricted maximum likelihood (REML).

Table 4.5. Summary table of GAMs results. Egg mass type: irradiated (I), refrigerated (R), and SIT (S).

Model	Adjusted R^2	Deviance explained	Component	Term	Estimate	Std Error	t-value	p-value
Proportion of emergence	30.70%	29%	A. parametric coefficients	(Intercept)	1.290	0.037	34.993	<0.001
				Type R	-0.272	0.052	-5.265	<0.001
				Type S	0.927	0.062	14.863	<0.001
			B. smooth terms	s(Day):Type I	4.536	4.867	625.785	<0.001
				s(Day):Type R	4.655	4.926	926.123	<0.001
				s(Day):Type S	4.005	4.521	212.335	<0.001
Sex ratio	6.93%	14%	A. parametric coefficients	(Intercept)	1.886	0.049	38.636	<0.001
				Type R	0.678	0.085	8.010	<0.001
				Type S	0.630	0.077	8.224	<0.001
			B. smooth terms	s(Day):Type I	4.731	4.955	169.552	<0.001
				s(Day):Type R	3.644	4.293	11.964	0.025
				s(Day):Type S	4.479	4.852	96.429	<0.001
Fecundity - 0 days	13.70%	15%	A. parametric coefficients	(Intercept)	25.966	0.355	73.043	<0.001
				Type R	0.992	0.470	2.109	0.036
				Type S	1.652	0.448	3.684	<0.001
			B. smooth terms	s(Day):Type I	1.001	1.001	38.540	<0.001
				s(Day):Type R	1.000	1.000	0.383	0.536
				s(Day):Type S	2.347	2.872	2.438	0.057
Fecundity - 12 days	26.40%	28%	A. parametric coefficients	(Intercept)	52.019	0.457	113.917	<0.001
				Type R	1.317	0.592	2.224	0.027
				Type S	3.373	0.565	5.972	<0.001
			B. smooth terms	s(Day):Type I	1.003	1.006	8.767	0.003
				s(Day):Type R	3.624	4.224	17.204	<0.001
				s(Day):Type S	2.073	2.540	1.984	0.102
Dry weight	19.50%	22%	A. parametric coefficients	(Intercept)	107.723	0.687	156.807	<0.001
				Type R	2.764	0.993	2.783	0.006
				Type S	6.092	0.951	6.405	<0.001
			B. smooth terms	s(Day):Type I	3.689	4.275	10.147	<0.001
				s(Day):Type R	4.209	4.705	3.543	0.004
				s(Day):Type S	1.000	1.000	2.335	0.127
Longevity	4.59%	6%	A. parametric coefficients	(Intercept)	115.147	3.612	31.875	<0.001
				Type R	-19.087	5.082	-3.755	<0.001
				Type S	-10.033	4.999	-2.007	0.045
			B. smooth terms	s(Day):Type I	1.000	1.001	0.079	0.779
				s(Day):Type R	2.995	3.613	3.946	0.009
				s(Day):Type S	1.003	1.006	0.015	0.913

4.4 Discussion

An effective pest management program based on CBC requires to release of an appropriate number of parasitoids (or other natural enemies) suitable for the establishment of a self-sustaining population of the biocontrol agent while reducing the population of the target pest (Van Driesche & Bellows, 2012).

Sterile eggs laid by wild females after mating with irradiated males can play an important role in the management of a BMSB population, either to facilitate and accelerate the natural multiplication of the released oophagous biocontrol agent or as sentinel eggs for field monitoring.

In this perspective, also sterile eggs obtained by irradiation of fresh eggs could play a role in multiplication and field monitoring of the egg parasitoids of the target species.

However, until now BMSB as well all the pentatomid bug species are not considered eligible for the classic SIT approach, because so far mass rearing studies were mainly addressed to holometabolous insect orders, such as Diptera and Lepidoptera. Moreover, sterile BMSB adults, since damage on plants is caused by the feeding activity mediated by the piercing-sucking mouthparts, can cause unsuitable damage on cultivated fruits and vegetables. Given the good results in all parameters evaluated, the setting up of a small-scale SIT laboratory colony of BMSB to produce large number SIT eggs could be considered a suitable approach for pre-release parasitoid multiplication or their field monitoring. Cristofaro et al. (2022) suggested the use of sterile eggs by SIT as a new concept of “sterile sentinel eggs” in which SIT eggs should be more attractive than frozen eggs because they are less manipulated. This concept could explain the difference observed between SIT eggs and the other two types of egg in this study for which some manipulations (irradiation or refrigeration) were required. Indeed, since egg parasitoid females are using kairomone for short range host locations

(Malek et al., 2021; Scala et al., 2022), direct irradiation and refrigeration of the eggs could affect chemical traces, ‘footprints’, released by BMSB females. Semiochemical interactions between sterile eggs (SIT, irradiated, and refrigerate) and egg parasitoids need further investigation.

Nonetheless, if well-managed, refrigerated and unfertilized eggs (produced by unmated females), can be successfully used for field monitoring and rearing colonies (Yang et al., 2018; Wong et al., 2021). Indeed, refrigerated eggs are easier to obtain and store for relatively long periods (two months in a refrigerator at 8 °C) before the emergence of *T. japonicus* significantly decrease (Wong et al., 2021). In our experiment, the data showed that during the first week the performance of BMSB refrigerated eggs as a suitable substrate for *T. japonicus* oviposition and larval development, was similar to SIT eggs, confirming the data reported by Wong et al. (2021) (Figure 4.1, Table 4.4). On the other hand, unfertilized eggs showed good parameters for both parasitization and larval development of *T. japonicus* up to 11 days (Yang et al., 2018). In this respect, irradiated eggs, which showed poorer performance than SIT eggs and no better than refrigerated eggs (for most of the parameters evaluated), are not to be recommended for monitoring; considering that a BMSB female may produce multiple egg masses during her lifetime (Nielsen et al., 2008; Costi et al., 2017; Yang et al., 2018) and that the production of SIT eggs or other abovementioned alternatives are less time-consuming and cheaper.

The results from the screening to assess egg sterility of BMSB by irradiation of fresh eggs (maximum 48 hours), showed that no eggs hatched when the irradiation was performed after 24 hours, while sporadic cases of egg eclosion were observed at 48 hours up to 32 Gy (Table 4.2). However, in many cases we observed immature and not malformed embryos, which are not a good substrate for the parasitoid larval development (Qiu et al., 2007) (Table 4.2), particularly at 16 Gy (30.95 % at 24 h and 25 % at 48 h) and at 24 Gy (30.36% at 24 h, and then 0% at 48 h). The 40 Gy dose did not show any egg hatching or development of embryos, thus was chosen as the optimal dose for the exposure to *T. japonicus* and probably suitable for the egg sentinel technique.

For the male irradiation response, at 50 Gy immature embryo formation was sporadic (3.86%) compared to 24 Gy (31.55%) and the percentage of eggs hatched was 0.81% while was 23.47% at 24 Gy (Table 4.3), indicating that 50 Gy was a reliable oviposition substrate for egg parasitoids and potentially suitable for the egg sentinel technique. Even if few SIT eggs hatched (at 50 Gy), as reported in other studies on BMSB irradiation, high mortality persisted in the progeny of irradiated males (Welch et al., 2017; Nguyen et al., 2021; Roselli et al., 2023) and cumulative sterility was observed when individuals born from not completely sterile egg masses reach the adult stage (Welch et al., 2017; Nguyen et al., 2021). Consequently, the poor hatching rate observed at 50 Gy does not represent a problem for applying SIT, which can be applied despite the partial sterility (Klassen, 1971), in our work, over 99%.

The exposure of BMSB sterile egg masses at different ages to *T. japonicus* showed that the SIT eggs remained a suitable substrate for oviposition for up to 20 days under the standard experimental conditions (L:D = 16:8, T = 20 °C, RH = 60%) and the subsequent conditions adopted for larval development (L:D =16:8; T = 24 °C; RH = 60%) (Figure 4.1; Table 4). On the other hand, irradiated and refrigerated eggs showed a higher proportion of emergence in 4-days-old eggs compared to 1-day-old, contrary to the expectation (Figure 4.1; Table 4). Probably the exposure to the preoviposition conditions of the eggs is responsible of these data. Further investigations are needed to establish the mechanism behind this. Moreover, none of the SIT eggs hatched or developed embryos during exposure at 20 °C constant temperature before exposition to *T. japonicus*, while, partially occurred at 27° C (Table 4.3). These data is also unexpected, considering that Nielsen et al. (2008) reported an average development time of 11.5 days at constant 20 °C eggs exposure with only 1 %

egg mortality. The data suggested that some possible developmental inhibition of *H. halys* embryos could occur in SIT eggs at 20 °C, with complete sterility as a result.

In SIT eggs the sex ratio of the progeny was slightly influenced by the age of the eggs compared to irradiated and refrigerated eggs (Table 4.4), suggesting that SIT eggs remain a better substrate for the larval development of *T. japonicus* than the other two types of eggs.

However, the influence of the egg age on the reduction of the emerging females (%) was statistically significant (Figure 4.2; Table 4.5). Fecundity (both F0 and F12) and dry weight confirmed that SIT eggs were in general less influenced by egg age (Table 4.5) and showed the highest value of egg load and dry weight among the treatments, especially when the egg age was more than 10 days (Table 4.4). The egg age did not also influence the longevity of the progeny emerged from SIT and irradiated eggs, while the progeny from refrigerated eggs was significantly influenced (Table 4.5). Particularly parasitoids developed in 20-days-old, refrigerated eggs, showed a significant reduction in longevity (Table 4.4).

The differences observed among treatments could depend also on the different nutrient content of the eggs. Skillman et al. (2017) reported alterations in nutrient content related to the length of time that the eggs were frozen. In a similar way, the nutrient content of irradiated and refrigerated eggs could be altered by the process of sterilization, differently by SIT eggs in which the male was irradiated, but the eggs were not exposed to direct γ irradiation or refrigeration. Nonetheless, our data confirmed also that refrigerated eggs are a good substrate for oviposition and larval development of *T. japonicus* up to 7 days without negative effects on the parameters evaluated, even if the proportion of emergence and sex ratio in one-day-old eggs was higher in 4- and 7-days-old eggs. The decreasing female offspring (sex ratio) observed in our experiment is coherent with the data reported on unfertilized eggs (Yang et al., 2020), showing a significant decrease in terms of sex ratio when exposed to seven day or older host-egg age. This highlighted a loss of quality as oviposition substrate for all the types of eggs we tested when the egg age increases, similar to which reported for fresh eggs (Qiu et al., 2017).

In a previous work (Roselli et al., 2023), we assessed the suitability of overwintering males for SIT as a potential use of irradiated wild males in the IPM strategy. To implement an AW-IPM or eradication program for managing BMSB in its invasive range, we considered the complementary effects that can be achieved through the combination of SIT and classical biological control methods (Knipling, 1979; Liebhold et al., 2016). In addition to reducing the population density of the target species as a suppression method (Klassen & Curtis, 2021), the outcome of SIT approach - sterile eggs laid by wild females mated with irradiated males - could provide a valid oviposition substrate useful to enhance the establishment and the multiplication of the selected egg parasitoids. In addition, sterile eggs derived by SIT could have an alternative role in their field monitoring or to support rearing colonies.

A complementary effect deriving by the combined use of SIT and biological control (Gurr & Kvedaras, 2010; Suckling et al., 2012) was considered for the suppression or eradication of a target species. In previous studies (Horrocks et al 2020; Horrock et al 2021), it was proposed the combined release of both sterile BMSB males and egg parasitoids as an alternative management strategy. However, this approach, even though it may be a more environmentally friendly solution (assuming there are no offspring from either the released target or from its parasitoid), does not provide the possibility for sterile eggs to multiply parasitoids. Indeed, as mentioned before, SIT eggs have the potential to be a suitable oviposition substrate for *T. japonicus* multiplication, available in the field after mating between sterile BMSB males with wild females. In this sense, the present study provides a base requirement to set up a potential control tactic that involves the use of SIT and biocontrol for

BMSB by releasing sterile BMSB males. Concerns about a long field exposition of SIT eggs remains in consideration of eggs predation. We expected that SIT eggs will be subjected to predation as well as the fresh and frozen sentinel eggs (Pezzini et al., 2018; Tillman et al., 2020). Although SIT eggs will be exposed to predation, they will be available to egg parasitoids as well for longer than fresh, frozen eggs, considering that fresh eggs hatch and frozen eggs showed a poorer performance as oviposition substrate compared to refrigerated eggs (Wong et al., 2021). Thus, the results obtained in this study can be considered a valuable starting point to implement a new strategy that combines SIT and biocontrol for BMSB. Nonetheless, field, and semi-field tests are needed to assess the real feasibility of this potential IPM strategy.

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CHAPTER 5. GENERAL DISCUSSION

This study aimed to provide new tools for managing BMSB that met environmental sustainability criteria and reduce consequently the impact of conventional control practices for this insect pest species. The need to find new control methods, was driven by the frequent use of broad-spectrum insecticides against BMSB (Leskey et al., 2012a; Leskey et al., 2012b), that not only represents a short-term strategy but also interferes with classical biological control, with negative effects on BCA (Lowenstein et al., 2019). On the other hand, the use of *T. japonicus*, the best candidate for biological control, applied alone, may not be sufficient for managing BMSB (Abram et al., 2020; Conti et al 2021). Therefore, it was essential to evaluate a strategy in which SIT was not an alternative tool but rather an integrative approach to implementing environmentally friendly practices.

In recent years, some authors have focused on evaluating the effects of irradiation on the physiology of newly emerged BMSB males (Welsh et al., 2017; Suckling et al., 2019) and fifth-instar nymphs (Nguyen et al., 2021). The competitive overflooding ratio has also been assessed, confirming that a ratio of sterile to fertile males of 5:1 result in good competitive performance by irradiated males to suppress BMSB (Suckling et al., 2019). The effects on the progeny of partially sterile males have also been evaluated, confirming high mortality and cumulative sterility (Welsh et al., 2017; Nguyen et al., 2021). However, a strategy that addresses the necessity to provide large numbers of insects for SIT application must be considered.

We have tried to meet this need, so the effectiveness of field collection of wild insects and their use in the application of SIT have been presented in Chapters 2 and 3.

In Chapter 2, a non-lethal trap-based approach was used, which also could have a high capture potential during pre-winter aggregation. These traps were much more effective than currently available sticky panels, capturing up to 15 times more adult BMSB with a mean improvement of about 7-fold. The concept of these traps could also be compatible with the requirements of eradicating a known and delimited population, avoiding the use of broad-spectrum insecticides and favouring the use of biological control. However, mass trapping would have costs for materials and labour (El-Sayed et al., 2006), which would be offset by the benefits of eradication (Brockerhoff et al., 2010).

The live traps developed in our work have introduced a mass trapping concept for BMSB (Anfora et al., 2019), which was previously mainly focused on insect monitoring and detection (Acebes-Doria et al., 2018; Anfora et al., 2019). Chen et al. (2020) modelled the pheromone plume in live traps (Suckling et al., 2019) comparing it with that dispersed by the A4-sticky-panel, observing that the live traps emit a narrower and more concentrated plume which seems to provide the bugs with a clearer path to follow. Starting from these models, traps are being developed that improve the design of live traps (Chen et al., 2020). Finally, the idea of using a wind vane proposed in the development of live traps was considered to increase aerodynamics and maximize lure dispersion of other trap designs in catching other insects (Welsh et al., 2022).

In Chapter 3, the concept of mass trapping was applied to overwintering individuals, followed by the evaluation of the effects of irradiation on the physiology of post-diapause male BMSB.

Wild overwintering males achieved a sterility level above 95% at a dose of 32 Gy without compromising parameters such as their longevity and the fecundity of the mated females. Although the sterility at this dose was not complete, it did not necessarily limit the use of SIT, especially in an integrated pest management (IPM) strategy (Klassen, 1971; Lance & McInnis, 2021).

Generally, SIT involves mass production of insects in artificial environments, followed by irradiation and shipment to distant facilities (Dowell et al., 2021). However, the difficulty of meeting the criteria for mass rearing (Lance & McInnis, 2021) and the need to irradiate highly mobile insects such as BMSB adults, led us to search for an alternative irradiation source that was more widely available. We identified a linear accelerator commonly used for human radiotherapy as an alternative to conventional radioactive cobalt sources, which are limited by safety, transport, and handling issues (Balestrino et al., 2016; Kutsaev et al., 2021; Wang et al., 2023). The introduction of additional control of unmanipulated and unirradiated insects demonstrated that the applied technique did not compromise or alter the physiological parameters of insects (except for fertility), allowing us to maintain good insect quality.

The irradiated insects needed to be able to successfully mate with wild females, as evaluated for species used in conventional SIT procedures (Parker et al., 2021). Therefore, the sexual behavior, which is a key parameter for SIT implementation (Hendrichs et al., 2002; Lance & McInnis, 2021), was evaluated in this study with wild BMSB males and females. The 32 Gy dose showed again the best results, showing mating times comparable with non-irradiated males under non-choice conditions.

When comparing all previous studies on the irradiation biology of BMSB (Welsh et al., 2017; Suckling ert al., 2019; Nguyen et al., 2021) and the present study, some differences were observed. Particularly in Welsh et al. (2017), the highest sterility was achieved at lower doses of irradiation. These differences could be due to several factors, including their provenance from different populations and their different phenological stage at the time of irradiation, or, as suggested by

Suckling et al. (2019), the different insect age and the differences in dosimetry among the irradiators. Furthermore, in the present work, for the first time, we used X-rays instead of gamma rays for BMSB irradiation, therefore, future investigations will be necessary to understand the differences observed and try to standardize the dose, timing and irradiation methods.

Trying to make a comparison with studies carried out on the irradiation biology of other pentatomid species, we realize that it is impossible to find a standard. If in this study the highest male radiation dose was 40 Gy, for a sterility of about 96%, in *N. viridula* a sterility of about 99% was already obtained at 16 Gy (Horrocks et al., 2020), while for *B. hilaris* 100 Gy was required to achieve complete sterility (Cristofaro et al., 2022).

Finally, in Chapter 4, the suitability of sterile eggs from BMSB obtained through SIT was evaluated as oviposition substrate for *T. japonicus* comparing them with refrigerated eggs which had shown good quality as sterile oviposition substrate (Wong et al., 2021). SIT eggs showed to be the best in all evaluated parameters, such as the proportion of emergence of *T. japonicus* and the sex ratio of the offspring. Furthermore, they also demonstrated the best results in parameters such as longevity, fecundity, and dry weight of emerged females, suggesting that SIT eggs are a suitable substrate for the development of *T. japonicus* while maintaining a significant difference compared to refrigerated eggs, especially when the eggs are over a week old and up to 20 days.

These findings suggest that a release of sterile males could potentially make SIT eggs available in the field after mating with wild females of BMSB. The eggs would theoretically remain a good substrate available for *T. japonicus* for a longer period compared to fresh eggs according to the developmental timeline of BMSB (Nielsen et al., 2008). Alternatively, SIT eggs can be considered for field monitoring of BMSB egg parasitoids in consideration of their high performance. A similar approach was already hypothesized for pentatomids, by Cristofaro et al. (2022), who suggested to produce sterile eggs of *B. hilaris* following a classic SIT approach in which sterile eggs obtained by mating between sterile males and fertile females could be exposed as sentinel eggs. The advantage of using a similar approach became fundamental in areas at risk of establishment, where the insect pest is not yet detected or in case of new colonization, preventing the accidental release of the pest using fertile eggs. The possibility of using SIT eggs for a long time in the field would allow the development of new strategies for monitoring and managing insect pest parasitoids. Sterile eggs of *N. viridula* and *B. hilaris* (Horrocks et al., 2020; Cristodaro et al., 2022) could be evaluated for a similar strategy that combine SIT and CBC.

Therefore, here we proposed an integrated control strategy based on the mass trapping of overwintering insects, their conservation under semi-natural conditions until spring, when they should be irradiated and released. This practice can be combined with classical biological control, which, for stink bugs (Abram et al., 2020), if not integrated into IPM tactics, hardly leads to complete control. Large-scale use of SIT for BMSB also remains unlikely. Indeed, SIT is most effective when used to suppress small pest populations to extremely low levels or even eliminate them from specific areas (Lance & McInnis, 2021).

Concerns about the release of harmful insects persist. However, it has been demonstrated on *Pistacia vera* (L.) (Sapindales: Anacardiaceae) that feeding damage caused by males is significantly lower than that caused by females (Lara et al., 2017). If similar data were recorded on other plant species of commercial interest, the use of SIT for BMSB would be more easily accepted by growers. In addition, the release of overwintering insects as we proposed, in case of consistent field damages could be somehow managed, considering that overwintering BMSB adults are easier to kill with conventional methods than F1 and F2 adults (Leskey et al., 2014). However, field, and semi-field tests are needed to evaluate the feasibility of the strategy proposed in this study.

In conclusion, our results provide the basic requirements for implementing novel IPM strategies in support of conventional control methods or biological control applied against BMSB, increasing the range of flexibility in choosing more sustainable management practices.

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CHAPTER 6. SATELLITE PROJECTS

THE COMPETITIVE MATING OF IRRADIATED BROWN MARMORATED STINK BUGS, *HALYOMORPHA HALYS*, FOR THE STERILE INSECT TECHNIQUE

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Abstract

The sterility of eggs and nymphs from gamma-irradiated male *Halyomorpha halys* was investigated to determine the potential for the sterile insect technique (SIT). Males irradiated at 0, 16, 24 and 32 Gy were placed with untreated virgin females and egg sterility was determined, showing 54.3 % at 16 Gy. The percentage of sterility from irradiation was 26 % lower than previous results from the USA and the variance was very high. Competitive overflooding ratio trials between irradiated virgin males and fertile virgin males at a 5:1 ratio resulted in the expected egg sterility, indicating competitive performance by irradiated males. By July and August, older, irradiated overwintered males were significantly less competitive than similar, non-irradiated males. There is a need to revisit the irradiation delivery method to achieve proper precision around the paternal dose required for an expected >80 % egg sterility and subsequent ~99 % endpoint sterility estimated at adult emergence in the F1 phase. These results suggest that the mating competitiveness and competency of males after irradiation at 16 Gy is not limiting to the sterile insect technique for suppression. A wild harvest of overwintering males using the aggregation pheromone, followed by irradiation and male release, might replace rearing. Mass-collected, sterilized bugs could be transported from an area of high *H. halys* density and shipped for release to enable suppression or eradication elsewhere. This concept is under development but further work is needed now to understand the difference in results between the US and Italian irradiators and increase the reliability of dosimetry.

Keywords: irradiation; stink bug; sterile insect technique; suppression; sterility; *Halyomorpha halys*; SIT; wild harvest

TRAPPING BROWN MARMORATED STINK BUGS: "THE NAZGÛL" LURE AND KILL NETS

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Abstract

Improvements to current brown marmorated stink bug (BMSB), *Halyomorpha halys*, surveillance and killing systems are needed to improve detection sensitivity and to reduce pesticide use. Detection of BMSB in New Zealand with traps is reliant on sticky panels with aggregation pheromone, which are low cost but inefficient compared with beating foliage. Trapping for BMSB adults and nymphs was conducted daily with lethal traps consisting of an aggregation pheromone-baited-coat hanger covered with dark-colored long-lasting insecticide-treated mesh, we termed "The Nazgûl", based on its sinister appearance. A deep tray lined with white plastic was attached centrally at the base for collecting the dead BMSB. The lethal traps killed and caught up to 3.5-fold more nymphs and adult BMSB than identically-baited sticky panels in the 3 weeks of deployment, and provided a snapshot of phenology by instar. We expect that lure-and-kill stations could contribute to the suppression of a delimited population and could be included as part of a semiochemical-based eradication program. Attracting and killing females and nymphs, thus removing future ffspring, could contribute to population suppression during an eradication.

Keywords: aggregation pheromone; *Halyomorpha halys*; trap; ghost trap; hedgerow; lure and kill