Universitat Autònoma de Barcelona

APPROACHES FOR THE BIOLOGICAL CONTROL OF STORED PRODUCT PESTS

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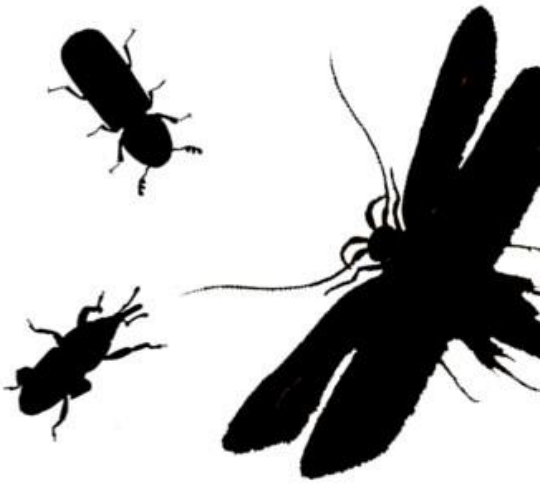

Doctoral thesis:

Approaches for the biological control of stored product pests

Mireia Solà Cassi

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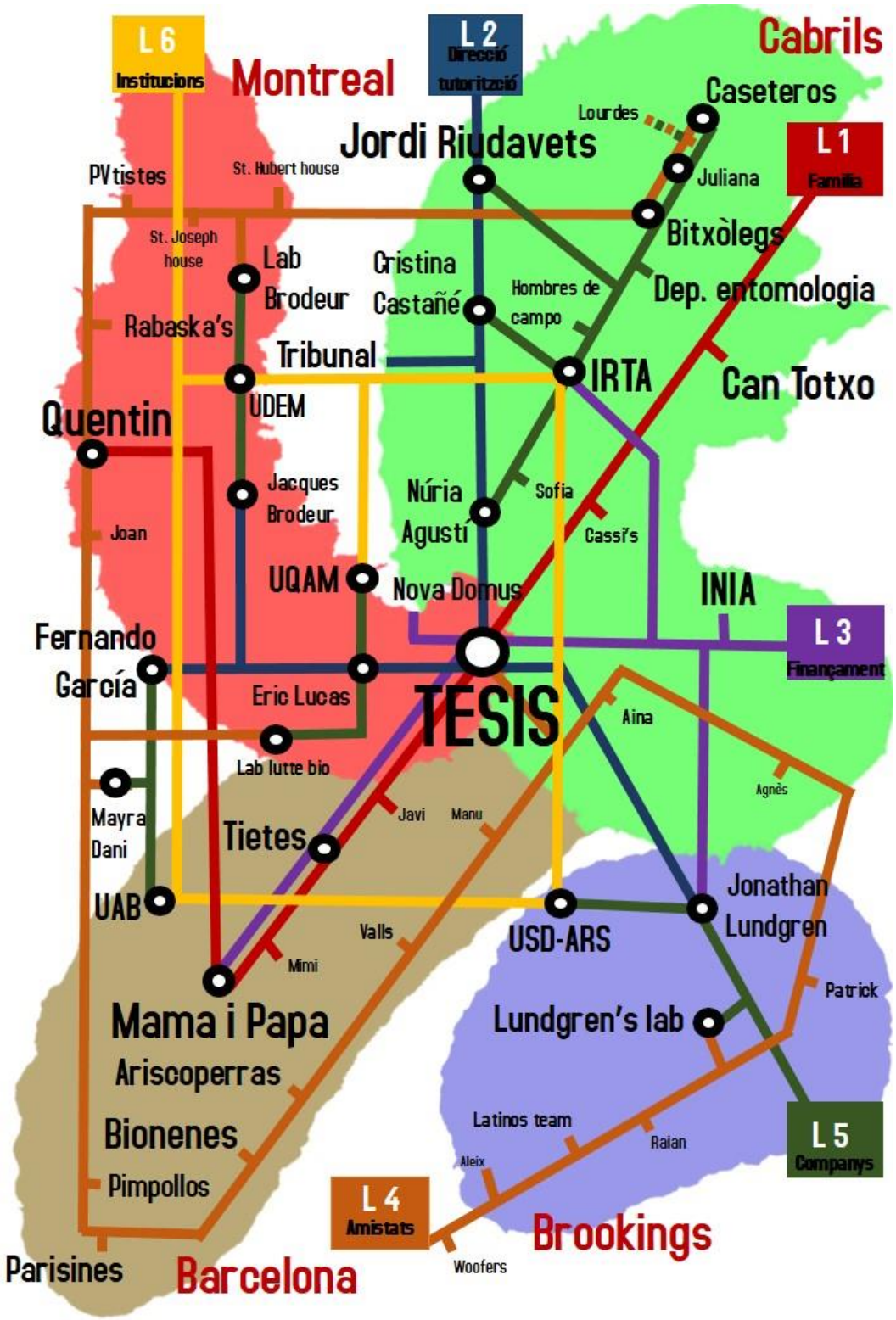
Perquè quan alguna cosa s’acaba n’hi ha una altra que comença...

A la familia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Abstract

ABSTRACT

Stored products include all postharvest agricultural foodstuffs that do not require refrigeration and that can be preserved for several months under proper conditions as cereal grain and other raw material or processed food.

Regrettably, in the Mediterranean region, the presence of insect pests such as the internal feeders of grain: *Rhyzopertha dominica* (F.) (Coleoptera: Bostrichidae), *Sitophilus* spp. (*S*. *granarius* (L.), *S*. *oryzae* (L.) and *S*. *zeamais* (Motschulsky)) (Coleoptera: Curculionidae) and *Sitotroga cerealella* (Olivier) (Lepidoptera: Gelechiidae) as well as the moth usually present in warehouses and grain mills, *Ephestia kuehniella* (Zeller) (Lepidoptera: Pyralidae), induce important quantitative and qualitative damage before consumption.

Among the integrated pest management strategies for the control of these concerning stored product pests, biological control with the introduction of parasitoids as natural enemies represents a good alternative to the use of pesticides. Unfortunately, for the correct implementation and success of this sustantaible approach, higher managmenet knowledge is required. For this reason, the aim of this thesis

was to assess different biological control approaches for the control of the most important stored product pests.

A major issue for the cereal industry is the early detection of insects during grain storage and processing, especially when immature stages of the pests are hidden inside the grain kernels, such as happens with the internal feeders. Then, the first two chapters of this thesis are dedicated to the development of two Polymerase Chain Reaction (PCR) methodologies for internal feeder's diagnosis. First, in chapter 1, a realtime PCR (qPCR) protocol for the detection and quantification of *R. dominica* in rice as a model system to quantify internal feeders in grain with a simple methodology is presented. For that purpose, a specific primer set was designed to amplify artificial infestations of this pest in rice. Then, using a regression model, a standard curve was generated that correlated individuals to adult equivalent DNA quantity (inverse of the C_t value). Results revealed that the designed primer set is specific for *R. dominica* and that the detection of its DNA was strongly associated with a given infestation size. The technique detected more than 73 % of the samples and presented high sensitivity (i.e. from 0.02 adults, 0.1 3rd instar to pupae or 13 egg to 2^{nd} instar detectable per kg of rice).

On the other side, in chapter 2, a multiplex PCR protocol for the simultaneous detection and identification of the five most prevalent

internal feeder pest species in different grains and processed food is described and then, also tested with commercial samples. Once the 5 pair of primers were designed and the optimal conditions for the reaction were set, it was demonstrated that the designed protocol can be used for the diagnosis of grain contamination with high sensitivity (0.1 pupa/ kilo of rice, except for *R. dominica:* 10 pupae/kilo) and especificitiy (51 species tested). Additionally, the technique can detect all developmental stages of *S. zeamais* in different kinds of grain and pasta even when the insects are dead.

For the effective use of natural enemies it is vital to understand and consider the interactions among physical, chemical and biological factors that take place when the grain is stored. For this reason, the third chapter of the thesis is focused on assessing the effectiveness of the parasitoid *Anisopteromalus calandrae* (Howard) (Hymenoptera: Pteromalidae) released in three different densities (10♀♀, 20♀♀ or 40♀♀ parasitoids) to control high infestations of the weevils *R. dominica* and *S. zeamais* in two kilos of rice under two risk temperatures (23ºC and 28ºC). Results showed that the effectiveness of control of the parasitoid increases with the increase of the number of parasitoids released and that the control is better at the highest tested temperature. Additionally, the suppression of both weevil's populations also leads to a reduction of rice

damage in terms of weight loss, frass production, or insect damaged kernels as well as in increase in mold presence.

The last chapter of the thesis is devoted to the optimization of an economic and easy to use device called Bankerbox for the control of the moth *E. kuehniella* by rearing and progressively releasing the parasitoid *Habrobracon hebetor* (Hüber) (Hymenoptera: Braconidae). In this perfectionated Bankerbox version, to avoid the risk of contamination of the stored products, *Galleria mellonella* (Lepidoptera: Pyralidae) larvae was chosen as host to rear the parasitoid. Then, three different treatments were tested, one with *E. kuehniella* 4^{th} instar larvae and two with *G. mellonella:* one containing 4^{th} instar larvae and the other with mixed larval stages (2^{nd} and 4^{th} instar larvae). Results showed that when the parasitoid was reared on *G. mellonella* in a mixture of two larval stages, the *H. hebetor* production was higher and the sex ratio of the parasitoids was female`s biased. The system released adult parasitoids over a period of approximately 25 days, allowing for their prolonged dispersion in the target location. Furthermore, 98% of the parasitoids left the rearing box and effectively attacked the *E. kuehniella* that was offered.

The research carried out in this thesis attends to increase the knowledge for the proper use of integrated pest management strategies

by providing feasible alternatives to the use of pesticides in the stored product industry for the control stored product pests.

Key words: Biological control, internal feeder, moth, parasitoid, PCR analysis, sampling, stored product

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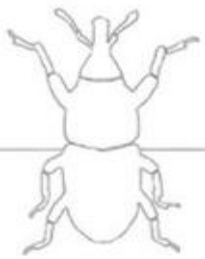
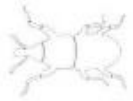


Introduction



INTRODUCTION

Stored products context and future trends in research of biological control by the introduction of parasitoids as natural enemies

1. Cereal losses

Cereals, with the increasing production of nearly 7 million tons in 2014, are the alimentary base for humans and livestock around the world (FAOSTATS, 2017). Particularly, rice, wheat, maize, millet, barley and rye are the dominant grain crops grown worldwide (Pimentel et al., 1997). However, according to the consumer or the region, there are specific differences in the preferred cereal. For example, wheat is the dominant crop supply in medium and high-income countries while rice is the preferred cereal in low-income regions, especially in the highly populated region of South and Southeast Asia (Gustavsson et al., 2011). Nevertheless, globally, before consumption, grain is transported, stored and often subject to an industrialized process to convert the raw material to a panoply of food products (Fig. 1).

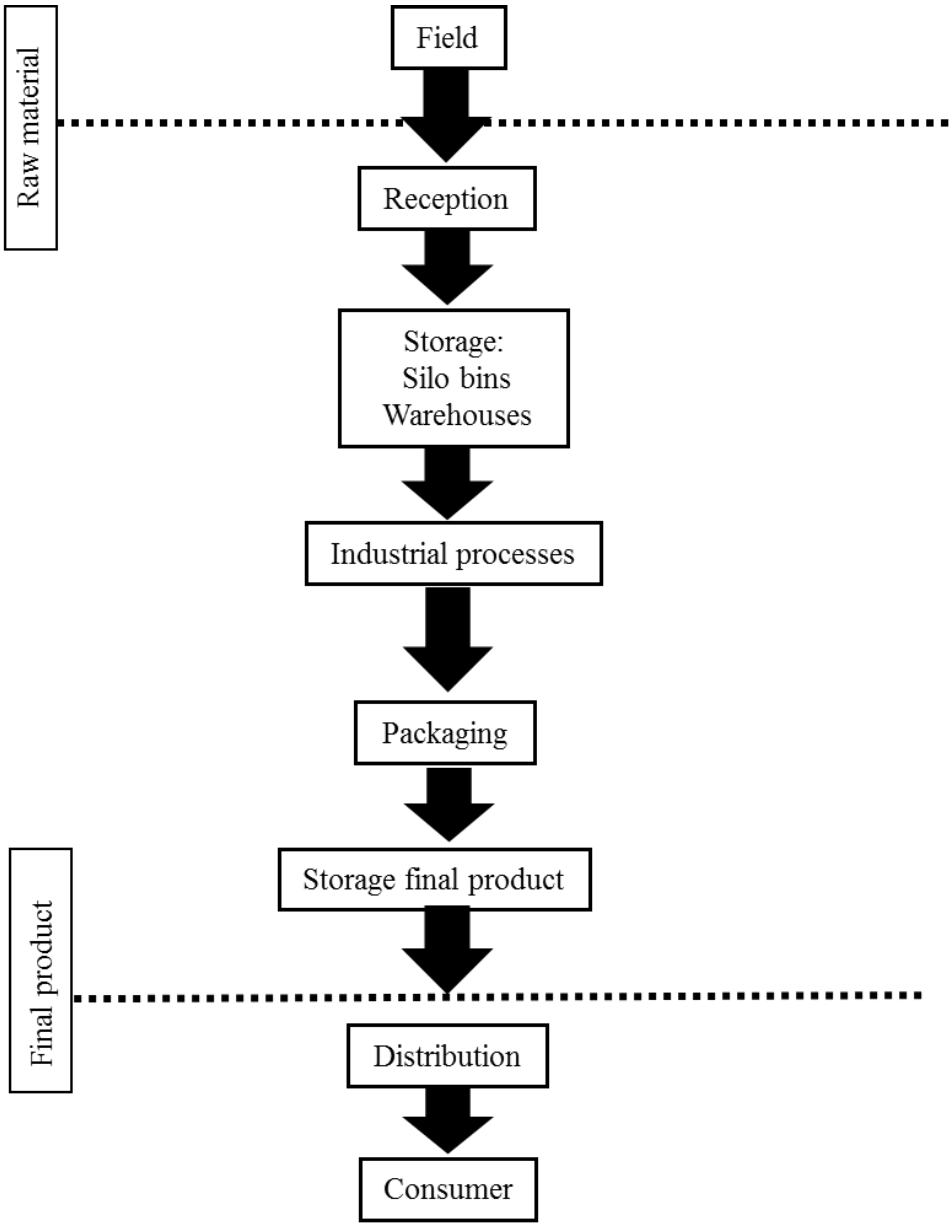


Figure 1. Diagram of the generalized food production chain

Unfortunately, according to the Food and Agriculture Organization (FAO), 1.3 billion tons of food, of which the majority are cereals, is lost per year. This roughly means that one-third of the edible part of the food gets lost or wasted. The loss occurs from the initial agricultural production down to final household consumption and reasons are variable (Fig. 1).

Interestingly, in the case of cereals, food losses in industrialized countries are as high as in developing countries, but in the last, more than 40% of the losses occur at post-harvest and processing levels, while in industrialized countries more than 40% of the food losses occur at retail and consumer levels which are often described as food waste (Gustavsson et al., 2011) (Fig. 2).

Notably, insects are culpable of 10% to 30% of value-added finished products losses even when packaged and ready for retail in developed and developing countries, respectively (Phillips and Throne, 2010; Pimentel, 2011; Hagstrum and Phillips, 2017). However, more dramatic occurrences may be found locally. For example, in Zimbabwe farms, a loss has been reported of 76% of maize stored without insecticide protection (Giga et al., 1991). In fact, insects are present during the whole processing chain however the most critical and

common point susceptible of insect infestation is during the storage period (Fig. 1) (Riudavets, 2017).

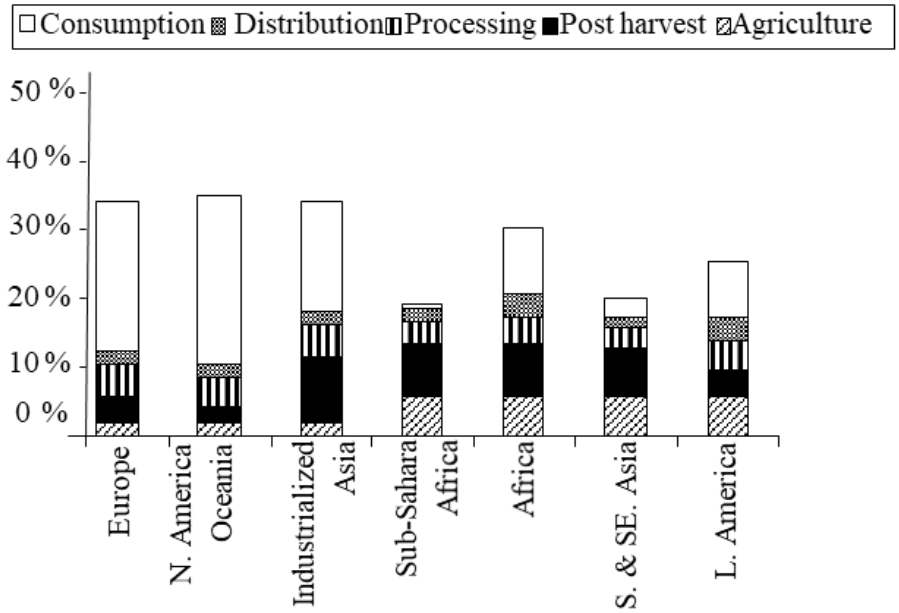


Figure 2. Part of the initial production lost or wasted at different stages of the food production stages, for cereals in different regions (Gustavsson et al., 2011).

2. Stored product pests

Despite the fact that the food production is increasingly following high standards of quality, the agro-alimentary industry harbours more than 1.500 insect species which 150 of them are considered actionable

pests (Hagstrum, 2009; Hagstrum and Phillips, 2017; White et al., 2011). The presence of living insects, insect body fragments or products of their metabolism such webbings or fecal material are unacceptable in the food industry (Fig.3.C, D, E and Fig. 4 and 5) (Arbogast et al., 1998; Athanassiou et al., 2017; Campbell et al., 2003). Unfortunately, the stored grain provides a unique sheltered environment with optimal temperature and humidity for insect infestations (Reichmuth, 2000). It also provides the potential for the local and global dispersal of these species through grain transportation and marketing networks (Nopsa et al., 2015).

Aside from their feeding which produces insect damaged kernels (IDK) (Fig. 3. C, D, E), frass (Fig. 3 E) and a consequent weight loss; insect oxygen consumption may lead to an increase in grain moisture content that may lead to a mold growth with possible mycotoxines production (Fig.3. C), worsening the security and safety of the food (Birch, 1947; Gram et al., 2002; Sinha and Sinha, 1992).

Figure 3. Grain industry and kernel damage due to insect presence. **A.** Cereal industry. **B.** Commercial rice package infested with *Sitophilus spp* **C.** Rice containers infested with *S*. *zeamais* and maintained at 23ºC for 71 days (from the experiment in Chapter 3). From the left container to the right, increasing number of parasitoid released (0, 10, 20, 40♀♀ parasitoids) **D.** Insect damaged rice kernel**. E.** Insect damaged wheat kernel with frass.

Among the species in stored grain, only 10–15 occur frequently (White et al., 2011). In the Mediterranean region, insect internal feeders such as the weevils *Rhyzopertha dominica* (Fabricius) (Coleoptera: Bostrichidae) (Fig.4.C, G), *Sitophilus spp*. (*S*. *granarius (L.), S*. *oryzae* (L.) and *S*. *zeamais* (Motschulsky)) (Coleoptera: Curculionidae) (Fig.4.A) and the grain moth *Sitotroga cerealella* (Olivier) (Lepidoptera: Gelechiidae) (Fig.4.B) are the most concerning ones (Athanassiou et al., 2017; Castañé and Riudavets, 2015; Stejkal, 2006; Trematerra et al., 2015) (Figure 3).

They are responsible for serious grain losses, not only because of being cosmopolitan species that are first colonizers of the grain, but mainly due to their development inside the grain kernel until the emergence of the adult, thus escaping from being detected with traditional sampling methods (Fig. 3. D, E, F) (Brader et al., 2002; Hubert et al., 2009).

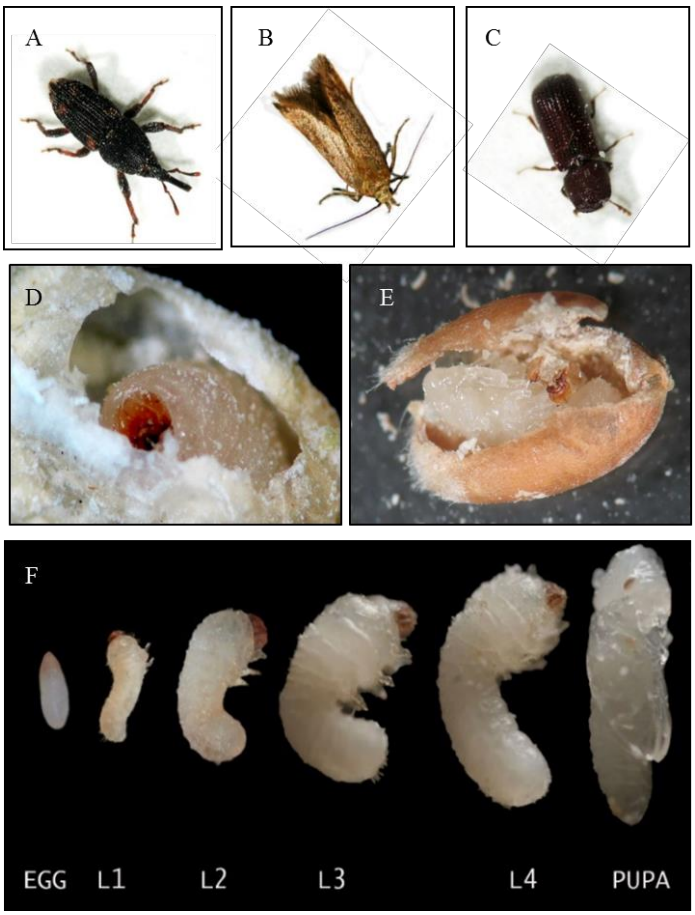


Figure 4. Internal feeders in the Mediterranean region. **A**. *Sitophilus spp*. (*S*. *granarius* (L.), *S*. *oryzae* (L.) and *S*. *zeamais*) (Motschulsky) (Coleoptera: Curculionidae). **B.** *Sitotroga cerealella* (Olivier) (Lepidoptera: Gelechiidae) **C.** *Rhyzopertha dominica* (Fabricius) (Coleoptera: Bostrichidae). **D.** Larva of *Sitophilus spp*. inside a rice kernel. **E.** Pupae of *Sitophilus spp*. inside a wheat kernel. **F.** Internal developmental stages of *R*. *dominicia* from egg to pupae (some **F** photos from J.G. Lundgren).

On the other side, apart from the internal feeders, stored-product moths are among the most serious and frequent pests in stored grain, mills, food processing industry and private households in Europe (Parkin, 1956; Trematerra and Gentile, 2010). Moth eggs (Fig. 5. E) are laid onto the surface of stored grain and the hatching larvae develop mainly in the upper 50 cm layer (Schöller et al., 1997). They produce webbing that may obstruct the flow of milling products in the food industrial equipment (Fig. 5. B). Particularly, in the Mediterranean region, the most abundant moth in alimentary industries is *E. kuehniella* (Zeller) (Lepidoptera: Pyralidae) (Fig. 5. A, B, C, D, F). As a representative example of this pest abundance, Belda and Riudavets (2013) reported *E. kuehniella* in 97.8% of the captures in pheromone traps in a pet food industry in the North-East of Spain.

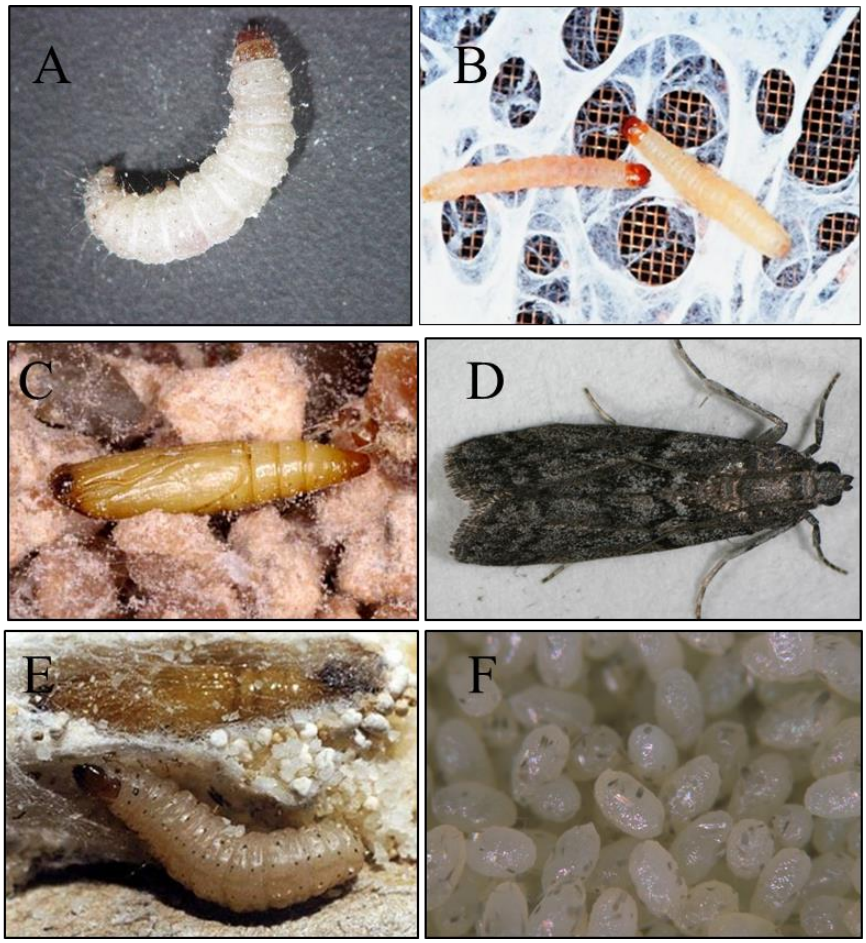


Figure 5. Stored product moths. **A.** Larvae of *Ephestia kuehniella* (Zeller) (Lepidoptera: Pyralidae). **B.** Some larvae of pyralidae with their weebing production (Photo from Jordi Riudavets). **C.** Pupae of *E. kuehniella* (http://www.ukmoths.org.uk). **D.** Adult of *E. kuehniella* (Photo from Jordi Riudavets) **E.** Larvae and pupae of pyralidae (https://housecentral.info/a/almond-moth.html). **F.** Eggs of *E. kuehniella* (Koppert biological systems).

3. Sampling strategies

In order to maintain food quality standards, raw material and processed food are checked for insect pest presence (Fig.6 & 7. A) (Subramanyam, 1995). Each industry and region applies their own procedure. However, figure 6 represents the generalized protocol for insect inspection in most food industries. When the grain arrives from the field, before unloading it for processing, several samples of 1 to 5 Kg. are taken and are visually inspected by the industry operators to check for insect presence. This step generally involves sieving the grain to separate insect adults or fragments and also to count the number of damaged kernels (IDK) due to the presence of insects. Then, if any insect adult or signs of its presence, such as molds (Fig. 3 C), frass (Fig. 3.E), insect fragments or insect damaged kernels (IDK) (3. D, E) are detected, a second sampling with 1kg. of grain is carried out. This sampling may be analyzed by one to several approaches. For example in Canada, the Berlese funnel method which works on the principle that insects moves away from heat, is the most commonly method for insect diagnosis in grain (Hubert et al., 2009; Karunakaran et al., 2003; Neethirajan et al., 2007). Alternatively, in USA, the most accepted methodologies for insect inspection are methods based on the insect fragments inspection (FDA, 1997). Insect fragments are generally counted under microscope

after cracking the grain and subjecting it to an acid hydrolysis where floating insect fragments are extracted with suction filtration (AOAC, 1996 cited from Toews et al., 2007). Although less common than insect fragment inspection, the X-ray is a widespread methodology in USA. This real time approach scans with a fluoroscope a series of single kernels and is able to detect insect internal infestations (Brader et al., 2002). Other techniques are the enzyme-linked immunosorbent assay (ELISA) based on the detection and quantification of myosin (Chen and Kitto, 1993) and the near infrared spectroscopy (NIRS) which is a non-destructive procedure for both qualitative and quantitative analysis that provides information based on the reflectance properties of the different substances present in the grain (Dowell, 1998; Maghirang and Dowell, 2003). Later, if any insect presence sign has been detected, cereal continues its path until consumption (Fig. 1 and 6). It may be worth noting that in general, a grade designation for insect presence in humans' food exists. Then, when it is overpassed, food can be used to feed the livestock.

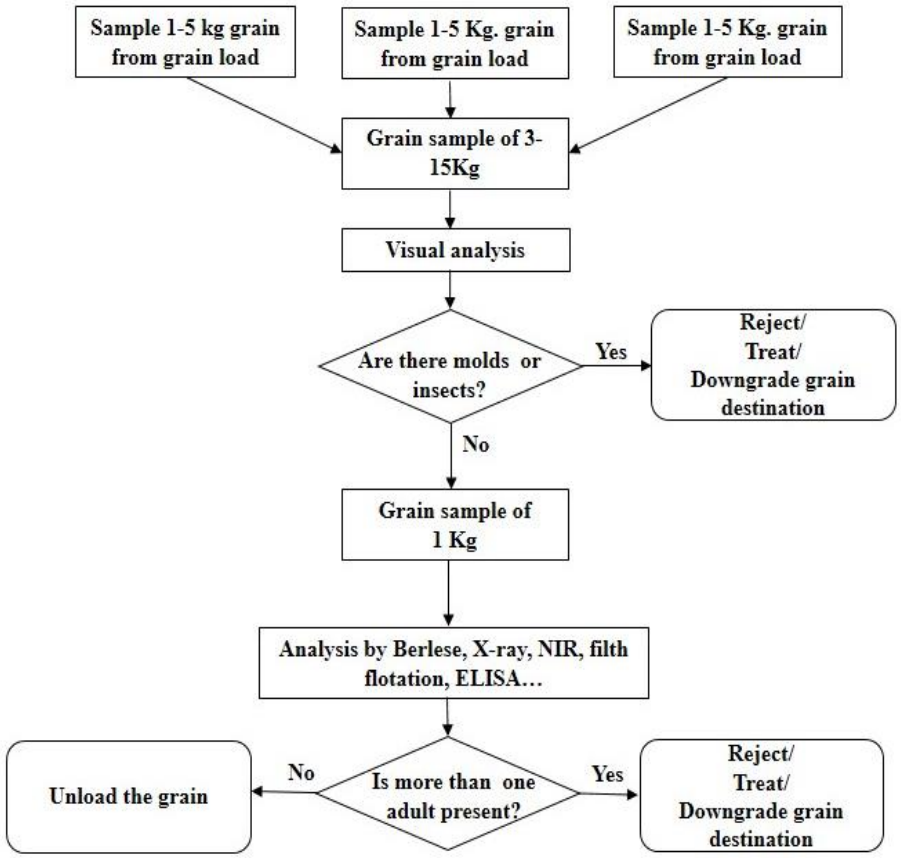


Figure 6. A common management protocol in a food industry for insect presence.

Although all the above techniques are currently used for insect diagnosis in food, they present one to several drawbacks (Table 1) (Brader et al., 2002; Neethirajan et al., 2007; Trematerra et al., 2011). While some techniques are not sensitive enough to detect low levels of pest or to detect internal stages of insect as reported by Hubert et al., (2009) when using sieving, filth flotation and Tullgren heat methods; other techniques such as X- ray or NIR, have the potential for use at the industry but are too expensive or labour intensive (Trematerra, 2013). As a result, often hidden insect infestation by immature internal feeders developing inside the grain kernels easily escape detection (Flinn et al., 2007; Toews et al., 2007). In fact, Storey et al., (1982) reported 12% of the export loads in the USA were infested but undetected due to the internal feeder's presence.

Table 1. Advantages and disadvantages of various detection techniques for stored-product insects in grain (adapted from Neethirajan et al., 2007).

Insect detection methods	Pros	Cons
Grain probes, pheromone traps, light coloured and electrocutor traps	Widely used for ecology studies, inexpensive, gives an indication of pest density, can be effective in indoor situations especially for flying insects	Labor intensive, limits the temporal availability of data, cannot detect internal infestations, restriction in the placement of traps, environmental factors affect trap catches, some of them not very specific, not quantitative
Sieving	Widely used, easy application, inexpensive, highly sensitive for external infestations, high accuracy when elevated infestation	Cannot detect internal stages, low efficacy when low infestations
Flith flotation	Widely used, can detect internal stages, qualitative and quantitative methodology, high accuracy of insect detection	Not efficient enough to extract adults and larvae, needs calibration, low efficacy when low infestations or small pest stages, low accuracy when quantifying
Acoustical methods based in insect feeding sounds	Internal infestation can be detected, detect insect densitites without removing grain samples	Cannot detect dead insects and infestations by early larval stages, requires quantitative understanding of several physical and biological factors

Electrical conductance based on differences in voltage across the kernel due to insect presence	Hidden internal infestation can be identified, inexpensive	Kernels with insect eggs and young larvae cannot be detected, low efficiency, time consuming
Near-infrared spectroscopy (NIR) based on the reflectance proprieties of different substances present in the product	Rapid method, no sample preparation, accurate, economic, qualitative and quantitative methodology, detect internal and external stages	Cannot detect low levels of infestation, cannot differentiate among dead or live insects, sensitive to moisture content in samples, calibration of equipment is complex and frequent
Machine vision based on visual reference images	Effective in detecting external insects, high accuracy	Subjective, complex device, time consuming, cannot detect internal insects
Enzime linked immunosorbent assay (ELISA)	Can detect immature stages, specific technique, high accuracy, qualitative and quantitative methodology	Requires sample preparation, not sensitive to small size larvae or eggs
X-ray imaging	Non-destructive, highly accurate, detect both internal and external insects, able to detect both live and dead insects inside grain kernels	Cannot detect insect eggs, very expensive

4. Insect control measures

Since 1940, chemical treatment with a broad-spectrum of pesticides has been the method of choice to control insect pests (Barratt et al., 2017; King et al., 1985; Van Lenteren, 2012; Zettler and Arthur, 2000). However, today the availability of effective insecticides has decreased drastically because the restrictions imposed by the domestic users, the government and the importing countries (Niedermayer and Steidle, 2013; Parrella et al., 1992). Some of the constraints to their use are environmental concerns, such as the presence of pesticide residues in food or the adverse effects on non-target organisms but especially the worring development of insect resistances to the long used protectants, which in addition, cause toxicity problems to the food workers (Zettler and Arthur, 2000; King et al., 1985). There are two types of pesticides: the fumigants and the contact insecticides. Among fumigants, three formulations are in widespread use: methyl bromide, phosphine or sulfuryl fluoride. They rapidly kill all life stages of stored product insects in a commodity or in a structure. Methyl bromide was the most used insecticide. However, due to the ozone depletion it has been banned, except for quarantine purposes, in 2005 in developed countries and by 2015 in most of the developing countries. Today, the most dominant fumigant worldwide, is the phosphine. However, this fumigant is under

regulation due to induce high toxicity and insect resistance (Hagstrum and Phillips, 2017; Phillips and Throne, 2010; Wilkin, 2000). Finally, sulfuryl fluoride is currently available for use only in some countries and mainly for treatment of structures. Among the contact insecticides most common in Europe are: chlorpyriphos-methyl and deltamethrin. These are sprayed directly on grain or structures, and provide protection from infestation for several months (Bell, 2000; Fields and White, 2002; Zettler and Arthur, 2000).

As a result of the lack of pesticides and the development of insect resistances, scientists have motivated research into various alternatives. Phillips and Throne (2010) reviewed the alternatives to the use of pesticides and classified them into two groups: strategies based on the manipulation of the physical environment or on biological based products. The first involve structure sanitation and insect exclusion; regulation of the temperature or moisture, or modifying the atmosphere of the system which directly affects the insect's vital needs; irradiation which acts by heating the product and insects by vibrating bonds in water molecules; and impact approach which consists in turning the grain and removing the insects. On the other side, the biological based approaches are divided into insect attractant pheromones and other semio-chemicals that are intraspecific chemical signals; botanical insecticides based on

plant extract repellents for the insects; varietal resistance of crops and foods; and finally, micro and macro organisms as natural enemies. These microorganisms are insect pathogens. The most common are the fungi *Beauveria bassiana* and *Metarhizium anisopliae* and the bacterium *Bacillus thuringiensis* (Bt). However, neither species is registered for stored product pest control in EU. The macroogranisms involve predators and parasitoids species (Flinn et al., 1996; Phillips and Throne, 2010). The parasitoid species are the subject of this thesis and are explained later

Biological control is a strategy that together with chemical and physical management tools constitutes Integrated Pest Management (IPM). These are ecological solutions to solve pest problems for an advanced food production system (MBTOC, 2014). Biological Control has been defined as the use of living organisms to suppress the population of a specific pest organism, making it less abundant or less damaging than it would otherwise be (Eilenberg et al., 2001). There are four different biological control strategies: the natural, whereby pest organisms are reduced by naturally occurring beneficial organisms as an ecosystem service; importation and introduction of new species; augmentation of beneficial species by mass rearing and periodic colonization; and conservation of natural enemies by manipulation of the

environment to make conditions more suitable for their survival (Hajek, 2004; Hokkanen, 1995; King et al., 1985; Van Lenteren et al., 2017).

Biological control has been successfully adopted for controlling pests on more than 30 million ha. worldwide. It has been proved to be a technically and economically feasible alternative to the use of chemical control (Van Lenteren et al., 2017). In consequence, an increasing commercial biological control industry has been maturating since 1900. This industry is well organized and has developed mass production, shipment and release methods as well as adequate guidance for users (Van Lenteren, 2012). Now, in 2017, almost 350 natural enemies targeting insect pests are commercially available on the global market. They are commercialized through about 500 producers, although concentrated in approximately 30 large companies. The biggest markets are the EU, US, Canada, Australia and New Zealand. However, Europe is still the largest market. This is partly due to political support of biological control within IPM programs, but also due to consumer demand, pressure by NGOs and a well-functioning, highly developed biological control industry and the lack of chemical tools (Barratt et al., 2017; Tracy, 2015; Van Lenteren, 2007; Van Lenteren, 2012). Around 80% of the global commercial revenue generated by biological control agents is attributed to their use in greenhouses (Pilkington, 2009 cited by

Tracy, 2015, Van Lenteren, 2017). However, the overall market of biocontrol is still very marginal representing around one to three percent of the worldwide annual turnover of plant protection products (Tracy, 2015; Waage, 2007). The most commonly sold beneficial agents belong to the following four groups: parasitoids, predatory insects, predatory mites, and entomopathogenic nematodes (Van Lenteren et al., 1997; Van Lenteren and Woets, 1988). To support the increasing market, these natural enemies are mass reared with the use of natural and artificial diets (Faal-Mohammad-Ali and Shishehbor, 2013; Ghimire and Phillips, 2010; King et al., 1985; Reichmuth, 2000; Schöller et al., 1997). Moreover, several experiences with mass releases of certain predators and larval parasitoids confirm the effectiveness of the augmentative releases (Keever et al., 1986; Lucas and Riudavets, 2002; Milonas, 2005; Niedermayer and Steidle, 2013).

5. Biological control of stored products

Stored products include all postharvest agricultural products that do not require refrigeration and can be stored for several months under proper conditions. Since usually the scientific literature (SCI) is biased toward the public concerns, in order to reveal the particular trends in biological control of stored product pests, a survey on the published SCI

in the last 10 years was realized (Solà & Riudavets in preparation). Filtering the terms, biological control, parasitoids, enthomopathogens or acari in the stored product or food industry, 137 SCI were found. The majority (51%) revealed parasitoids as the principal studied natural enemy, while 21% of the SCI works targeted enthomopathogens and 18% predators. As expected from the specific abundances in these environments, more than half of the literature targeted coleopteran species (63%), while 29% targeted lepidoptera and 8% acari. The major part of the studies were performed in Europe, revealing again the interest and pressure from the public for alternatives to insecticides. Interestingly, only 12% of the studies corresponded to field work in contrast to an 81% of laboratory work. Moreover, from the field work, only 8% of the published work (corresponding to a simple manuscript) targeted biological control efficacy while the remaining 92% corresponded to insect species surveys.

Among the macroscopic natural enemies, the survey revealed 8 different predator species as the target of stored product research. Predatory mites were the subject of 52% of the SCI publications targeting predator species in stored products. These included three representants of the Blattisocious family (*B. dendriticus* (Berlese), *B. keegani* (Fox) and *B. tarsalis* (Berlese), (Acari: Ascidae); and the species

Cheyletus malaccesis (Oudemans) (Acari: Cheyletidae). Alternatively, 24% of the SCI dealing with predator species concerned to Heteropteran species of the genera Anthocoridae (*Dufouriellus ater* (Dufour) and *Xylocoris flavipes* (Reuter). Then, of those SCI which were published, 8% (representing 2 SCI manuscripts each) referred to the genera Reduviidae (Hemiptera) (*Alloeocranum biannulipes* (Montrouzier & Signoret) and *Amphibolus venator* (Klug) or the species *Teretrius nigrescens* (Lewis) (Coleoptera: Histeriidae). Finally, the Pseudoscorpionida Withiidae, *Withius piger* (Simon), was the target of 1 of these SCI (4% of SCI concerned to predators).

On the other side, the survey highlighted 14 parasitoid species as subject of the scientific research about the stored product, suggesting these species as potential control agents for stored products (Table 2). Moreover, among the publications of parasitoids in stored product (73 SCI), more than 70% dealt with the genus Pteromalidae and Braconidae (Figure 7. C & D). The first are parasitoids of coleopteran species that are internal feeders, while the second only includes *Habrobracon hebetor* as a potential control species of stored product moths. The species of the genus Bethilidae, Ichenumonidae and Trichogramatidae represented 24% of the SCI publications about parasitoids. Finally, the

families Chalcididae and Eulophidae were the target of only 3% of the SCI publications targeting parasitoid species (Table 2).

Although biological control industries are growing fast and expanding their market, they have demonstrated little interest in developing biological solutions for pest control in stored products (Riudavets, 2017). This is particularly observed when regarding the list of commercialized parasitoids reported by Van Lenteren in 2012 and complemented by the same author in 2017. Among the 182 commercialized parasitoids worldwide, only 7 corresponded to natural enemies with interest for protection of stored products. There are only 2 commercializated predator species: *X. flavipes* targeting among others, stored coleopteran species, and *Cheyletus eruditus* (Schrank), a sibling species of *C. malaccesis*, predator of stored product mites that revealed to be the subject of 40% of the SCI published research focused on stored product predators. Moreover, there are 9 parasitoid species that have proved to be efficient in controlling storage pests but are not commercially available (Table 2).

Stored product environment offers favorable conditions for the presence of macroscopic natural enemies (Schöller and Flinn, 2000; Schöller et al., 2006). Thus, there are several predators and parasitoids suitable to being used as natural enemies. Nevertheless, this thesis is

centred in the release of parasitoids according to the augmentative biolgical control strategy.

5.1. The use of parasitoids as natural enemies

In augmentative biological control, adult parasitoids and predators are released in the storage facility. Then, free living females forage for hosts for their progeny, spreading around and locating pests even when hidden inside a grain kernel deep within a grain mass (Schöller et al., 2006). This method of control is based on the immature stages that develop in a single host individual, which is killed in the course of feeding but also due to lethal injuries caused by the females.

As long as hosts are available and environmental conditions are suitable, the parasitoids will continue to reproduce without needing new introductions. Some of the advantages of the use of parasitoids as natural enemies for biocontrol control is that are really small and can easily be removed from bulk grain using normal cleaning procedures before milling (Arbogast, 1998; Schöller and Flinn, 2000; Schöller et al., 1997).

Additionally, they leave no harmful residues and since the procedures used to apply biological control are very safe to the user and the environment, the risk to nontarget species is generally very low (Delfosse, 2005), particularly in the stored product environments.

Moreover, since it is not harmful for humans, there is no waiting period after release of agents (Riudavets 2017, Van Lenteren 2017.).

Table 2. Number of scientific publications (SCI) targeting parasitoid species for the use against the stored product pests in the last 10 years. The family and genus of the species as well as the study region of the research laboratory are noted (Solà & Riudavets in preparation). Note: Species in bold are commercially available according to Van Lenteren (2017).

Parasitoid			**Target species**				
Family	**Genus**	**Species**	**Family**	**Genera**	**Species**	**Region**	**SCI**
Hymenoptera	Chalcididae	*Antrocephalus mitys* (Walker)	Lepidoptera	Pyralidae	*Ephestia cautella* (Walker)	South America	1
	Bethylidae	***Cephalonomia tarsalis* (Ashmead)**	Coleoptera	Sylvanidae	*Oryzaephilus surinamensis* (L.)	Europe	3
		Cephalonomia waterstoni (Gahan)	Coleoptera	Cucujidae	*Cryptolestes ferrugineus* (Stephens)	Europe	1
		Holepyris sylvanidis (Brethes)	Coleptera	Tenebrionidae	*Tribolium confusum* (Jacquelin du Val)	Europe	2
	Braconidae	***Habrobracon hebetor* (Say)**	Lepidoptera	Pyralidae	*Amyelois transitella* (Walker), *Corcyra cephalonica* (Stainton), *Ephestia cautella* (Walker), *Ephestia kuehniella* (Zeller), *Galleria mellonella* (L.), *Plodia interpunctella* (Hubner)	Africa, Asia, Europe, USA	15

Eulophidae	*Trichospilus diatraeae* (Cherian & Margabandhu)	Coleoptera	Tenebrionidae	*Tenebrio molitor* (L.)	South America	1
Ichneumonidae	*Venturia canescens* (Gravenhorst)	Lepidoptera	Pyralidae	*Corcyra cephalonica* (Stainton), C*adra figulilella* (Gregson), *Ephestia kuehniella*(Zeller), *Plodia interpunctella* (Hubner)	Europe, Asia	5
Pteromalidae	***Anisopteromalus calandrae* (Howard)**	Coleoptera	Bostrichidae, Curculionidae	*R. dominica (Fabricius), Sitophilus granarius (L.), Sitophilus oryzae (L.)*	Africa, Asia, Europe, USA	10
	Dinarmus basalis (Rondani)	Coleoptera	Bruchidae	*Acanthoscelides obtectus* (Say), *Callosobruchus analis* (F.), *Callosobruchus chinensis* (L.)	Africa, South America	7

	***Lariophagus distinguendus* (Forster)**	Coleoptera	Bruchidae, Bostrichidae, Curulionidae	*Acanthoscelides obtectus* (Say), *Rhyzopertha dominica* (Fabricius), *Sitophilus granarius* (L.), *Sitophilus oryzae* (L.), *Sitophilus zeamais* (Motschulsky)	Africa, Asia, Europe	9
	Pteromalus cerealellae (Boucek)	Coleoptera	Bruchidae	*Callosobruchus maculatus* (F.)	Africa	6
	Theocolax elegans (Westwood)	Coleoptera	Curulionidae	*Sitophilus oryzae* (L), *Sitophilus zeamais* (Motschulski)	Asia, Europe, South America	3
Trichogrammatidae	***Trichogramma evanescens* (Westwood)**	Lepidoptera	Pyralidae	*Corcyra cephalonica* (Stainton), *Plodia interpunctella* (Hubner)	Europe, USA	4
	Uscana lariophaga (Steffan)	Coleoptera	Bruchidae	*Callosobruchus maculatus* (F.)	Europe	1

6. Legislation for insect presence in stored products

Throughout the food industry, strict standards regarding insect presence are imposed to regulate food quality and hygiene. In consequence, pest species but also parasitoids and predators are subject to strict regulations to their presence (Schöller et al., 1997). Whereas during the production in the field a certain amount of pest individuals can be tolerated, during food storage the threshold is close to zero (Jian and Jayas, 2012). Notwithstanding the efforts of the International Organisation for Biological Control (IOBC) to harmonize and simplify the registration of biocontrol products, the variations in national approaches to regulate biocontrol are quite significant, especially in Europe (Bale, 2011; Droby et al., 2009; Van Lenteren, 2007). Nevertheless, often consumers and retailers will impose their own, higher conditions (FDA, 1997; Stejskal et al., 2015).

In the United States, it is legally permissible to add insect parasitoids and predators to a bulk of grain and to food warehouses under regulations passed by the Food and Drug Administration (FDA) and the U.S. Environmental Protection Agency (EPA) (Phillips and Throne, 2010). In short, since 1991, insect natural enemies in USA were technically designated as insecticides so they could be regulated, and then exempted from a requirement of a tolerance level in food (Schöller

et al., 1997). Nevertheless, only certain species of hymenoptera parasitoids and predators are included in the EPA ruling. (Fields and White, 2002; King et al., 1985). On the other side, in the European Union, parasitoids and predators can be released as long as they are indigenous (Fields and White, 2002; Reichmuth, 2000) but there are no general regulations for authorization of beneficial insects for biological control (Reichmuth, 2000).

7. Biological control constrains

Despite the extended guild of natural enemies associated with stored-product and the existence of several studies which demonstrate that the releases of predators and parasitoids are effective, the use of beneficial insects to combat insect pest in food storage and industries is still quite controversial and it is far from being adopted by the food industry (Hansen, 2007; King et al., 1985; Phillips and Throne, 2010; Schöller et al., 1997; Winston et al., 2014). In fact, the use of biological control has long been neglected because of general concerns about deliberately contaminating foodstuff with insects, even if natural enemies; the low tolerance limit for insects in food industries; the availability and quality of parasitoids and predators; and the complexity of use that requires more information and careful timing compared to

traditional chemical insecticide (Arbogast et al., 1998; Collier and Van Steenwyk, 2004; Droby et al., 2009; Fields and White, 2002; Niedermayer and Steidle, 2013; Parrella et al., 1992; Wilkin, 2000).

Additionally, biological control may not eliminate a pest completely and this is often perceived as a requirement with food products at or close to the point of consumption (Wilkin, 2000). Furthermore, all introduced natural enemies present a degree of risk to non-target species although this is particularly limited in stored product environments. Another main constraint for the use of biological control is the high price of the natural enemies, especially when multiple releases are needed (Parrella et al., 1992). However, in the stored product context, traditional chemical treatments are already very expensive. Thus, the use of biological control may not represent an increase in price.

8. Improvements in biological control

In this thesis, research has focused on the improvement of sampling and monitoring approaches for the insect diagnosis in order to overcome the problems of detection of internal infestation and on the use of biological control by introduction of parasitoids that attack the most troubling coleopteran and lepidopteran pest species of stored products.

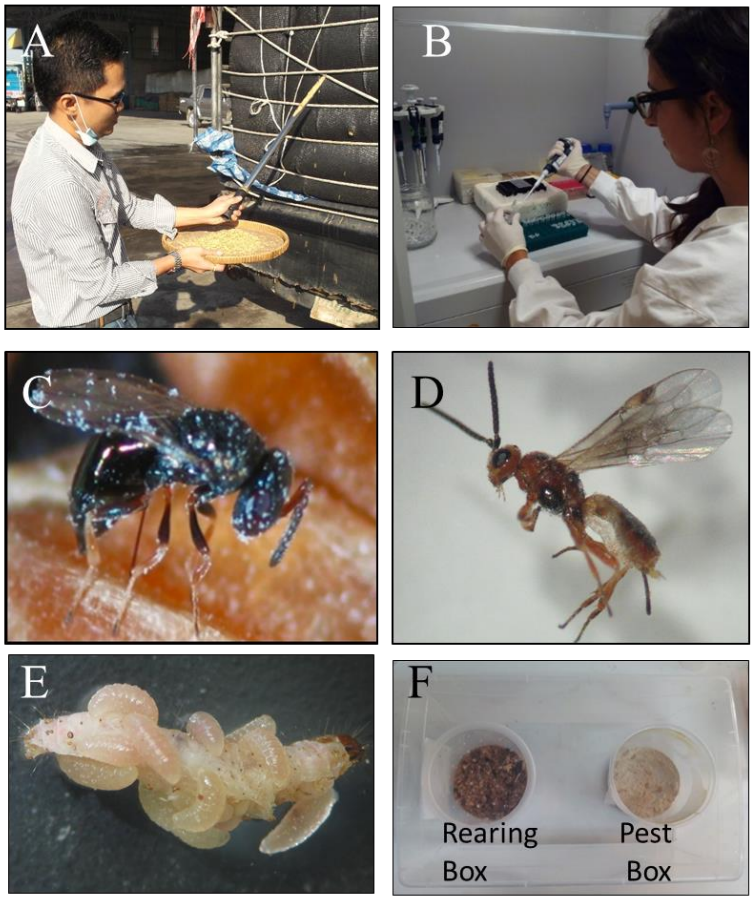


Figure 7. Sampling strategies and biological control of stored product pests. **A.** Sampling the grain for insect presence before loading it for food processing. **B.** Sampling the grain for insect immature stages in grain with molecular diagnosis. **C.** *Anisopteromalus calandrae* (Howard) (Hymenoptera: Pteromalidae) (Photo by Irene Fraile). **D.** *Habrobracon hebetor* (Say) (Hymenoptera: Braconidae). **E.** Larva of *E. kuehniella* parasited by more than 10 larvae of *H. hebetor*. **F**. Bankerbox system used in Chapter 4.

8.1. Pest monitoring with PCR approaches

Polymerase chain reaction (PCR) approaches represent an interesting sampling alternative for insect diagnosis. Although PCR is routinely used in agro-alimentary industries to detect genetically modified organisms (GMOs) in food, it is not used yet to detect insect presence in food. Different PCR approaches are suitable for the use in insect detection in food industries depending on the needs. Conventional PCR would be useful to detect a particular insect species hidden in grain (singleplex PCR), while its variant, Multiplex PCR, would be the most appropriate approach to detect multiple insect species even if a limited quantity of material is available (Gariepy et al., 2007) (Chapter 2). Alternatively, in cases where the quantification of the insect presence is needed, Real-time PCR (qPCR), an approach that uses a fluorescent dye that intercalates with the double-stranded DNA, would be the most suitable approach (Chapter 1).

8.2. Biological control with the introduction of parasitoids

The interaction of several physical, chemical and biological factors (Figure 8) in the stored products complicate the implementation of the biological control. Thus, it is vital to obtain detailed information on the biology, behavior and life history of the pest and its natural

enemies under the effect of these factors in order to know how and when to release the biocontrol agent and at what volume under each circumstance (Hodges, 1998; Tracy, 2015). Because of this, there is a huge need for well-designed experiments to study the interaction of the several factors implied in the stored grain to identify the systems in which biocontrol can work in a cost-effective manner (Droby et al., 2009) (Chapter 3).

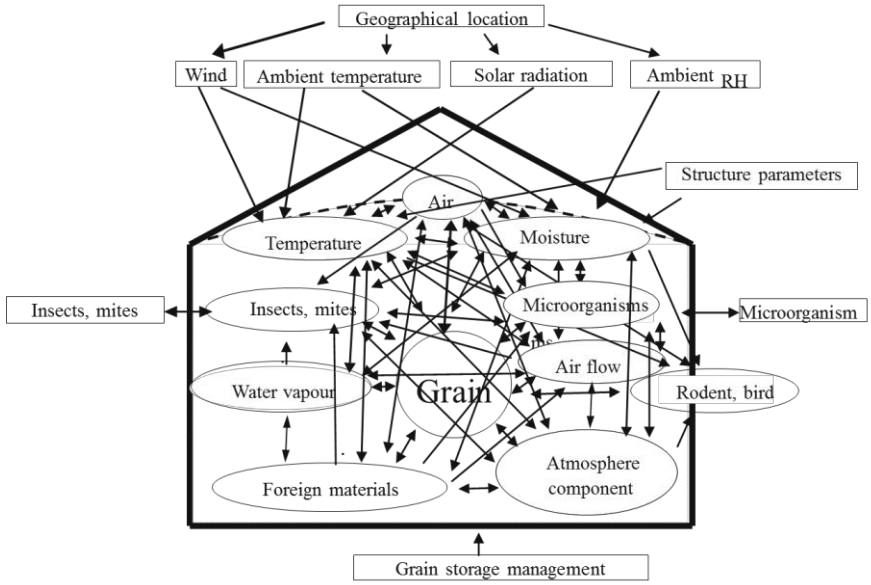


Figure 8. Factors in a stored grain ecosystem and the interactions among factors in non-aerated and unsealed bulk grain (Jian and Jayas, 2012).

Moreover, the efficacy of several natural enemies of stored products pests has been studied under laboratory conditions but very

little information is available of their performance in semi-practical or commercial situations (only 1 SCI in 10 years). In consequence, there is a need to improve studies on the feasibility of the use of natural enemies that are mass reared to control real infestations in stored grain (Chapter 4).

References

Arbogast, R.T., Weaver, D.K., Kendra, P.E., Brenner, R.J., 1998. Implications of spatial distribution of insect populations in storage ecosystems. Environmental Entomology 27**,** 202-216.

Athanassiou, C.G., Kavallieratos, N.G., Campbell, J.F., 2017. Competition of three species of Sitophilus on rice and maize. Plos One 12, 3.

Bale, J., 2011. Harmonization of regulations for invertebrate biocontrol agents in Europe: progress, problems and solutions. Journal of applied entomology 135**,** 503-513.

Barcenas, N.M., Unruh, T.B., Neven, L.G., 2005. DNA diagnostics to identify internal feeders (Lepidoptera : Tortricidae) of pome fruits of quarantine importance. Journal of Economic Entomology 98**,** 299-306.

Barratt, B.I.P., Moran, V.C., Bigler, F., Van Lenteren, J.C., 2017. The status of biological control and recommendations for improving uptake for the future. BioControl, 1-13.

Belda, C., Ribes-Dasi, M., Riudavets, J., 2011. Improving pest management in pet food mills using accurate monitoring and spatial analysis. Journal of Stored Products Research 47, 385-392.

Belda, C., Riudavets, J., 2012. Reproduction of the parasitoids Anisopteromalus calandrae (Howard) and Lariophagus distinguendus (Forster) on arenas containing a mixed population of the coleopteran pests Sitophilus oryzae and Rhyzopertha dominica. Journal of Pest Science 85, 381-385.

Belda, C., Riudavets, J., 2013. Natural enemies associated with lepidopteran pests in food and feed processing companies. Journal of Stored Products Research 53, 54-60.

Bell, C.H., 2000. Fumigation in the 21st century. Crop Protection 19, 563-569.

Birch, L.C., 1947. The oxygen consumption of the small small strain of Calandra Oryzae and Rhyzopertha Dominica Fab as affected by temperature and humidity. Ecology 28, 17-25.

Brader, B., Lee, R.C., Plarre, R., Burkholder, W., Kitto, G.B., Kao, C., Polston, L., Dorneanu, E., Szabo, I., Mead, B., Rouse, B., Sullins, D., Denning, R., 2002. A comparison of screening methods for insect contamination in wheat. Journal of Stored Products Research 38**,** 75-86.

Campbell, J.F., Mullen, M.A., Dowdy, A.K., 2002. Monitoring stored-product pests in food processing plants with pheromone trapping, contour mapping, and mark-recapture. Journal of Economic Entomology 95**,** 1089-1101.

Campbell, J.F., Prabhakaran, S., Schneider, B., Arbogast, R.T., 2003. Critical issues in the development and interpretation of pest monitoring programs for food processing facilities. Advances in Stored Product Protection**,** 121-127.

Castañé, C., Riudavets, J., 2015. Sampling arthropod pests and natural enemies in stored barley. Journal of Stored Products Research 64, 54-61.

Chen, W.M., Kitto, G.B., 1993. Species-specific immunoassay for Sitophilus granarius in wheat. Food and Agricultural Immunology 5**,** 165-175.

Collier, T., Van Steenwyk, R., 2004. A critical evaluation of augmentative biological control. Biological Control 31**,** 245-256.

Delfosse, E.S., 2005. Risk and ethics in biological control. Biological control 35, 319-329.

Dowell, F.E., 1998. Automated color classification of single wheat kernels using visible and near-infrared reflectance. Cereal Chemistry 75, 142-144.

Droby, S., Wisniewski, M., Macarisin, D., Wilson, C., 2009. Twenty years of postharvest biocontrol research: is it time for a new paradigm? Postharvest biology and technology 52, 137-145.

Eilenberg, J., Hajek, A., Lomer, C., 2001. Suggestions for unifying the terminology in biological control. BioControl 46, 387-400.

Faal-Mohammad-Ali, H., Shishehbor, P., 2013. Biological parameters of Bracon hebetor (Hym.: Braconidae) parasitizing Ephestia kuehniella (Lep.: Pyralidae): effect of host diet. Journal of Crop Protection 2, 411-419.

[FDA] Food and Drug Administration (1997), 578.450, Wheat & flour adulteration with insect fragments androdent hairs (CPG 7104.06). Compliance Policy Guides Manual, Chapter 5, Foods, color and cosmetics, Subchapter 578 - processed grain. (http://www.fda.gov/ora/compliance_ref/cpg/default.htm#1998)

Fields, P.G., White, N.D.G., 2002. Alternatives to methyl bromide treatments for stored-product and quarantine insects. Annual review of entomology 47, 331-359.

Flinn, P.W., Hagstrum, D.W., McGaughey, W.H., 1996. Suppression of beetles in stored wheat by augmentative releases of parasitic wasps. Environmental Entomology 25, 505-511.

Flinn, P.W., Hagstrum, D.W., Reed, C.R., Phillips, T.W., 2007. Stored Grain Advisor Pro: Decision support system for insect management in commercial grain elevators. Journal of Stored Products Research 43, 375-383.

Gariepy, T.D., Kuhlmann, U., Gillott, C., Erlandson, M., 2007. Parasitoids, predators and PCR: the use of diagnostic molecular markers in biological control of Arthropods. Journal of Applied Entomology 131, 225-240.

Ghimire, M.N., Phillips, T.W., 2010. Mass rearing of Habrobracon hebetor Say (Hymenoptera: Braconidae) on larvae of the Indian meal moth, Plodia interpunctella (Lepidoptera: Pyralidae): effects of host density, parasitoid density, and rearing containers. Journal of Stored Products Research 46, 214-220.

Giga, D.P., Mutemerewa, S., Moyo, G., Neeley, D., 1991. Assessment and control of losses caused by insect pests in small farmers' stores in Zimbabwe. Crop protection 10**,** 287-292.

Gram, L., Ravn, L., Rasch, M., Bruhn, J.B., Christensen, A.B., Givskov, M., 2002. Food spoilage—interactions between food spoilage bacteria. International Journal of Food Microbiology 78**,** 79-97.

Gustavsson, J., Cederberg, C., Sonesson, U., Van Otterdijk, R., Meybeck, A., 2011. Global food losses and food waste. FAO Rome.

Hagstrum, D., 2009. Stored-product insect resource. Elsevier.

Hagstrum, D.W., Phillips, T.W., 2017. Evolution of Stored-Product Entomology: Protecting the World Food Supply. In: Berenbaum, M.R., (Ed.), Annual Review of Entomology 62, 379-397.

Hajek, A.E., 2004. Natural enemies: an introduction to biological control. Cambridge University Press.

Hansen, L.S., 2007. Potential for widespread application of biological control of stored-product pests—the European perspective. Journal of stored products research 43**,** 312-313.

Hodges, R. J., 1998. Biological control methods for insect pests of stored grain in the Tropics-constraints and prospects for developing countries. In Proceedings of the 7th International Working

Conference on Stored Product Protection, Beijing, China 2, 1193–1204.

Hokkanen, H.M.T., 1995. Biological control. Wiley Online Library.

Hubert, J., Nesvorna, M., Stejskal, V., 2009. The efficacy of sieving, filth flotation and Tullgren heat extraction for detecting various developmental stages of Tribolium castaneum and Ephestia kuehniella in samples of wheat grain, flour and semolina. Journal of Stored Products Research 45**,** 279-288.

Jian, F., Jayas, D.S., 2012. The Ecosystem Approach to Grain Storage. Agricultural Research 1**,** 148-156.

Karunakaran, C., Jayas, D.S., White, N.D.G., 2003. X-ray image analysis to detect infestations caused by insects in grain. Cereal Chemistry 80**,** 553-557.

Keever, D.W., Mullen, M.A., Press, J.W., Arbogast, R.T., 1986. Augmentation of natural enemies for suppressing two major insect pests in stored farmers stock peanuts. Environmental Entomology 15**,** 767-770.

King, E.G., Hopper, K.R., Powell, J.E., 1985. Analysis of systems for biological control of crop arthropod pests in the US by augmentation of predators and parasites.

Lucas, É., Riudavets, J., 2002. Biological and mechanical control of Sitophilus oryzae (Coleoptera: Curculionidae) in rice. Journal of Stored Products Research 38**,** 293-304.

Maghirang, E.B., Dowell, F.E., 2003. Hardness measurement of bulk wheat by single-kernel visible and near-infrared reflectance spectroscopy. Cereal Chemistry 80**,** 316-322.

MBTOC. 2014: Methyl Bromide Technical Committee Assessment Report. United Nations Environmental Programme. http://ozone.unep.org/en/assessment-panels/technology-and-economic-assessment-panel.

Mendoza, A., Fernandez, S., Cruz, M.A., Rodríguez-Perez, M.A., Resendez-Perez, D., Saldaña, H.A.B., 2006. Detection of the genetically modified maize food products by the polymerase chain reaction. Ciencia y Tecnologia Alimentaria 3, 175-181.

Menon, A., Flinn, P.W., Dover, B.A., 2002. Influence of temperature on the functional response of Anisopteromalus calandrae (Hymenoptera: Pteromalidae), a parasitoid of Rhyzopertha dominica (Coleoptera: Bostrichidae). Journal of Stored Products Research 38**,** 463-469.

Milonas, P.G., 2005. Influence of initial egg density and host size on the development of the gregarious parasitoid Bracon hebetor on three different host species. BioControl 50, 415.

Neethirajan, S., Karunakaran, C., Jayas, D.S., White, N.D.G., 2007. Detection techniques for stored-product insects in grain. Food Control 18, 157-162.

Niedermayer, S., Steidle, J.L.M., 2013. The Hohenheimer Box - A new way to rear and release Lariophagus distinguendus to control stored product pest insects. Biological Control 64, 263-269.

Nopsa, J.F.H., Daglish, G.J., Hagstrum, D.W., Leslie, J.F., Phillips, T.W., Scoglio, C., Thomas-Sharma, S., Walter, G.H., Garrett, K.A., 2015. Ecological Networks in Stored Grain: Key Postharvest Nodes for Emerging Pests, Pathogens, and Mycotoxins. Bioscience 65, 985-1002.

Parkin, E.A., 1956. Stored product entomology (the assessment and reduction of losses caused by insects to stored foodstuffs). Annual Review of Entomology 1, 223-240.

Parrella, M.P., Heinz, K.M., Nunney, L., 1992. Biological control through augmentative releases of natural enemies: a strategy whose time has come. American Entomologist 38, 172-180.

Phillips, T.W., Throne, J.E., 2010. Biorational Approaches to Managing Stored-Product Insects. Annual Review of Entomology. Annual Reviews, 55, 375-397.

Pimentel, D., Wilson, C., McCullum, C., Huang, R., Dwen, P., Flack, J., Tran, Q., Saltman, T., Cliff, B., 1997. Economic and environmental benefits of biodiversity. BioSci. 47**,** 747–757.

Pimentel, D., 2011. Food for Thought: A Review of the Role of Energy in Current and Evolving Agriculture. Critical Reviews in Plant Sciences 30**,** 35-44.

Reichmuth, C., 2000. Biological control in stored product protection. IOBC WPRS BULLETIN 23**,** 11-24.

Riudavets, J., 2017. Biological Control of stored products pests. IOBC WPRS BULLETIN

Schatzki, T.F., Wilson, E.K., Kitto, G.B., Behrens, P., Heller, I., 1993. Determination of hidden Sitophilus- Granarius (Coleoptera, Curculionidae) in wheat by myosin ELISA. Journal of Economic Entomology 86**,** 1584-1589.

Schöller, M., Flinn, P.W., 2000. Parasitoids and predators. Alternatives to pesticides in stored-product IPM. Springer, 229-271.

Schöller, M., Flinn, P.W., Grieshop, M.J., Zdarkova, E., 2006. Biological control of stored-product pests. Insect management for food storage and processing, 67-87.

Schöller, M., Prozell, S., Al-Kirshi, A.G., Reichmuth, C., 1997. Towards biological control as a major component of integrated pest management in stored product protection. Journal of Stored Products Research 33, 81-97.

Sinha, K.K., Sinha, A.K., 1992. Impact of stored grain pests on seed deterioration and aflatoxin contamination in maize. Journal of Stored Products Research 28, 211-219.

Stejskal, V., Hubert, J., Aulicky, R., Kucerova, Z., 2015. Overview of present and past and pest-associated risks in stored food and feed products: European perspective. Journal of Stored Products Research 64, 122-132.

Storey, C.L., Sauer, D.B., Ecker, O., Fulk, D.W., 1982. Insect infestations in wheat and corn exported from the United-States. Journal of Economic Entomology 75, 827-832.

Subramanyam, B., 1995. Integrated management of insects in stored products. CRC Press.

Toews, M.D., Perez-Mendoza, J., Throne, J.E., Dowell, F.E., Maghirang, E., Arthur, F.H., Campbell, J.F., 2007. Rapid

assessment of insect fragments in flour milled from wheat infested with known densities of immature and adult Sitophilus oryzae (Coleoptera : Curculionidae). Journal of Economic Entomology 100**,** 1714-1723.

Tracy, E.F., 2015. The promise of biological control for sustainable agriculture: a stakeholder-based analysis. Journal of Science Policy & Governance 5, 1

Trematerra, P., 2013. Aspects related to decision support tools and Integrated Pest Management in food chains. Food Control 34**,** 733-742.

Trematerra, P., Gentile, P., 2010. Five years of mass trapping of Ephestia kuehniella Zeller: a component of IPM in a flour mill. Journal of Applied Entomology 134**,** 149-156.

Trematerra, P., Ianiro, R., Athanassiou, C. G., & Kavallieratos, N. G., 2015. Behavioral interactions between Sitophilus zeamais and Tribolium castaneum: The first colonizer matters. Journal of Pest Science, 88, 573-581.

Van Lenteren, J.C., 2007. Biological control for insect pests in greenhouses: an unexpected success. Biological control: a global perspective. CAB Int, Wallingford**,** 105-117.

Van Lenteren, J.C., 2012. The state of commercial augmentative biological control: plenty of natural enemies, but a frustrating lack of uptake. BioControl 57**,** 1-20.

Van Lenteren, J.C., Bolckmans, K., Köhl, J., Ravensberg, W.J., Urbaneja, A., 2017. Biological control using invertebrates and microorganisms: plenty of new opportunities. BioControl**,** 1-21.

Van Lenteren, J.C., Roskam, M.M., Timmer, R., 1997. Commercial mass production and pricing of organisms for biological control of pests in Europe. Biological Control 10**,** 143-149.

VanGuilder, H.D., Vrana, K.E., Freeman, W.M., 2008. Twenty-five years of quantitative PCR for gene expression analysis. Biotechniques 44**,** 619.

Waage, J., 2007. The sustainable management of biodiversity for biological control in food and agriculture: status and needs. FAO, Commission on genetic resources for food and agriculture, Background study paper**,** 54.

White, N.D.G., Fields, P.G., Demianyk, C.J., Timlick, B., Jayas, D.S., 2011. Arthropods of stored cereals, oilseeds, and their products in Canada: artificial ecosystems on grasslands. Arthropods of Canadian grasslands 2**,** 267-289.

Wilkin, R., 2000. Options for new approaches to pest control in food. IOBC WPRS BULLETIN 23**,** 1-10.

Winston, R.L., Schwarzländer, M., Hinz, H.L., Day, M.D., Cock, M.J.W., Julien, M.H., 2014. Biological control of weeds: a world catalogue of agents and their target weeds. Biological control of weeds: a world catalogue of agents and their target weeds.

Zettler, J.L., Arthur, F.H., 2000. Chemical control of stored product insects with fumigants and residual treatments. Crop Protection 19**,** 577-582.

Objectives

OBJECTIVES

The aim of this thesis is to move forward the knowledge needed for the successful implementation of integrated pest management for the control of the most troubling stored product pests in the Mediterranean region by improving the sampling techniques and the biological control strategies with the introduction of natural enemies. To reach the main objective, two specific objectives were set.

Objective 1. To develop a pest monitoring protocol based on Polymerase chain reaction (PCR) for the routine detection of internal feeders in grain and processed food at any step of the commercial sequence.

Objective 1.1. To create a model system, using infestations in rice of *Rhyzopertha dominica* in all developmental stages, to specifically detect and quantify with Real-time PCR, grain contaminated with this internal feeder (**Chapter 1**).

Objective 1.2. To develop and optimize a multiplex PCR protocol to exclusively detect and simultaneously identify the five most common internal feeders in different grains and processed food (**Chapter 2**).

Objective 2. To assess the effectiveness of two biological control strategies with the release of naturally occurring parasitoids for the control of the coleopteran internal feeders or the stored product moths, respectively.

Objective 2.1. To evaluate the effectiveness of *Anisopteromalus calandrae* released in three different densities to control *Rhyzopertha dominica* & *Sitophilus zeamais* and their associated rice damage at 23ºC and 28ºC (**Chapter 3**).

Objective 2.2. To optimize a bankerbox system for rearing and releasing *Habrobracon hebetor* for the control of *Ephestia kuehniella* with the use of *G. mellonella* in two different sizes as an alternative host (**Chapter 4**).

1

Detection and quantification of the insect pest *Rhyzopertha dominica* (F.) (Coleoptera: Bostrichidae) in rice by qPCR

PEST MONITORING WITH qPCR

Introduction

Cereal grains can be stored for periods longer than one year before being processed or transported. During this interval, grain becomes susceptible to being attacked by insects, which cause direct quantitative and qualitative losses to the product (HLPE, 2014 and Gorham, 1979). As a consequence, a significant portion of this food never reaches its final destination and is never consumed. For example, in maize 30 % of grain can be lost due to post-harvest pests (HLPE, 2014). External feeding pests can be removed from grain by sieving before milling. However, grain internal feeders cannot be easily disassociated. Because internal pests are difficult to detect and remove from grain, they are generally regarded as the most pestiferous ones (Flinn et al., 2007; Toews et al., 2007). Storey et al. (1982) reported that 12 % of wheat samples from export loads in USA have hidden internal insects which are undetected during the standard grain inspection process.

The lesser grain borer, *Rhyzopertha dominica* (F.) (Coleoptera: Bostrichidae) is a grain internal feeder that is a major pest of rice and other cereals around the world (Castañé and Riudavets, 2015). It can be

present alone or together with four other common internal feeders: *Sitophilus granarius* (L), *S. oryzae* (L.), *S. zeamais* (Motschulsky) (Coleoptera: Curculionidae) and the moth *Sitotroga cerealella* (Olivier) (Lepidoptera: Gelechiidae) (Chanbang et al., 2008). Also, in some tropical areas of America and Africa, *R. dominica* can be found together with the closely related grain internal feeder *Prostephaus truncatus* (Horn) (Coleoptera: Bostrichidae). *Ryzopertha dominica* eggs are laid outside of the grain kernel and newly hatched larvae feed voraciously inside the kernels, where they develop until the emergence of the adult (Edde, 2012). Both larvae and adults consume grain-based products resulting in fragmented kernels, powdery residues and a characteristic pungent odour (Toews et al., 2006) which can facilitate the entrance of secondary pests and fungi (Shah and Khan, 2014). Sittisuang and Imura (1987) reported weight losses of up to 40 % in brown rice after 14 weeks of artificial exposure to *R. dominica*.

Insect detection, identification and quantification are important to maintain standards of quality for grain. However, the legislation for detecting undesirable insects, frass and exuviae differs significantly among countries and industries (Obrepalska-Steplowska et al.; 2008, Flinn et al., 2007; Neethirajan et al., 2007; Toews et al., 2007). In the EU there are no systematic surveillance programs or any scientific risk

assessment concerning storage pests on the agenda of the European Food Safety Authority (EFSA) panel (Stejskal et al., 2014). In contrast, in the USA, the Food and Drug Administration (FDA) has developed a set of pest action thresholds that serves as a regulatory standard for food quality control. For example, thresholds of 75 insect fragments per 50 g of wheat flour, or 225 insect fragments per 225 g of macaroni and noodle products have been presented. Also, samples with more than 31 insect damaged kernels (IDK) per 100 g of wheat have been classified as unfit for human consumption (FDA, 1997).

Several techniques are currently available to detect insect infestations in stored grain (Neethirajan et al. 2007). Some of these methodologies are based on sieving, grain flotation, Berlese funnel extractions (Hubert et al., 2009), Enzyme-Linked ImmunoSorbent Assay (ELISA) (Kitto et al., 1993), the acoustic emission impact of insect movements within grain (Fleurat-Lessard et al. 2006, Hagstrum et al. 1997;), Near Infrared (NIR) (Dowell, 1998) or soft X-rays (Shah and Khan, 2014; Fornal et al., 2007; Karunakaran et al., 2003). Although some of these technologies are effective for detecting insect infestations of stored grain, they are either expensive, time consuming, unable to detect and identify low levels of pests, or not able to quantify internal insect infestations (Trematerra, 2013; Neethirajan et al., 2007). For

example, NIR methodology and soft X-ray can discriminate among developmental stages, but the first requires frequent calibration and cannot detect low levels of pests (Perez-Mendoza et al., 2003), while the second is tedious and cost prohibitive (Neethirajan et al. 2007).

Real-time PCR or quantitative PCR (qPCR) represents a reliable and fast way to detect, identify and quantify all life stages of an organism, based on the presence of its DNA (Mendoza et al., 2006). For this reason, over the last decade this technology has increasingly been used in the field of applied entomology to detect significant quarantine pests (Barcenas et al., 2005).

The objective of the present study was to develop a qPCR method to identify and quantify *R*. *dominica* individuals in rice using a DNA detection approach, as a model system for detecting rice contaminated with stored grain pests.

Material & Methods

1. Biological material

1.1. Insect specimen collection and rearing

We used *Rhyzopertha dominica* from two different origins. For the detection and quantification of *R*. *dominica* in grain, adults were shared from a colony maintained at the USDA-ARS Stored Pest Insect Research

Unit (Manhattan, KS, USA). Then, they were reared at the USDA-ARS North Central Agricultural Research Services on organic brown short rice (Lundberg Family Farms, Richvale, CA, USA) at 30° C and 70% relative humidity (RH). On the other side, the insects used for the specificity test: *R. dominica* together with the four other internal feeders in our area (*S. granarius, S. oryzae, S. zeamais* and *S. cerealella*) came from a laboratory colony maintained at IRTA Cabrils (Spain). In this case, coleopteran species were reared on organic brown rice (Eco-Salim, Maquefa, Spain) at 28º C and 70% RH and 16L: 8D while lepidopteran were reared on maize (Crit d'or, Spain) at 23ºC and 70% RH and 16L: 8D.

1.2. Insect DNA extraction procedures

DNA was extracted from whole adult individuals using the DNeasy Blood & Tissue extraction kit (Qiagen, Valencia, CA, USA; following the protocol for animal tissues). Total DNA was eluted in 400 µl AE buffer (10 mM Tris-Cl 0.5 mM, EDTA; pH 9.0) provided by the manufacturer. However, for the DNA extractions destined to the specificity test, the DNeasy Tissue Kit (QIAGEN, Hilden, Germany; protocol for animal tissues) was used and DNA was eluted in 100 µl of AE buffer.

1.3. Rice collection and processing

Rice used in the experiments was from two different origins. For the detection of *R. dominica* in infested grain kernels, we used organic brown short rice (Lundberg Family Farms, Richvale, CA, USA). To detect and quantify *R. dominica* in grain we used the same brown rice as was used for rearing the insects in IRTA Cabrils. In order to ensure that the brown rice was insect-free, a sample of 500 g of brown rice was maintained at 28ºC and 70% RH and three months later, it was sieved in a 2 mm mesh to check for insect adult presence.

1.4. Rice DNA extraction

Ten grams of pest-free brown rice was ground in a coffee grinder (Laboratory mill 3303, Perten-Instruments, Hägersten, Sweden) and DNA was extracted using the Extragen® Alimentos extraction kit (Sistemas Genómicos, Valencia, Spain). Total rice DNA was eluted in 100 µl of HPLC-grade water, and 50 µl of this elution was finally added to 150 µl of AE buffer. These DNA rice extractions were carried out in IRTA (Cabrils, Spain) and 50 µl of each were sent to North Central Agricultural Research Laboratory (NCARL) (Brookings, USA) for the qPCR analysis.

A negative control was added to all DNA extraction set (insect, rice and infested rice). Final DNA extractions were stored at -20° C.

2. Primer design and specificity analyses

A *R*. *dominica*-specific primer set was designed to target part of the Cytochrome Oxidase I (COI) mitochondrial region. For this, COI sequences of *R*. *dominica* and the mentioned four other common internal grain pests (*S*. *granarius, S*. *oryzae, S*. *zeamais* and *S*. *cerealella)* were aligned using ClustalW2 (www.ebi.ac.uk/Tools/msa/clustalw2) and compared for regions specific to *R*. *dominica* (GenBank accession numbers: JQ989165, DQ200131, AY131101, AY131099 and AY131100, respectively).

DNA samples from all insect strains and rice origins were amplified in a Stratagene MX3000P thermal cycler (Stratagene, La Jolla, CA, USA). Reaction PCR volumes (25 µl) contained 12.5 µl Quantitect SYBR Green PCR Master Mix (Qiagen, Valencia, CA, USA), 1 µl of each *R*. *dominica*-specific forward and reverse primers (225 nM) and 2 µl of template DNA (whole insect DNA or pest free brown rice DNA). Amplification conditions consisted of a single cycle of 95° C, followed by 50 cycles of 94° C for 15 s, 52° C for 30 s, and 72° C for 30 s. Each plate also had a positive control series (n = 5 wells) and no-template control series (n = 3 wells). The positive control consisted of a DNA extraction of five *R*. *dominica* larvae.

The designed *R*. *dominica* primer set was tested for specificity by

amplifying DNA of the four other internal feeder pest species described above under the same qPCR conditions. Also, we ensured that our PCR process did not detect prior infestations with a preliminary set of PCRs where the DNA extraction of the brown rice used, a positive control (five *R. dominica* larvae), a negative control (water) and a mixture of the positive control and the rice, were ran (n= 3 each). To ensure that only the target DNA was amplified, the dissociation temperature of the *R. dominica* qPCR product (melting) temperature was determined by incubating the reaction mix at 95 ºC for 60 s and then monitoring the fluorescence every 0.5ºC from 55 to 94 ºC

3. *R. dominica* detection in grain by qPCR analyses

Rhyzopertha dominica eggs were sieve-separated (500 µm mesh size) from the grain and placed into small vials containing brown rice using a fine paintbrush. Eggs were 24 h old and larvae were allowed to develop for 10, 15, 19 and 25 d to obtain 1st, 2nd, 3rd and 4th instars, respectively. Then, to ensure a single insect infestation per kernel, rice kernels were visually inspected and selected.

DNA extractions from individual rice kernels artificially infested with a single larva of *R. dominica* (1st, 2nd, 3rd or 4th instar) were performed (n= 5 per each larval stage). The DNA amplification was performend by qPCR under the same conditions used for the specificity

test. However, in this case, as a template, 2 µl of DNA from a rice kernel infested with a single *R. dominica* were used.

4. *R. dominica* quantification and sensitivity of the technique

For each *R. dominica* developmental stage (egg, larvae, pupae and adult), artificial infestations of 10, 5, 1 and the equivalent of 0.1, 0.01 and 0.001 individuals in 10 g of brown rice were set up. For this purpose, several mixtures of rice and pest DNA were prepared. Except for eggs, 1st instars and adults, all larval stages were gently removed from inside of infested rice kernels. Then, DNA from 10, 5 or 1 *R. dominica* individuals was extracted. The lowest insect infestations (the equivalent of 0.1, 0.01 and 0.001 individuals/10 g of rice) were obtained by serial dilutions with AE buffer of DNA extractions of 1 *R. dominica* individual. Ten different replicates, each with a different *R. dominica* specimen, were performed per each infestation concentration and developmental stage, with a total of 420 samples. Again, for the DNA amplification, qPCR conditions used were the same as described above. In this case, DNA mixtures consisting on 1 µl of insect DNA and 1 µl of brown rice DNA were used as templates.

5. Data analysis

For each sample, the resulting PCR cycle at which the fluorescence could be observed above background (C_t value) was obtained, being

inversely related to the quantity of original template DNA in the sample. Only samples showing a simple peak of the expected PCR product in the dissociation curve were counted as positive. To evaluate if the DNA detection (positive sample) was sensitive to the DNA amount, the frequencies (%) of positive samples were estimated and Chi-squared tests were performed. All C_t values were standardized according to the mean deviation of the positive control series on that plate from the overall interplate positive control mean. Then, the mean and the standard error of the standardized C_t values per each infestation dose and developmental stage were calculated. An ANCOVA was performed with JMP® (Version 8.0.1) and significantly different mean C_t values were separated using Tukey HSD and plotted with R (R- Core Team, 2014) (Fig. 9). In order to obtain a single standard curve for the quantification of unknown insect infestations, data were transformed to adult equivalents. For that purpose, data were pooled into three age categories (from egg to 2nd instar, from 3rd instar to pupae, and adult stage). The ANCOVA analysis revealed that age classes within these grouping were statistically similar (Fig 9). The mean C_t value of each age category per infestation dose was divided by the mean C_t value of the adult at the same infestation dose. Then, a mean of the obtained values per each age category was used as coefficient to transform the data of each age

category and infestation dose to adult equivalent. For the prediction of the relationship between adult equivalent C_t and infestation dose a linear regression was performed. The best fitting equation and the generated standard curve was plotted with the 95% of confidence intervals. Then, the inverse prediction with the 95% confidence interval was used to predict a range of infestation size according to an age category that might be found in 1 kg of brown rice related to a randomly sampled C_t value using JMP® (Version 8.0.1). In statistical literature, according to Wells and LaMotte (1995), the inverse prediction is the model relating a response (C_t adult equivalent) to a factor (insect infestation size) and fitted to experimental data. This information is then used to infer the factor value of a subject based on its measured response. Thus, to infer the inverse confidence intervals according to a specific age category in relation to an expected response (C_t adult equivalent), we first transformed the C_t adult equivalent value to C_t value per each age category using the above coefficient to transform the data of each age category and infestation dose to adult equivalent. Then, to obtain the insect infestation range in 1 kg of rice, the inferred confidence intervals were antilog transformed and multiplied by 100.

Results

The designed *R*. *dominica*-specific pair of primers (forward and reverse) had the following sequences: RdF1 5'-GCTTCTTCCACCCTCCTTAACC-3' and RdR1 5'-AGATAATAATAAAAGCAAAGC-3'. This primer set showed to be specific for *R*. *dominica* when tested against the other 4 internal feeders (*S*. *granarius*, *S*. *oryzae*, *S*. *zeamais* and *S*. *cerealella*) since only those samples containing *R*. *dominica* specimens alone or mixed with rice DNA, presented the target dissociation peak at 77º C. Moreover, none of the four non-target pests tested, nor the samples with uninfested rice, shared the dissociation peak with *R*. *dominica* but all of them presented a melting temperature of 70º C. Additionally, no insect adults were observed in the 500 g of brown rice maintained under controlled conditions for 3 months after sieving. The qPCR analysis for the detection of *R*. *dominica* in infested rice revealed that the designed primers set successfully detected all larval stages (1^{st}, 2^{nd}, 3^{rd} and 4^{th} instar) of this pest in one kernel of rice in all samples tested (n= 20).

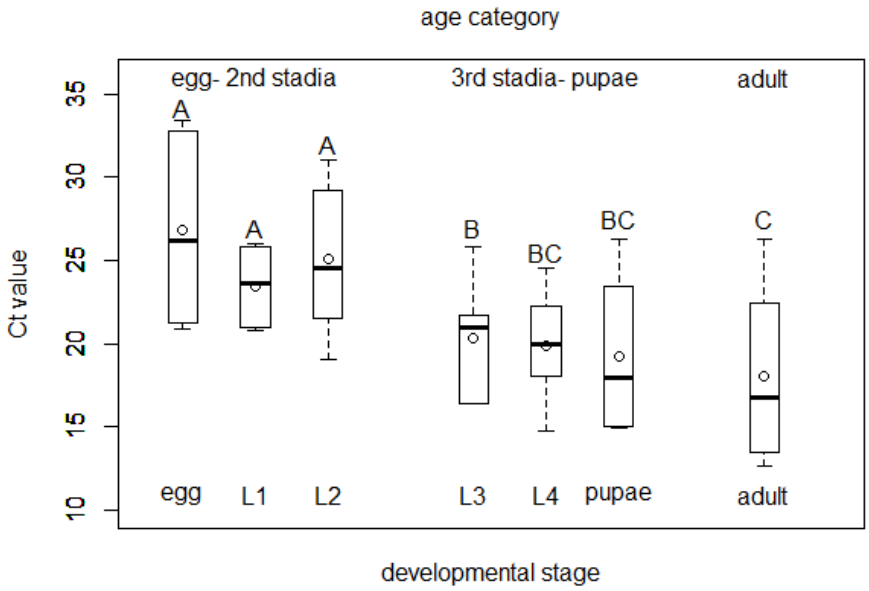


Figure 9. Box plot of the C_t values obtained for each developmental stage (egg, L1, L2, L3, L4, pupae and adult). The horizontal line within the box indicates the median, boundaries of the box indicate the 25th- and 75th- percentile, and the whiskers indicate the highest and lowest values of the results. The empty dots in the box indicates the mean.

The ANCOVA analysis performed on the C_t values revealed that the DNA amount was related with the infestation dose (R^2=0.89, $F_{7,32}$=37.26; P<0.0001) and with the developmental stage as covariant (F_6=20.96; P<0.0001). Then, the Tukey HSD means separation test

classified the developmental stages in 3 age categories (egg- 2nd instar; 3rd instar- pupae, and adult). Different letters above higher obtained values indicate significant differences among C_t values at different developmental stages.

The qPCR analysis for the quantification of *R. dominica* in rice revealed that the DNA amount detected was related with the infestation dose (ANCOVA: R^2=0.89, $F_{7,32}$=37.26; P<0.0001) and with the developmental stage as covariant (F_6=20.96; P<0.0001). However, the mean comparison done by Tukey HSD revealed no statistical differences between eggs and small larvae (1st and 2nd instars) or large larvae (3rd and 4th instars) and pupae (Fig. 9). For this reason, we classified the developmental stages in three different age categories (from egg to 2nd instars; from 3rd instars to pupae; and adult). The correlation between *R. dominica* population size and detectable pest DNA amount was analyzed as three categories not only because of the statistical significance, but also because earlier instars (egg- 2nd instar) are less damaging than older larval stages (i.e., 3rd instar- pupa) and the adult stage can be easily detected by sieving.

Table 3. Frequencies (%) of DNA detection at different age categories and insect densities in 10g of rice. For the age categories egg-2nd instar and 3rd instar - pupae, n=30 replicates were used. For adults, n=10 were used.

Insect density	Age category			% of DNA detection by insect density
	egg-2nd instar	3rd instar-pupae	adult	
0.001	10	90	100	57
0.01	73	97	90	86
0.1	73	93	90	84
1	77	100	100	90
5	80	97	100	90
10	87	97	100	93
DNA detection by age category	67	96	97	83

R. dominica DNA detection was sensitive to the quantity of *R. dominica* DNA present in the sample. Thus, the Chi-squared test revealed that the DNA detection was significatively related to *R. dominica* age category (X^2_2=43.85, P<0.001) and infestation density (X^2_5=63.05, P<0.001). The frequency of detection increased with the developmental stage or the infestation dose (Table 3).

Overall, 83% of the samples tested were successfully detected. As expected, the lowest DNA detection rate (10% of samples) corresponded

to the youngest insects (egg-2nd instar) at the lowest infestation dose (0.001 insects/10g of rice) (Table 3). For this age category, fluorescence above the background (C_t value) was detected in more than 70% of the samples at infestation sizes larger than 0.01 insects/10g of rice. For 3rd instar to adult, all doses tested (from 0.001 to 10 insects/10g) were successfully detected in more than 90% of the samples.

Significant correlations between infestation dose and adult equivalent DNA quantity was found when performing the linear regression to the data ($F_{1,38}$=166.4, P<0.0001, R^2=0.92) (Fig. 10). The DNA amount decayed (and C_t value increased) as the infestation size decreased, and this relationship was described by the following equation (SE in brackets):

$$y = 15.92\ (\pm 0.30) - 2.53\ (\pm 0.19)\ \mathrm{x}$$

Were *y* stands for adult equivalent C_t value and the x is the insect infestation dose in 10 g of brown rice.

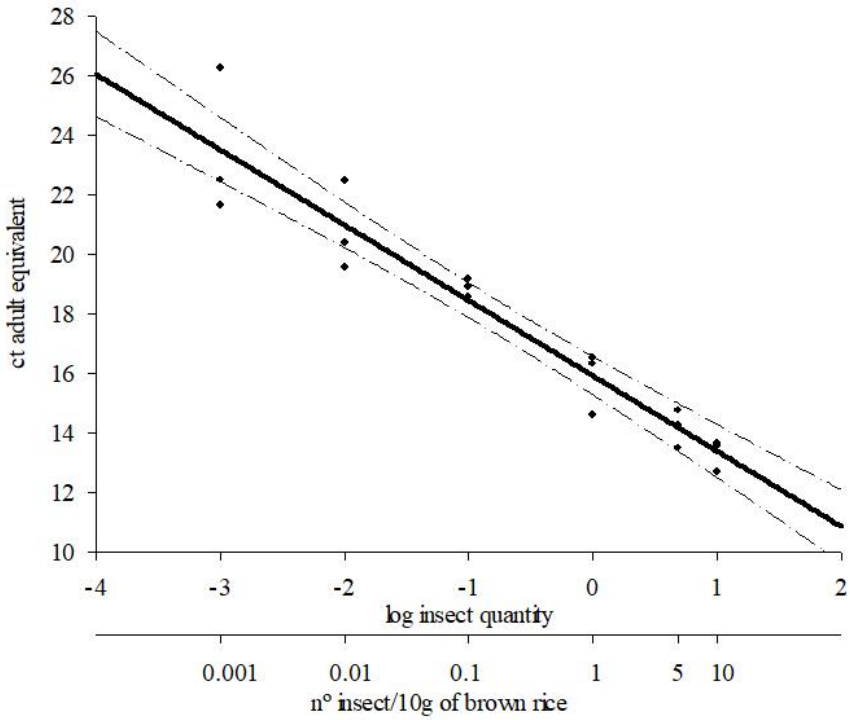


Figure 10. Correlation of C_t adult equivalent and insect infestation dose in 10 g of rice represented in a logarithmic scale using qPCR (n= 420). Each point represents the mean of the obtained C_t value per each age category (n=30 for eggs to 2nd instar, 3rd instar to pupae and n=10 for adult). The 95% confidence intervals for the regression are represented by the dashed lines.

Discussion

In the present study, a protocol to detect *R*. *dominica* DNA and quantify infestation sizes of this species in rice was developed using qPCR. Our results revealed that the designed pair of primers are able to detect any life stage of *R*. *dominica* in brown rice. This work also revealed that the quantity of *R*. *dominica* DNA present is strongly associated with a given infestation size and with a determinated age category. This suggests that this technique can precisely and accurately diagnose pest populations of all developmental stages in rice. A major consideration is that this approach is highly specific to *R*. *dominica* since neither of the four target species tested dissociates at 77º C.

Previous research has shown that *R*. *dominica* rarely reaches infestations higher than 10 individuals per kg of grain in commercial grain elevators (Flinn et al., 2010). However, hidden infestations by pre-emergent populations may be 10 times higher than free-living adults (Fleurat-Lessard, 1988). For this reason, in order to detect realistic populations of pests in grain, a very sensitive technology is required. Comparative studies of screening methods for insect contamination in grain have demonstrated that the sieving approach showed the highest sensibility for insect presence (Hubert et al. 2009). Unfortunatelly, this methodology is not able to detect immature stages of internal feeders. On

the other hand, Brader et al. (2002) showed that their ELISA approach presented the highest detection for immature insects. This screening method detected the equivalent of 40 *S*. *granarius* larvae per kg of wheat flour. Nevertheless, a much higher sensitivity has been reached using molecular approaches. Obrepalska-Steplowska et al. (2008) successfully detected the equivalent of 0.01 *S*. *granarius* individuals per kg of wheat flour purposely infested with insect adults using qPCR. In the present study, the sensitivity threshold reached, also performed with artificial insect infestations in rice, was of 0.02 *R*. *dominica* adults, 0.1 3rd instar to pupae or 13 eggs -2nd instar per kg of rice (Table 4). Although Obrepalska-Steplowska et al. (2008) and the present work used different coleopteran species; both studies proved the highest potential of detection using molecular techniques.

The accuracy of detection is another important feature to keep in mind when choosing the best approach for insect detection in grain. Predictably, accuracies of detection are dependent on the infestation size and the developmental stage of the pest. Pearson et al. (2003) obtained 24% and 89% of accuracy of the detection of small and large larvae of *S*. *oryzae* and *R*. *dominica*, respectively, using electrical conductance.

Table 4. Predicted range of *R*. *dominica* individuals at each age category in 1 kg of brown rice related to a given C_t adult equivalent with the 95% of confidence interval. A C_t value higher than 26 would be out of rank while a C_t< 18 would represent an infestation higher than 25 adults per kg of rice, which is improbable. Only infestation levels tested (between 0.1 and 1000 individuals/kg) are represented.

Adult equivalent C_t value	egg-2nd instar	3rd instar -pupae	Adult
26	13-38	0.1-0.4	.
24	44-140	0.4-2	0.02-0.2
22	142-551	3-8	0.2-0.8
20	436-2230	14-39	0.8-4
18	.	65-215	9-25

On the other side, Maghirang et al. (2003) obtained accuracies of 62 to 94% for small larvae and pupae, respectively, with NIR system. At the moment, among the panoply of screening technologies available for the detection of internal stages of pests, the X ray approach has presented the highest accuracy by successfully identifying more than 98% of wheat kernels infested with a single *S*. *oryzae* or *R*. *dominica* larvae (Karunakaran et al., 2003). Nevertheless, the current study reached a 100% of accuracy when detecting a single *R*. *dominica* larvae within an infested rice kernel. Furthermore, the methodology developed here

increased the detection threshold when increasing the sample size from one kernel to 10 g of grain, reaching accuracies of 73% to 100% when 0.01 eggs-small larvae and 1 adult are tested, respectively (Table 3).

The presence of insects or their fragments and products in food is in general not acceptable. For this reason, in the USA, the FDA uses insect fragments in grain as a regulatory standard control for food quality. Unfortunatelly, hidden infestations by pre-emergent stages within the kernels are not always well correlated with insect fragments (Perez-Mendoza et al., 2003; Brader et al., 2002; Sachdeva, 1978) and this could lead to underestimations of the real infestation size. For example, Throne et al., (2007) determined using NIR-spectrometry, that wheat infested with a single pre-emergent adult lesser grain borer, contributed 28 and 10 times as many fragments as wheat infested with a single larva or pupae, respectively. Also, they predicted that 1 kg of wheat with more than 20 kernels infested with pre-emergent adult borers would be above the FDA defect action level for insect fragments. Therefore, according to them, a C_t value of 17.7 (aprox. 20 adults) in 1 kg of rice would be the threshold to comply the federal requirement. On the other side, it is common knowledge that many European food-processing factories fumigate at thresholds of 1 adult per kg of grain in order to comply with the quality standards for insect presence in food.

Thus, we predict that a C_t value of 21, which is the equivalent to a mean of 0.9 adults, 10 big larvae/pupae or 482 eggs/small larvae in 1 kg of rice, would represent the action threshold for chemical application.

This protocol, although being able to detect any life stage of *R. dominica*, is not able to discriminate among life stages in mixed populations. This situation is shared with qPCR analysis, as well as the protein detection techniques such as NIR or ELISA. Nevertheless, with the protocol showed here it is possible to obtain a C_t value threshold to consider wether a grain sample is infested or not by transforming C_t values to adult equivalent. Moreover, since the DNA amount can be closely correlated with insect mass, this technique can be used to estimate the biomass of *R. dominica* in a grain sample. Edde & Phillips (2006) stated that the mean of the fresh body weight of one *R. dominica* adult is about 1.20 mg. Then, the C_t value of 18 (9-25 adults/kg of rice) would represent an insect biomass between 10.8 and 30 mg in 1Kg of grain or a C_t value of 24 (0.02-0.2 adults/kg of rice) would represent an insect biomass between 0.024 and 0.24 mg in one Kg of grain. This value would give an idea of the *R. dominica* contamination in the analyzed grain. Despite it is not essential to determine the insect species and developmental stage for grain grading, the detection and identification of insect developmental stages could help to make most of the

management decisions for grain cleaning (Dowell et al 1998). For this reason, we propose the aplication of inverse predictions of the resulting C_t values. Then, estimations of insect infestations regarding age categories will be obtained, facilitating the management decisions.

In this work we have simulated *R. dominica* infestations in rice. This technique showed high sensitivity and accuracy of detection. However, in order to be able to integrate this approach in the routine of the alimentary industry for the detection of insect presence, a futher analysis of real field infestations by internal feeders in grain with different insect densities and developmental stages would be advisable. Additionally, before using this qpCR protocol in other regions, where other internal feeding species could be present, we suggest a previos step by completing the specificity test with those susceptible species. Also, further experiments are needed to assess the detection range among different post mortem periods. The discrimination between death and alive insects is another consideration for the application of management measures, otherwise an overestimation of the insect population and of the control measures can be reached.

The detection and quantification of *R. dominica* populations can be critical to grain and milling factories. The qPCR protocol described in this study is of a great importance while being species-specific and

sensitive enough to detect low pest populations in all developmental stages. This protocol can be used in all steps of the food production chain with an easy interpretation of the results. This technology should be further used to detect other common internal feeders in grain, as also done by Obrepalska-Steplowska et al. (2008) with *S. granarius* on wheat flour. Considering that PCR, and even qPCR analysis, are already used for detecting other quality attributes in the milling and grain processing industry, the qPCR techinque developed here can be used in parallel to detect grain also infested grain with internal insects.

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References

Barcenas NM, Unruh TB and Neven LG (2005) DNA diagnostics to identify internal feeders (Lepidoptera: Tortricidae) of pome fruits of quarantine importance. J Stored Prod Res 98:299-306.

Brader B, Lee RC, Plarre R, Burkholder W, Kitto GB, Kao C, Polston L, Dorneanu E, Szabo I, Mead B, Rouse B, Sullins D, Denning R (2002) A comparison of screening methods for insect contamination in wheat. J Stored Prod Res 38:75–86.

Castañé C and Riudavets J (2015) Sampling arthropod pests and natural enemies in stored barley. J Stored Prod Res 64:54-61.

Chanbang Y, Arthur FH, Wilde GE and Throne JE (2008) Hull characteristics as related to susceptibility of different varieties of rough rice to Rhyzopertha dominica (F.) (Coleoptera: Bostrichidae). J Stored Prod Res 44:205-212.

Dowell FE (1998) Automated color classification of single wheat kernels using visible and near-infrared reflectance. Cereal Chem 75:142-144.

Edde PA, Phillips TW (2006) Longevity and pheromone output in stored-product Bostrichidae. B Entomol Res 96:547-554.

Edde PA (2012) A review of the biology and control of Rhyzopertha dominica (F.) the lesser grain borer. J Stored Prod Res 48:1-18

[FDA] Food and Drug Administration (1997), 578.450, Wheat & flour adulteration with insect fragments androdent hairs (CPG 7104.06). Compliance Policy Guides Manual, Chapter 5, Foods, color and cosmetics, Subchapter 578 - processed grain. (http://www.fda.gov/ora/compliance_ref/cpg/default.htm#1998)

Fleurat-Lessard F (1988) Determination of insect infestation. In Preservation and Storage of Grains, Seeds and their By-products, Multon, J.L. (Ed.), Lavoisier Tec & Doc, New York, 495-515.

Fleurat-Lessard F, Tomasini B, Kostine L and Fuzeau B (2006) Acoustic detection and automatic identification of insect stages activity in grain bulks by noise spectra processing through classification algorithms. Lorini I, Bacaltchuk B, Beckel H, Deckers D, Sundfeld E, Dos Santos JP,. Biagi JD, Celaro JC, D'A Faroni LR., Bortolini L.de OF., Sartori MR, Elias MC,. Guedes RNC, Da Fonseca RG, Scussel VM (eds.), Proceedings of the 9th International Working Conference on Stored Product Protection, pp. 476–186.15 to 18 October 2006, Campinas, São Paulo, Brazil. Brazilian Post-harvest Association - ABRAPOS, Passo Fundo, RS, Brazil, 2006. (ISBN 8560234004).

Flinn PW, Hagstrum DW, Reed CR and Phillips TW (2007) Stored Grain Advisor Pro: Decision support system for insect management in commercial grain elevators: J Stored Prod Res 43:375-383.

Flinn PW, Hagstrum DW, Reed C and Phillips TW (2010) Insect population dynamics in commercial grain elevators. J Stored Prod Res 46:43-47.

Fornal J, Jelinski T, Sadowska J, Stanislaw G, Nawrot J, Niewiada A, Warchalewski JR and Blaszczak W (2007) Detection of granary weevil Sitophilus granarius (L.) eggs and internal stages in wheat grain using soft X-ray and image analysis. J Stored Prod Res 43:142-148.

Gorham JR (1979) The significance for human health of insects in food. Ann Rev Entomol 24:209-24.

Hagstrum DW, Subramanyam BH and Flinn PW (1997) Nonlinearity of a generic Variance-Mean Equation for Stored-Grain Insect Sampling Data. Environ Entomol 26(6):1213-1223.

HLPE (2014) Food losses and waste in the context of sustainable food systems. A report by the High Level Panel of Experts on Food Security and Nutrition of the Committee on World Food Security, Rome 2014 (www.fao.org/cfs/cfs-hlpe).

Hubert J, Nesvorna M and Stejskal V (2009) The efficacy of sieving, filth flotation and Tullgren heat extraction for detecting various developmental stages of Tribolium castaneum and Ephestia kuehniella in samples of wheat grain, flour and semolina. J Stored Prod Res 45:279–288.

JMP®, Version 8.0.1. SAS Institute Inc., Cary, NC, 1989-2007.

Karunakaran C, Jayas DS and White NDG (2003) X-ray image analysis to detect infestations caused by insects in grain. Cereal Chem 80:553-557.

Kitto GB, Quinn FA, Burkholder WE (1993) Development of immunoassays for quantitative detection of insects in stored products. In: Highley E, Wright EJ, Banks HJ, Champ BR, editors. Proceedings of the 6th International Working Conference on Stored-Product Protection; 17–23 April 1993; Canberra, Australia. p. 415–420.

Maghirang EB, Dowell FE, Baker JE, Throne JE (2003) Automated detection of single wheat kernels containing live or dead insects using near-infrared reflectance spectroscopy. Transactions of the ASAE 46:12-1282.

Mendoza A, Fernández S, Cruz MA, Rodríguez-Perez MA, Resendez-Perez D and Saldaña HAB (2006) Detection of the genetically

modified maize food products by the polymerase chain reaction. Cienc Tecnol Aliment 3:175-181.

Neethirajan S, Karunakaran C,. Jayas DS and White NDG (2007) Detection techniques for stored-product insects in grain. Food Control 18:157-162.

Obrepalska-Steplowska A, Nowaczyk K, Holysz M, Gawlak M and Nawrot J (2008) Molecular techniques for the detection of granary weevil (Sitophilus granarius L.) in wheat and flour. Food Addit and Contam. Part a-Chemistry Analysis Control Exposure & Risk Assessment 25:1179-1188.

Pearson TC, Brabec DL, Schwartz CR (2003) Automated detection of internal insect infestations in whole wheat kernels using a Perten SKCS 4100. Appl Eng Agric 19:727–733.

Perez-Mendoza J, Throne JE, Dowell FE, Baker JE (2003) Detection of insect fragments in wheat flour by near-infrared spectroscopy. J Stored Prod Res 39:305–312.

R Core Team (2014) R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL http://www.R-project.org/

Sachdeva AS (1978) Effect of infestation stage, form and treatment on fragment count in flour. M.S. thesis, Kansas State University, Manhattan.

Shah MA and Khan AA (2014) Imaging techniques for the detection of stored product pests. Appl Entomol Zool 49:201-212.

Sittisuang P and Imura O (1987) Damage of rough and brown rice by four stored product insect species. Appl Entomol Zool 22:585–593.

Stejskal V, Hubert J, Aulicky R, Kucerova Z (2014) Overview of present and past and pest-associated risks in stored food and feed products: European perspective. J Stored Prod Research 1-11.

Storey CL, Sauer DB, Ecker O and Fulk DW (1982) Insect infestations in wheat and corn exported from the United States. J Econ Entomol 75 (5):827-832.

Throne JE, Perez-Mendoza J, Maghirang EB, Dowell FE and Baker JE (2007) Insect fragments in flour: Relationship to Lesser Grain Borer (Coleoptera: Bostrichidae) infestation level in wheat and rapid detection using near-infrared spectroscopy, Integrated Protection of Stored Products IOBC/wprs Bulletin 30 (2), p. 99-109.

Toews MD, Campbell JF, Arthur FH, Ramaswamy SB (2006) Outdoor flight activity and immigration of Rhyzopertha dominica into seed wheat warehouses. Entomol Exp Appl 121:73–85.

Toews MD, Perez-Mendoza J, Throne JE, Dowell FE, Maghirang E, Arthur FH and Campbell JF (2007) Rapid assessment of insect fragments in flour milled from wheat infested with known densities of immature and adult Sitophilus oryzae (Coleoptera : Curculionidae). J Econ Entomol 100:1714-1723.

Trematerra P (2013) Aspects related to decision support tools and Integrated Pest Management in food chains. Food Control 34:733-742.

Wells JD and LaMotte LR (1995) Estimating Maggot Age from weight Using Inverse prediction. J Forensic Sci 40(4):585-590.

2

Detection and identification of five common internal grain insect pests by multiplex PCR

PEST MONITORING WITH MULTIPLEX PCR

Introduction

Cereal grain, either as raw or processed material, constitutes 80% of consumed food (Pimentel et al., 1997). Unfortunately, since the routine procedures before food consumption harbor several pest species, the safety and security of this food are susceptible to being affected when grain is stored, transported and processed (Hagstrum, Reed, & Kenkel, 1999; Nopsa et al., 2015; Stejskal, Hubert, Aulicky, & Kucerova, 2015). Phillips and Throne (2010) estimated post-harvest losses due to stored-product insects of between 9% and 20% or more in developed and developing countries, respectively. Among insect pests, internal feeders, which are primary pest species that develop and feed inside the grain kernels, have generally been regarded as the most damaging pests of stored cereals (Toews, Campbell, Arthur, & Ramaswamy, 2006). These cereal grain, either as raw or processed material, constitutes 80% of consumed food (Pimentel et al., 1997). Unfortunately, since the routine procedures before food consumption harbor several pest species, the safety and security of this food are susceptible to being affected when

grain is stored, transported and processed (Hagstrum, Reed, & Kenkel, 1999; Nopsa et al., 2015; Stejskal, Hubert, Aulicky, & Kucerova, 2015). Phillips and Throne (2010) estimated post-harvest losses due to stored-product insects of between 9% and 20% or more in developed and developing countries, respectively.

Among insect pests, internal feeders, which are primary pest species that develop and feed inside the grain kernels, have generally been regarded as the most damaging pests of stored cereals (Toews, Campbell, Arthur, & Ramaswamy, 2006). These species not only consume large quantities of grain, but are hidden inside the grain kernels during their preimaginal development. Furthermore, these insects facilitate grain contamination by secondary pests, which might increase the damage to the food by depositing faeces and cast skins. These may cause localized increases in heat and moisture that might lead to accelerated mold growth and mycotoxin production threatening the grain quality and human health (Beti, Phillips, & Smalley, 1995; Phillips & Throne, 2010; Shah & Khan, 2014).

Because these internal feeders are not easily detected and removed during routine cleaning or processing practices, a situation where contamination is underestimated can often occur (Perez-Mendoza, Throne, Maghirang, Dowell, & Baker, 2005; Toews et al., 2006). Hence,

Storey, Sauer, Ecker, and Fulk (1982) reported that 12% of wheat samples from export loads contained hidden internal insects in the United States. Consequently, it is not surprising that primary pests are mainly present in filth contamination of finished cereal products (Trematerra, Stejskal, & Hubert, 2011). The most concerning internal feeders in grain worldwide are the following five species: *Rhyzopertha dominica* (F.) (Coleoptera: Bostrichidae); three species of the genus *Sitophilus* (*S. granarius* (L.), *S. oryzae* (L.) and *S. zeamais* (Motschulsky) (Coleoptera: Curculionidae)) and *Sitotroga cerealella* (Olivier) (Lepidoptera: Gelechiidae) (Castañé & Riudavets, 2015; Toews et al., 2007; Trematerra, Ianiro, Athanassiou, & Kavallieratos, 2015). Also, *Prostephanus truncates* (Horn) (Coleoptera: Bostrichidae), which is an important internal feeder of stored maize and cassava, has also become a serious pest in tropical and subtropical areas (CABI, 2017).

The increased consumer concerns about food safety and wholesomeness have produced a general trend toward a decrease in tolerance of live insects in food (Hagstrum et al., 1999; Trematerra, 2013). This situation has brought changes in grain standards in terms of food quality, which has emphasized the need for regulative approaches in the commercial sequence from the growers to consumers, driving market changes, politically and industrially (FDA, 1997; Stejskal,

Aulicky, & Kucerova, 2014). For example, domestic flour millers generally report zero tolerance for live insects, while the national agency in charge of food safety in the US, the Food & Drug Administration (FDA), has produced administrative guidelines that set maximum levels for natural or unavoidable defects in food for humans (FDA, 1997). Because failure to control insect infestations when they initially occur in storage (or in the field) can lead to extensive contamination of the stored grain that could affect food security (Nopsa et al., 2015), the importance of establishing strategies for early diagnosis of insect contamination is evident.

With the purpose of detecting insect contamination, hazard analyses are routinely conducted in grain industries. At the moment, grain is inspected with sieves and all sorts of methods to crack kernels for the identification of insect adults, damaged kernels or insect fragments. However, when those visual methods are used alone, internal infestations are not evident (Brader et al., 2002; Hubert, Nesvorna, & Stejskal, 2009). Additionally, insect fragments produced are not equivalent at each development stage of the pest (immature stages and eggs have low to no chitin content, respectively), highlighting the need for other analysis approaches (Brabec, Pearson, Flinn, & Katzke, 2010; Perez- Mendoza et al., 2005).

Nowadays, there is a panoply of techniques available for insect detection (Hagstrum & Subramanyam, 2014; Neethirajan, Jayas, & White, 2007; Parkin, 1956; Phillips & Throne, 2010; Trematerra, 2013). Unfortunately, although acoustic emissions, ELISA, NIR and X-ray are diagnostic techniques that are capable of detecting hidden infestations (Chen & Kitto, 1993; Fleurat-Lessard, Tomasini, Kostine, & Fuzeau, 2006; Fornal et al., 2007; Maghirang, Dowell, Baker, & Throne, 2003; Perez-Mendoza et al., 2005), they also present some limitations. Among their main drawbacks, some of these approaches do not accomplish the cost-time compromise, while others are less sensitive to low population densities (Neethirajan et al., 2007; Nowaczyk et al., 2009).

In recent years, the application of molecular techniques has gained importance in food diagnostics because of their simplicity, speediness and specificity (Obrepalska-Steplowska, Nowaczyk, Holysz, Gawlak, & Nawrot, 2008; Solà, Lundgren, Agusti, & Riudavets, 2017). DNA-based approaches such as PCR have become relevant for the analysis of genetically modified organisms (GMOs) in food (Ciabatti, Froiio, Gatto, Amaddeo, & Marchesi, 2006; Datukishvili, Kutateladze, Gabriadze, Bitskinashvili, & Vishnepolsky, 2015), as well as for identifying insect species (Barcenas, Unruh, & Neven, 2005; Zhang et al., 2016), providing an excellent method for both adult and immature

forms even for sibling species (Correa, de Oliveira, Braga, & Guedes, 2013; Hidayat, Phillips, & FrenchConstant, 1996; Peng, Lin, Chen, & Wang, 2002). Among PCR approaches, the multiplex is the most suitable technique for screening multiple species because it is able to simultaneously identify all species present in a sample within a single PCR reaction (King et al., 2011; Solà, Agusti, & Riudavets, 2015). It also offers simplicity of execution, a reduction of carryover errors and time saving, compared to the traditional singleplex PCR (Bai et al., 2009).

A multiplex PCR approach was here developed and described as a reliable molecular method for routine detection and identification of the five main internal feeders in grain samples, namely: the lesser grain borer (*R*. *dominica*), the three grain weevils species (*S*. *granarius*, *S*. *oryzae*, and *S*. *zeamais*) and the Angoumois grain moth (*S*. *cerealella*). One major consideration was to perform a large specificity test covering a wide range of species potentially present in stored grain facilities. The sensitivity of this protocol has been determined taking into consideration all developmental stages of the insect pests (egg to adult), the post-mortem time, different grain types and the potential of a grain treatment with modified atmospheres. Finally, some real commercial samples have been analyzed using the developed method.

Material and methods

1. Biological material

Five target pest species (*R. dominica*, *S. granarius*, *S. oryzae*, *S. zeamais* and *S. cerealella*) were maintained in laboratory cultures at IRTA (Barcelona, Spain). Coleopteran species were grown on organic rice (Eco-Salim, Maquefa, Spain), while the Lepidoptera species was reared on maize (Crit d'or, Granollers, Spain). All insect cultures were maintained in climatic chambers at 28º C, 70% RH, and 16L: 8D.

Forty-six species were tested in the specificity test of the designed primers. The specimens of these non-target species were found in alimentary factory surveys since 1997 or came from laboratory colonies (Table 5). Identification of all species was performed using morphological keys before storing the specimens in alcohol 96º or frozen at -20º C until DNA extraction.

The following insect-free grain and pasta were also tested for the characterization of the protocol: brown rice and wheat (Eco- Salim, Maquefa, Spain), maize (Crit d'or, Granollers, Spain), spelt (Biogrà, Polinyà, Spain), barley and oat (Celnat, Saint-Germain- Laprade, France) and macaroni pasta (Castagno Bruno, Giaveno TO, Italy). In order to ensure that the food samples used in the analyses were insect-free, a sample of 125 g of each grain and pasta was maintained at 28º C,

and 70% RH for three months and checked for insect adult presence by sieving it with a 2 mm mesh. Also, for the same purpose, three samples of 5 g of each grain type and pasta were first ground with a laboratory grinder (Laboratory Mill 3303, Perten Instruments, Hägersten, Sweden) to be then analyzed for insect presence with the multiplex PCR described below.

2. DNA extraction and multiplex PCR

Two different DNA extraction protocols were performed: one for the insect DNA extraction and another for the grain (infested or not). Insect DNA was extracted from whole individuals using a SpeedTools Tissue DNA extraction kit (Biotools, Madrid, Spain) and eluted in 100 µl of AE buffer. In addition, 5 g (or 10 g in the case of the sensitivity test) of homogenized infested grain and pasta DNA was extracted with the Extragen Alimentos extraction kit (Sistemas Genómicos, Valencia, Spain) following the manufacturer's instructions and eluted in 1 ml of purified water. One negative control was included in each DNA extraction group. DNA was stored at -20 °C until PCR.

Multiplex PCR reaction volumes (10 µl) contained 5 µl of 2x Multiplex PCR Master Mix (Qiagen), 2 µl of primer mix, 2 µl of DNA template, 1 µl of purified water and 0.05 µl of BSA [100 mg/ml]. Primer concentrations in the primer mix were different depending on the species

(Table 6). Samples were amplified in a 2720 thermal cycler (Applied Biosystems, CA, USA) for 35 cycles at 94 °C for 30 s, 60 °C for 90 s and 72 °C for 60 s. An initial denaturation step was carried out at 95 °C for 15 min and a final extension step was performed at 72 °C for 10 min. Targeted DNA and water were always included as positive and negative control in the PCR, respectively. Obtained PCR products were run by electrophoresis in 1.5% agarose gel stained with ethidium bromide and visualized under UV light.

3. Primer design and specificity

Four pairs of species-specific primers were designed to target the mitochondrial cytochrome oxidase I (COI) region of the three *Sitophilus* species (*S*. *granarius*, *S*. *oryzae* and *S*. *zeamais*) and the moth *S*. *cerealella* (Table 6).

Table 6. Specific primer pairs designed for each target species. The corresponding primer concentration (μM) used in the primer mix and the number of base pairs (bp) amplified are also indicated.

Target species	Primer name and sequence (5'-3')	Primer concentration (μM)	Amplicon size (bp)
S. cerealella	**SCF4:** GATACTTATTACGTAGTTGCTC	0.4	93
	SCR4: TAAGGGGTATCAATGAATG	0.4	
S. zeamais	**SZF2:** CTCCCTCCATCATTAATTC	0.6	151
	SZR3: TACCTGCTATATGAAGAC	0.6	
S. oryzae	**SOF4:** TGGAAACTGATTAATCCCAT	0.1	213
	SOR2: CTGAAAATGGCCAGATCAAC	0.1	
*R. dominica**	**RDF1:** GCTTCTTCCACCCTCCTTAACC	0.6	286
	RDR1: AGATAATAATAAAAGCAAAGC	0.6	
S. granarius	**SGF1:** CGTTACTGCTCACGCATTT	0.2	452
	SGR1: TAGTAATTGCTCTAGCTAAG	0.2	

*Designed by Solà et al. (2017).

For that purpose, we first searched all the sequences present in the GenBank for each target species. When more than one sequence was found, they were aligned with ClustalW2 (www.ebi.ac.uk/Tools/msa/clustalw2). Since all sequences showed a 100% of homology, we chose the largest one. Sequences selected for primers design of the four target species corresponded to the accession numbers: DQ200131, AY131101, AY131099 and AY131100 for *S. cerealella*, *S. granarius*, *S. oryzae* and *S. zeamais*, (JQ989165) were aligned and compared for non-conserved regions. In the case of *R. dominica*, a previously developed pair of primers (RdF1/RdR1), which amplified a fragment of 286 bp, was used (Solà et al., 2017).

Specificity was assessed by testing at least 10 individuals of each target species from our laboratory rearings. In order to confirm the detection of other populations of the target species, additional analysis of individuals (n= 3) from other origins were also performed. Populations tested were: three of *R. dominica* (one from Portugal, one from Rumania and one from Turkey); and four of *S. oryzae* (one from Andalusia (Spain), one from Portugal, one from France and one from Greece). Also, three individuals from the other 46 non-target species, except two individuals in two of them and one individual in one of them were tested (Table 5). To ensure the presence of DNA in those samples

that gave a negative result, they were also amplified using universal primers as a positive control. The following universal pairs of primers were used depending on the species (see Table 5): ZBJ-ArtF1C/ZBJ-ArtR2C (Zeale, Butlin, Barker, Lees, & Jones, 2011), Uni-MinibarF1/Uni-MinibarR1 (Meusnier et al., 2008) or LCO1490/HCO2198 (Folmer, Black, Hoeh, Lutz, & Vrijenhoek, 1994). The DNA was amplified following the protocols described in those studies. If the expected fragment obtained using these universal pair of primers was not amplified, the specimen was not considered in the specificity analysis. The designed primers sequences were also compared by performing a BLAST (www.blast.ncbi.nlm.nih.gov/Blast.cgi) in order to find potential cross-reactions with other species.

Table 5. Insect species potentially present in stored products tested for specificity with the developed Multiplex PCR protocol. In bol the five target species (n>10). The order, the family, the origin, the collection year and the universal primer set used for the diagnosis DNA presence of each sample is indicated. Three samples of each non target species were analyzed except for *Trogoderma glabru* (n=2) or *Dinerella agra* and *Alphitobius laevigatus* (n=1).

Order	Family	Species	Origin	collection year	Universal Primer set*
Coleoptera	Anobiidae	*Lasioderma serricorne* (Fabricius)	lab colony (IRTA), Spain	2013	ZBJ-ARTF1c/ ZBJ-ArtR2c
		Stegobium paniceum (Linnaeus)	field sample, Spain	2002	ZBJ-ARTF1c/ ZBJ-ArtR2c
	Bostrychidae	*Lyctus brunneus* (Stephens)	field sample, Spain	2003	Uni-MinibarF1/ Uni-MinibarR1
		Dinoderus minutus (Fabricius)	field sample, Vietnam	2002	Uni-MinibarF1/ Uni-MinibarR1
		***Rhyzopertha dominica* (Fabricius)**	lab colony (IRTA), Spain**	2013	ZBJ-ARTF1c/ ZBJ-ArtR2c
		Prostephanus truncatus (Horn)	field sample, Mexico	2010	ZBJ-ARTF1c/ ZBJ-ArtR2c
	Bruchidae	*Acanthoscelides obtectus* (Say)	lab colony (IRTA), Spain	2013	ZBJ-ARTF1c/ ZBJ-ArtR2c
		Zabrotes subfasciatus (Boheman)	lab colony (IRTA), Spain	2013	ZBJ-ARTF1c/ ZBJ-ArtR2c
	Chrysomelidae	*Callosobruchus maculatus* (Fabricius)	lab colony (IRTA), Spain	2013	ZBJ-ARTF1c/ ZBJ-ArtR2c
	Cleridae	*Necrobia rufipes* (Fabricius)	field sample, Spain	2010	ZBJ-ARTF1c/ ZBJ-ArtR2c
	Curculionidae	***Sitophilus granarius* (Linnaeus)**	lab colony (IRTA), Spain**	2013	ZBJ-ARTF1c/ ZBJ-ArtR2c
		***Sitophilus oryzae* (Linnaeus)**	lab colony (IRTA), Spain**	2013	ZBJ-ARTF1c/ ZBJ-ArtR2c
		***Sitophilus zeamais* (Motschulsky)**	lab colony (IRTA), Spain**	2013	ZBJ-ARTF1c/ ZBJ-ArtR2c
	Dermestidae	*Dermestes haemorrhoidalis* (Küster)	field sample, Spain	2006	ZBJ-ARTF1c/ ZBJ-ArtR2c
		Dermestes maculatus (DeGeer)	field sample, Spain	2011	ZBJ-ARTF1c/ ZBJ-ArtR2c
		Trogoderma glabrum (Herbst)	lab colony (IRTA), Spain	2013	Uni-MinibarF1/ Uni-MinibarR1
		Trogoderma granarium (Everts)	lab colony (IRTA), Spain	2013	LCO1490/ HCO2198
		Trogoderma inclusum (Leconte)	lab colony (IRTA), Spain	2013	Uni-MinibarF1/ Uni-MinibarR1
	Laemophloeidae	*Cryptolestes ferrugineus* (Stephens)	lab colony (IRTA), Spain	2013	ZBJ-ARTF1c/ ZBJ-ArtR2c
		Cryptolestes pusillus (Schonherr)	lab colony (JKI), Germany	2015	ZBJ-ARTF1c/ ZBJ-ArtR2c
		Cryptolestes turcicus (Grouvelle)	lab colony (JKI), Germany	2015	ZBJ-ARTF1c/ ZBJ-ArtR2c

	Latridiidae	*Dinerella arga* (Reitter)	field sample, Spain	2000	ZBJ-ARTF1c/ ZBJ-ArtR2c
		Dinerella fillum (Aubé)	field sample, Russia	2004	ZBJ-ARTF1c/ ZBJ-ArtR2c
	Silvanidae	*Ahasversus advena* (Waltl)	field sample, Spain	2006	Uni-MinibarF1/ Uni-MinibarR1
		Oryzaephilus mercator (Fauvel)	field sample, Spain	2004	Uni-MinibarF1/ Uni-MinibarR1
		Oryzaephilus surinamensis (Linnaeus)	lab colony (IRTA), Spain	2013	Uni-MinibarF1/ Uni-MinibarR1
	Ptinidae	*Niptus hololeucus* (Faldermann)	field sample, Spain	2015	ZBJ-ArtF1c/ ZBJ-ArtR2c
	Tenebrionidae	*Alphitobius diaperinus* (Panzer)	field sample, Spain	2007	Uni-MinibarF1/ Uni-MinibarR1
		Alphitobius laevigatus (Fabricius)	field sample, Spain	2013	ZBJ-ArtF1c/ ZBJ-ArtR2c
		Gnathocerus cornutus (Fabricius)	field sample, Spain	2006	Uni-MinibarF1/ Uni-MinibarR1
		Latheticus oryzae (Waterhouse)	lab colony (JKI), Germany	2015	ZBJ-ARTF1c/ ZBJ-ArtR2c
		Tenebrio molitor (Linnaeus)	lab colony (JKI), Germany	2015	ZBJ-ARTF1c/ ZBJ-ArtR2c
		Tribolium confusum (Jaqueline du Val)	lab colony (IRTA), Spain	2013	ZBJ-ARTF1c/ ZBJ-ArtR2c
		Tribolium castaneum (Herbst)	lab colony (IRTA), Spain	2013	ZBJ-ARTF1c/ ZBJ-ArtR2c
	Trogossitidae	*Tenebroides mauritanicus* (Linnaeus)	field sample, Spain	1999	ZBJ-ARTF1c/ ZBJ-ArtR2c
Hymenoptera	Bethylidae	*Cephalonomia spp*.	field sample, Spain	2015	LCO1490/ HCO2198
	Braconidae	*Habrobracon hebetor* (Say)	lab colony (IRTA), Spain	2013	ZBJ-ARTF1c/ ZBJ-ArtR2c
	Ichneumonidae	*Venturia canescens* (Gravenhorst)	lab colony (IRTA), Spain	2013	ZBJ-ARTF1c/ ZBJ-ArtR2c
	Pteromalidae	*Anisopteromalus calandrae* (Howard)	lab colony (IRTA), Spain	2013	ZBJ-ARTF1c/ ZBJ-ArtR2c
		Lariophagus distinguendus (Förster)	lab colony (IRTA), Spain	2013	ZBJ-ARTF1c/ ZBJ-ArtR2c
Lepidoptera	Pyralidae	*Ephestia cautella* (Walker)	lab colony (JKI), Germany	2015	ZBJ-ARTF1c/ ZBJ-ArtR2c
		Ephestia elutella (Hübner)	lab colony (JKI), Germany	2015	ZBJ-ARTF1c/ ZBJ-ArtR2c
		Ephestia kuehniella (Zeller)	lab colony (IRTA), Spain	2013	ZBJ-ARTF1c/ ZBJ-ArtR2c
		Plodia interpunctella (Hübner)	lab colony (IRTA), Spain	2013	ZBJ-ARTF1c/ ZBJ-ArtR2c
	Gelechiidae	***Sitotroga cerealella* (Olivier)**	lab colony (IRTA), Spain**	2013	ZBJ-ARTF1c/ ZBJ-ArtR2c
	Tineidae	*Nemapogon granella* (Linnaeus)	field sample, Spain	1999	ZBJ-ARTF1c/ ZBJ-ArtR2c
Mesostigmata	Ascidae	*Blattisocius tarsalis* (Berlese)	lab colony (IRTA), Spain	2001	Uni-MinibarF1/ Uni-MinibarR1
Pseudoscorpionida	Withiidae	*Withius piger* (Simon)	field sample, Spain	2011	ZBJ-ARTF1c/ ZBJ-ArtR2c
Psocoptera	Liposcelididae	*Liposcelis botrichophila* (Badonnel)	field sample, Spain	1997	LCO1490/ HCO2198
Sarcoptiforme	Acaridae	*Tyrophagus perniciosus* (Zakhvatkin)	lab colony (IRTA), Spain	2013	ZBJ-ARTF1c/ ZBJ-ArtR2c
		Tyrophagus putrecentiae (Schrank)	field sample, Spain	1997	LCO1490/ HCO2198

* ZBJ-ArtF1c and ZBJ-ArtR2c (Zeale et al., 2011); Uni-MinibarF1 and Uni-MinibarR1 (Meusnier et al., 2008); LCO1490 and HCO2198 (Folmer et al., 1994)

**The species coming from laboratory colonies (IRTA) were originally from Tarragona, Spain.

4. Characterization of the multiplex PCR: sensitivity, post-mortem detection, detection in different grains and in treated grain

To characterize the multiplex PCR method developed here, four tests were conducted: determination of the sensitivity threshold, determination of the post-mortem detection period, detection of larvae in different grains, and analysis of treated and untreated rice using *S. zeamais* eggs. In all experiments, insects were maintained in climatic chambers at 28ºC, 70% RH and 16L: 8D. Three replicates consisting of three independent DNA extractions were tested in all experiments and each independent DNA extraction was tested up to three times, being considered positive if at least one of them was positive. In the sensitivity test, only one DNA extraction was conducted, which was also tested three times.

The sensitivity threshold of the multiplex PCR developed here was determined by performing artificial infestations with the equivalent of 100, 10, 1 and 0.1 pupae/kg of rice. For that purpose, 20 g of rice infested with two pupae of each specieswas ground and used as a base for preparing all the insect infestation doses. The highest infestation dose tested (100 pupae/kg of rice) corresponded to a subsample of 10 g of this infested and ground grain. The remaining insect doses were obtained through serial mixtures of 90 g of ground insect-free rice homogenized

with 10 g of ground infested grain from the preceding infestation dose. Therefore, the highest infestation dose corresponded to a sample of 10 g of infested grain, while the rest consisted in subsamples of 10 g extracted from 100 g of infested grain.

In order to determine the post-mortem detection period, five adults (one of each target species) killed by freezing at -80º C for 20 min were maintained for different periods in small vials with some rice at 28º C, 70% RH and 16L: 8D to allow DNA degradation. After, 0, 30, 90, 150, 365, 548 and 760 days, insects were frozen at -20º C until DNA extraction to stop their degradation.

Insect detection in different kinds of grain was tested by conducting artificial infestations of *S. zeamais* adults in six different grains: barley, maize, oat, spelt, rice and wheat, as well as in pasta (macaroni). For that purpose, 250 g of organic cereal or pasta was infested with 10 adults of *S. zeamais* and maintained for 15 days in the climatic chamber in the same conditions described above. Then, grain was sieved with a 2 mm mesh to collect the adults and divided into two portions of 125 g; one was ground and frozen for molecular analysis, while the other one was maintained in the climatic chamber (same conditions) for 40 days. The *S. zeamais* adults that emerged from the second portion of grain were counted after sieving as a way to estimate

the number of hidden larvae present in the first portion used for molecular analysis.

In order to determine whether the developed multiplex PCRwas able to detect *S*. *zeamais* eggs in treated, as well as in untreated, grain, 1.5 kg of brown rice was infested with 10 *S*. *zeamais* adults. One week later, the infested rice was sieved to eliminate the introduced adults and divided into three equal parts. Two portions were treated with a modified atmosphere of 90% CO_2 for 12 days before grinding. This CO_2 dose is known to be efficient for killing eggs of these species (Riudavets, Castañé, Alomar, Pons, & Gabarra, 2009). The third portion remained untreated. This one and one of the previously treated portions were ground and frozen at -20º C until DNA extraction. The other treated portion was maintained for 40 days under the same controlled conditions as above to check for the presence of adults.

5. Analysis of commercial samples

Some commercial grain samples from a real Spanish industry were analyzed for the presence of the five target species using the developed multiplex PCR method. These grain samples came from the routine procedure of this industry when newgrain arrives from the field to be processed. This procedure consists in taking a portion of 1 kg of grain and sieving it to check for insect presence. Then, the same 1 kg of

grain samples were sent to our laboratory for further analyses. Once in the laboratory, all samples were first sieved with a 2 mm mesh and the obtained insects were counted and identified. Then, each sample was divided into two equal portions of 500 g; one was ground and frozen at -20º C for molecular analysis, while the other one was maintained for at least 40 days in the climatic chamber (same conditions as above) to check for adult insect presence after this period of time. Five commercial samples originally from France (one from 20th May 2015, another one from 29th May 2015, two from different silos from 14th March 2016 and one from 31st May 2016) were analyzed in total. Three replicates consisting of three independent subsamples of 5 g were analyzed by multiplex PCR per each of the samples received, except for one of the samples from 14th March 2016, where only 2 multiplex PCR where carried out. Each sample was considered positive when at least one out of three of these subsamples was positive for insect presence.

6. Data analysis

DNA amplification observed in the agarose gels was scored as 1 or 0 according to the presence or absence of the expected band, respectively. Then, the frequency of the positive amplification was calculated. For the sensitivity test and the analysis of post-mortem detection, a logistic regression to the data was performed with JMP®

(Version 8.0.1). In the sensitivity test, pest species and infestation dose were used as sources of variation, while in the post-mortem analysis, time and species were the selected factors. The relationship between the results obtained by sieving and by multiplex PCR to diagnose insect presence in commercial samples was studied with a Pearson's correlation using SigmaPlot (Systat Software, San Jose, CA).

Results

1. Primer specificity

The multiplex PCR developed here using the five species specific primer pairs successfully amplified the expected amplicons (Fig. 11) when our laboratory rearing specimens were tested. In the case of *S. oryzae*, some specimens amplified two bands, the expected one of 213 bp and a faint one smaller than 151 bp. Nevertheless, the amplification of all *S. oryzae* specimens tested led to the same pattern and did not interfere with the identification of the other four target species. When specimens of *S. oryzae* and *R. dominica* from other origins (different populations) were tested, all of them were also amplified.

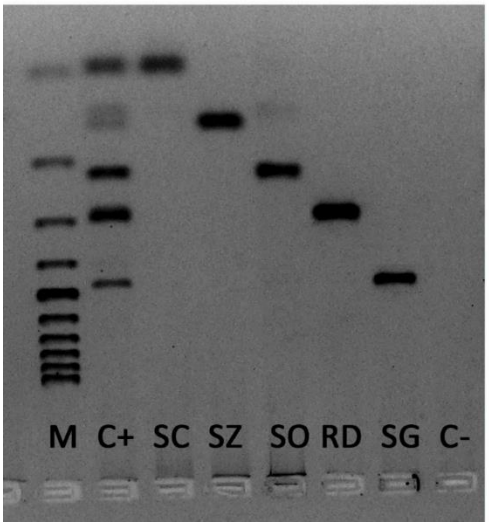

Figure 11. Agarose gel electrophoresis of the PCR products amplified with the designed multiplex PCR. **M**: molecular marker (100 bp ladder). **C+**: Positive control of the 5 target species (mixture of 1 µl of DNA extraction of each of the target species in 15 µl of purified water). **SC**: *S. cerealella*, **SZ**: *S. zeamais*, **SO**: *S. oryzae*, **RD**: *R. dominica*, **SG**: *S. granarius*, **C-**: Negative control (purified water).

When the other 46 insect species were tested with the designed multiplex PCR, only the five target species showed the expected band, proving a high specificity for the five target species (Table 5). It is a major consideration that when those 46 species that gave a negative amplification with the designed protocol were tested using insect universal primers, they all gave a positive amplification, thereby

demonstrating the presence of insect DNA. When the potential cross-reactivity of the designed primers with sequences of other species was tested by performing a BLAST, the only species identified using both forward and reverse designed primers were the target species with a 100% of matches and an e-value<1. The only exceptionwas the pair of primers of *S*. *granarius*, which also matched *Ichneumonidae sp*., which are not pest species of stored products.

2. Characterization of the designed multiplex PCR: sensitivity, post-mortem detection, detection in different grains and in treated grain

When different artificial infestation doses (100, 10, 1 and 0.1 pupae of each species/kg of rice) were tested to determine the sensitivity of the multiplex PCR, the sensitivity threshold was determined on the doses of 0.1 pupa per kilo of rice for the three *Sitophilus* species and *S*. *cerealella*, while *R*. *dominica* was detected up to 10 pupae per kilo of rice (Table 7; Fig. 12). DNA amplification among infestation doses did not present statistical differences (X^2=5.99, DF= 3, P=0.112). However, the DNA diagnosis differed among the internal feeder species (X^2=14.92, DF= 4, P= 0.005).

Table 7. Frequencies (%) of DNA amplification per each insect species at four different infestation doses and 6 post mortem periods

of time. Two replicates have been use for the infestation dose whereas 3 to analysis the time post mortem.

	infestation				Time post mortem					
species	**100**	**10**	**1**	**0,1**	**30**	**90**	**150**	**365**	**548**	**760**
S. cerealella	100	100	100	100	100	100	100	100	100	100
S. zeamais	100	100	100	100	100	100	100	100	100	100
S. oryzae	100	100	100	100	100	100	100	100	100	100
S. granarius	100	100	100	100	100	100	100	100	67	0
R. dominica	100	100	0	0	100	100	100	100	33	0

The analysis of a mixture of five adults (one from each target species) killed at 0, 30, 90, 150, 365, 548 and 760 days showed a post-mortem detection period of 365 days. After 548 days, this molecular method was less able to detect DNA from *S. granarius* and *R. dominica*. However, *S. cerealella*, *S. zeamais* and *S. oryzae* were still detected up to 760 days (more than two years) after insect death (Table 7; Fig. 13). The logistic regression performed showed that the time post-mortem and the insect species affected significantly the insect diagnosis (X^2= 22.23, DF= 5, P= 0.0005 and X^2= 18.28, DF= 4, P= 0.0011, respectively).

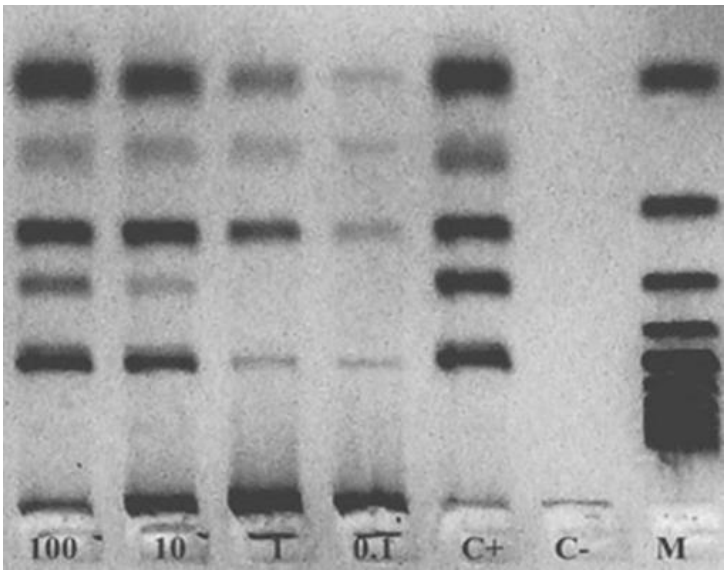

Figure 12. PCR products obtained when testing the sensitivity of the designed multiplex PCR with the five internal feeders (*S. cerealella, S. zeamais, S. oryzae, R. dominica, S. granarius*) in different artificial pupae infestation doses in rice (**100:** 100 pupae/Kg, **10:** 10 pupae/Kg, **1:** 1 pupae/Kg, **0.1:** 0.1 pupae/kg, **C+:** positive control of the 5 target species (mixture of 1 µl of DNA extraction of each of the target species in 15 µl of purified water), **C-:** negative control (purified water), **M:** molecular marker (100 bp ladder).

The DNA of *S. zeamais* was successfully amplified in all the artificial infestations conducted in 250 g of different grains (barley,

maize, oat, spelt, rice and wheat) and pasta (macaroni) with 10 adults of this species for 15 days. These positive results were corroborated when 121,104,147,135 and 156 *S. zeamais* adults were obtained in rice, wheat, oat, barley and spelt, respectively, after sieving the portion maintained under controlled conditions for 40 days. Because no insect adults were obtained in maize, three subsamples of 5 g of a ground mixture of 130 *S. zeamais* adults (the average of the insects' offspring obtained in the other grains) in 125 g of this maize were analyzed by multiplex PCR.

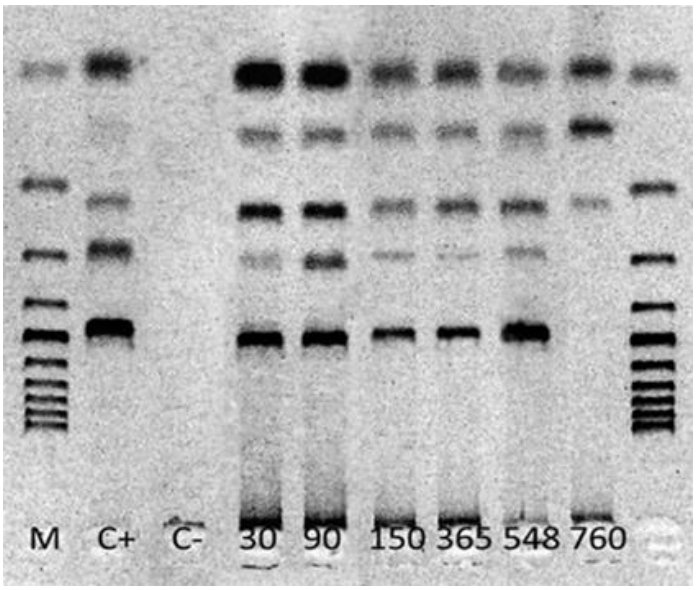

Figure 13. PCR products amplified from the five internal feeders (*S. cerealella, S. zeamais, S. oryzae, R. dominica, S. granarius*) after different post-mortem periods (**M:** molecular marker (100 bp ladder); **C+:** positive control of the 5 target species (mixture of 1 µl of DNA

extraction of each of the target species in 15 µl of purified water); **C-:** negative control purified water); **30, 90, 150, 360, 548** and **760**: number of days postmortem).

The analysis of the maize showed *S. zeamais* DNA amplification (Fig. 14). Similarly, no adults, and only seven small larvae, were obtained in the macaroni pasta. For this reason, we replaced seven insect-free macaroni from the molecular portion with these seven infested macaroni from the climatic chamber portion.

The analysis of the portion destined to molecular diagnosis by multiplex PCR confirmed the ability of the technique to detect the immature *S. zeamais* in the artificially infested pasta (Fig. 14). On the other hand, when the non-infested 250 g portion maintained under controlled conditions was sieved, no insect adult was observed. Also, when the non-infested 250 g portion used for the molecular analysis was tested by multiplex PCR, no DNA amplification was obtained either. This confirmed that before manipulation, cereals and pasta where insect free. Finally, when brown rice infested with *S. zeamais* and treated with 90% CO2 for 12 days; and not treated brown rice infested with *S. zeamais* were analyzed by multiplex PCR, *S. zeamais* infestations were detected in both cases. As expected, no *S. zeamais* adults were obtained

from the treated grain portion maintained in the climatic chamber for 40 days.

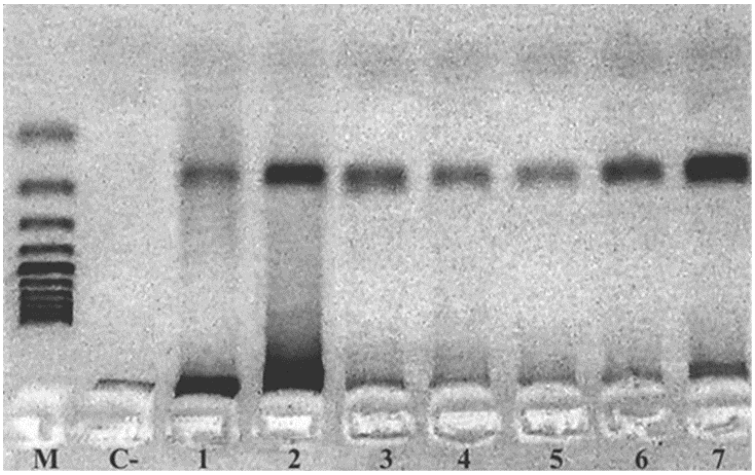

Figure 14. Agarose gel electrophoresis of the DNA amplification of *Sitophilus zeamais* larvae in different cereals (**M**: molecular marker (100 bp ladder); **C-**: negative control (purified water); **1:** spelt, **2:** wheat, **3:** rice, **4:** oat, **5:** barley, **6:** pasta, **7:** maize).

3. Analysis of commercial samples

The molecular analysis of the grain samples from a Spanish industry was coherent with the results obtained by sieving in the same industry. The five samples which were received the following dates: 20th May 2015, 29th May 2015, two from 14th March 2016, and 31st May

2016, were again sieved in the laboratory and divided into two portions: one for adult emergence and the other one for molecular analysis. We were informed by the industry that two of them were positive for Sitophilus spp. adults. They were the samples from 29th May 2015 and 31st May 2016. After sieving these two samples in the laboratory, one and six *Sitophilus* spp. adults were obtained, respectively. Forty days later, another sieving was performed and two Sitophilus spp. adults were observed in both samples. The molecular analysis of these samples showed that they were *S*. *oryzae*. More specifically, in the first sample, the three subsamples gave a positive result for S. oryzae, while in the second sample, two positives were obtained for this species out of three subsamples. The rest of the samples (20th May 2015, 14th March 2016 a and b) were negative for insect presence in the industry, as well as in our laboratory, after sieving twice and after performing the multiplex PCR. Moreover, results obtained by sieving (either in the industry or in the laboratory) were highly correlated with the results obtained by multiplex PCR ($r= 0.86$, $DF= 12$, $P< 0.0001$). In fact, when a sample was considered negative after sieving, was always negative by multiplex PCR. On the other hand, a positive result obtained by sieving, was also positive by multiplex PCR in the 83% of the occasions.

Discussion

In the present study, a multiplex PCR protocol has been developed to detect primary pest species in grain, offering significant advantages for routine analysis. This protocol showed high sensitivity by successfully detecting 0.1 pupa from *S. granarius*, *S. oryzae*, *S. zeamais* and *S. cerealella* per 1 kilo of rice (1 pupa per 10 kilos) and 10 pupae per kilo of rice in the case of *R. dominica*. This sensitivity threshold is similar to or even overpasses the regulatory standards for insect presence in food factories or commercial trade standards using the most common detection techniques. These defect action levels are commonly based on macro-analytical visual detection of adults, insect fragments or insect-damaged kernels (IDKs) (Chen & Kitto, 1993). In addition, Toews et al. (2007) and Perez-Mendoza et al. (2005) demonstrated that near-infrared reflectance spectroscopy (NIRS), one of the techniques used for insect detection, shows an important variability when analyzing samples with fewer than 100 insect fragments per kilo of wheat flour, and was unable to reach the quality standard set by the FDA (75 insect fragments per 50 g of wheat flour) (Brabec et al., 2010). On the other hand, X-ray, which is an official standard method in the USA (Fornal et al., 2007), despite appearing to have the greatest potential for being introduced in the food industry for insect detection (Neethirajan et al., 2007), is not sensitive

enough to accurately detect eggs and small larvae (Karunakaran, Jayas, & White, 2003). Similarly, note that in the diagnosis of insect presence based on IDKs, the damage caused by insect eggs or small larvae is null or inappreciable.

Previous studies have demonstrated the potential of introducing molecular techniques in grain industries as a tool for diagnosing insect presence. Solà et al. (2017) reached sensitivity thresholds of 13 eggs/small larvae, 0.1 big larvae/pupae and 0.02 adults of *R*. *dominica* per kilo of rice, using quantitative PCR (qPCR). Alternatively, Obrepalska-Steplowska et al. (2008) reached a sensitivity threshold equivalent to 0.01 *S*. *granarius* adults per kilo of wheat flour using qPCR. This information might help managers from the food industry make decisions about rejecting batches, storing grain, using control measures, processing grain or transporting it to another market outlet with less stringent standards (Brabec et al., 2010; Hagstrum et al., 1999). However, molecular approaches, although they are able to detect all life stages of the target primary pests, are not able to discriminate among life stages in mixed populations. This could be a drawback since stored grain usually has insect pests of mixed ages. Although it is not essential to determine the developmental stage of the pests for grain grading, the

precision of insect developmental stages could help to make the most of management decisions on processing the grain (Dowell, 1998).

Degradation of the DNA of dead organism's increases with time post-mortem and this might hamper a successful DNA amplification. For this reason, the detection range of the five target insect species has been determined by analysing several periods after insect death, showing that the developed multiplex PCR was able to detect adults of the five pest species even one year after death. After this period of time, the technique was not able to detect DNA from *S*. *granarius* or *R*. *dominica*, but was still able to detect *S*. *cerealella*, *S*. *zeamais* and *S*. *oryzae* even after two years. The bigger size of the amplified amplicons for *S*. *granarius* and *R*. *dominica* could be the reason for losing their detection earlier. The detection of immature *S*. *zeamais* DNA in CO_2-treated grain samples also shows the ability of the technique to detect dead immature stages of *S*. *zeamais*, and suggests that this might be the case for the other target species. The fact that dead insects can be detected for long periods of time has positive and negative aspects. On the one hand, the detection of dead insects provides an idea of the contamination in the analyzed grain, even in the past. On the other hand, the inability to discriminate between dead and alive insects could lead to an overestimation of the control

measures needed with a consequent overtreatment of the grain (Solà et al., 2017).

This method enhances the accuracy of the identification of insects based on their specific detection. Because different species have different behaviors and cause different levels of grain loss requiring different approaches to control them (Cao et al., 2015), in this work we have developed a multiplex PCR protocol rather than a singleplex PCR approach (Solà et al., 2015). This enhanced the specific and simultaneous identification of the five target pest species by easily recognizing the precise bands of different molecular weights in the agarose gels (Fig. 11). The universality of the designed primers is suggested by the positive detection of other populations of *S. oryzae* and *R. dominica* with different origins, as well as for the high homology that presented the sequences of all populations of each target species present in GenBank. Nevertheless, in the case that in future tests other populations of the five target species different from those tested in this study may be present, we recommend to conduct a previous specificity test in order to confirm the correct amplification of the target species population. The cross-reactivity test performed with the 51 species potentially present in stored and agroecosystem environments (Table 5) ensured the specific identification of only the target species. The potential cross-reactivity of

the designed primers tested by performing a BLAST also demonstrated their high specificity.

The immature stages of some of these species are particularly difficult to recognize, as in the case of the three *Sitophilus* species studied here. Although most identification procedures rely on the morphological characterization of the adults, in the case of sibling species, such as *S. oryzae* and *S. zeamais*, this is tedious and needs the expertise of a taxonomist and the use of microscopy techniques (Hidayat et al., 1996; Peng et al., 2002). Using the multiplex PCR designed here, we succeeded not only in distinguishing the *Sitophilus* adults, as achieved in other studies (Correa et al., 2013; Hidayat et al., 1996; Peng et al., 2002), but also simultaneously recognizing immature stages of these sibling species in a single PCR reaction.

On the other hand, it is well known that *S. oryzae* is more resistant to phosphine, which is one of the most commonly used chemical insecticides in stored grain worldwide, than its sibling species, *S. zeamais* (Hagstrum et al., 1999). Therefore, the use of the present PCR method would help managers use appropriate control measures according to the species present. Usually, only insect eggs or first-instar larvae are present after fumigation (Brabec et al., 2010). Thus, since routine analysis techniques are based on visual lures, those infestations

may evade diagnosis and then the storability of the grain may be underestimated.

We have also demonstrated success in detecting *S. zeamais* eggs in rice after a treatment with CO_2 for 12 days, suggesting that this method would also detect other developmental stages of the other four target species after a treatment of this kind, thereby avoiding future increases of pest populations and therefore decreasing the grain downgrade. In this respect, the use of the present protocol would help managers to fumigate only once infestations reached a critical density (commonly considered to be more than two insects/ kg of grain) (Flinn, Hagstrum, Reed, & Phillips, 2010) and avoid unnecessary treatments based on standard calendars (Trematerra, 2013). This would be in line with the increasing public concerns beyond the overuse of agricultural chemicals that are harmful to the environment and human health (Bulathsinghala & Shaw, 2013) and would prevent the development of insect resistance due to continuous fumigation (Hagstrum & Subramanyam, 2014; Phillips & Throne, 2010).

The dominant grain crops grown worldwide are rice, wheat, maize, millet, barley and rye (Pimentel et al., 1997), and internal feeders are frequently found in all of them when grain is stored. For this reason, we have tested the detection of the five target insect pests in most of

those grains. As a model, we tested the detection of larvae of *S. zeamais* in rice, wheat, maize, oat, spelt and barley. Since these grains are usually processed before consumption, in order to ensure that insects are also detected in processed grain we analyzed the presence of this weevil in macaroni pasta. The results obtained showed that the method was able to detect immature stages of *S. zeamais* in all these grains and in pasta, thereby suggesting that this multiplex PCR method would also detect all developmental stages of the five target species.

The fact that no weevil offspring was observed in the infested maize highlights the global effort to select varieties resistant to insect presence in the most valuable grain crops (Abebe, Tefera, Mugo, Beyene, & Vidal, 2009). Nevertheless, the ability of the present method to detect insects in maize was demonstrated with the amplification of the expected band for *S. zeamais* in the agarose gel when analyzing samples consisting in a ground mixture of weevil adults and maize. On the other hand, only seven small larvae were obtained from the macaroni pasta when sieving. A comparison of this number with the others obtained from the rest of the grains (an average of 132 insect adults) reveals that although *S. zeamais* can lay eggs inside pasta, this substrate is not the most suitable for the development of this pest in comparison to the other grain cereals tested.

Insect infestations can occur during the storage process in manufacturing facilities, warehouses, general stores and retail shops, but insects can colonize food at any processing step, providing situations where insects might reach consumers (Jayas, White, & Muir, 1995). The stability of DNA, which can withstand temperatures of pasteurization and sterilization (Laube et al., 2007), suggests that the use of molecular approaches as a diagnostic technique in food factories would enable unambiguous identification of insects in food at any processing point. However, further studies should be conducted in order to corroborate this statement, particularly after the manufacturing process of pasta. This might be an advantage, particularly ahead of approaches based on proteins such as ELISA, where false-positive situations can occur due to the denaturation of proteins at temperatures above 56º C (Velebit, Markovic, Jankovic, & Borovic, 2009).

After analyzing commercial samples from a grain industry, results obtained by multiplex PCR were in accordance with those obtained by the operator of the industry. This demonstrates the potential of this molecular method for being introduced in processing industries for diagnosing insect presence. PCR-based methods are commonly accepted and recommended for food quality control, such as the detection of GMOs or for food traceability (Bai et al., 2009; Laube et al.,

2007). In this sense, the detection of non-desired insects in food by molecular tools is suitable as a food control measure as has already been suggested by Obrepalska-Steplowska et al. (2008), Solà, Riudavets, & Agusti (2015) and Solà et al. (2017).

The determination of the correct sampling of the grain in order to detect infestations is often inaccurate because insect infestations are not homogeneous in grain facilities. Nevertheless, the present method identifies insect infestations with high accuracy and sensitivity when grain is thoroughly homogenized. However, in order to ensure that the obtained information is representative of the real grain contamination, it is important to establish an adequate number of samples of a determinate size (Jian, Jayas, & White, 2014a, b). Once samples are defined, the transmission of this information to grain managers in food industries should help them to implement IPM practices, develop economic thresholds and set up decision-making strategies aimed at using pesticides more selectively and thus be more environmentally friendly while at the same time preventing the undesired presence of insects in food. Additionally, the combination of the present multiplex PCR with a qPCR protocol for the detection of particular pest species, such as those developed by Solà et al. (2017) and Obrepalska- Steplowska et al. (2008), would provide an improved screenshot of the grain status.

Therefore, further work is needed to combine simultaneous identification of concerned primary pests with the quantification of real populations.

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References

Abebe, F., Tefera, T., Mugo, S., Beyene, Y., & Vidal, S. (2009). Resistance of maize varieties to the maize weevil Sitophilus zeamais (Motsch.) (Coleoptera: Curculionidae). African Journal of Biotechnology, 8(21), 5973- 5943.

Bai, W., Xu, W., Huang, K., Yuan, Y., Cao, S., & Luo, Y. (2009). A novel common primer multiplex PCR (CP-M-PCR) method for the simultaneous detection of meat species. Food Control, 20, 366-370.

Barcenas, N. M., Unruh, T. B., & Neven, L. G. (2005). DNA diagnostics to identify internal feeders (Lepidoptera: Tortricidae) of pome fruits of quarantine importance. Journal of Economic Entomology, 98, 299-306.

Beti, J. A., Phillips, T. W., & Smalley, E. B. (1995). Effects of maize weevils (Coleoptera: Curculionidae) on production of aflatoxin B1 by Aspergillus flavus in stored corn. Journal of Economic Entomology, 88(6), 1776-1782.

Brabec, D., Pearson, T., Flinn, P., & Katzke, D. (2010). Detection of internal insects in wheat using a conductive roller mill and estimation of insect fragments in the resulting flour. Journal of Stored Products Research, 46, 180-185.

Brader, B., Lee, R. C., Plarre, R., Burkholder, W., Kitto, G. B., Kao, C., et al. (2002). A comparison of screening methods for insect contamination in wheat. Journal of Stored Products Research, 38, 75-86.

Bulathsinghala, A. T., & Shaw, I. C. (2013). The toxic chemistry of methyl bromide. Human & Experimental Toxicology, 33, 81-91.

CABI. (2017). Available from: http://www.cabi.org/isc/datasheet/44524.

Cao, Y., Zhang, C., Chen, Q., Li, Y., Qi, S., Tian, L., et al. (2015). Identification of species and geographical strains of Sitophilus oryzae and Sitophilus zeamais using the visible/near-infrared hyperspectral imaging technique. Pest Management Science, 71, 1113-1121.

Castañé, C., & Riudavets, J. (2015). Sampling arthropod pests and natural enemies in stored barley. Journal of Stored Products Research, 64, 54-61.

Chen, W., & Kitto, G. B. (1993). Species-specific immunoassay for Sitophilus granarius in wheat. Food and Agricultural Immunology, 5, 165-175.

Ciabatti, I., Froiio, A., Gatto, F., Amaddeo, D., & Marchesi, U. (2006). In-house validation and quality control of real-time PCR methods for GMO detection: A practical approach. Developments in Biologicals, 126, 79-86.

Correa, A. S., de Oliveira, L. O., Braga, L. S., & Guedes, R. N. C. (2013). Distribution of the related weevil species Sitophilus oryzae and S. zeamais in Brazil. Insect Science, 20, 763-770.

Datukishvili, N., Kutateladze, T., Gabriadze, I., Bitskinashvili, K., & Vishnepolsky, B. (2015). New multiplex PCR methods for

rapid screening of genetically modified organisms in foods. Frontiers in Microbiology, 6, 757.

Dowell, F. E. (1998). Automated color classification of single wheat kernels using visible and near-infrared reflectance. Cereal Chemistry, 75, 142-144.

[FDA] Food and Drug Administration. (1997). 578.450, Wheat & flour adulteration with insect fragments and rodent hairs (CPG 7104.06). Compliance Policy Guides Manual, Chapter 5, Foods, color and cosmetics, Subchapter 578-processed grain http://www.fda.gov/ora/compliance_ref/cpg/default.htm#1998.

Fleurat-Lessard, F., Tomasini, B., Kostine, L., & Fuzeau, B. (2006). Acoustic detection and automatic identification of insect stages activity in grain bulks by noise spectra processing through classification algorithms. In I. Lorini, B. Bacaltchuk, H. Beckel, D. Deckers, E. Sundfeld, J. P. Dos Santos, et al. (Eds.), Proceedings of the 9th international working conference on stored product protection (Vols. 476e186). Passo Fundo, RS, Brazil: Brazilian Post-harvest Association - ABRAPOS, 15 to 18 October 2006, Campinas, S~ao Paulo, Brazil. (ISBN 8560234004).

Flinn, P. W., Hagstrum, D. W., Reed, C., & Phillips, T. W. (2010). Insect population dynamics in commercial grain elevators. Journal of Stored Products Research, 46, 43-47.

Folmer, O., Black, M., Hoeh, W., Lutz, R., & Vrijenhoek, R. (1994). DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. Molecular Marine Biology and Biotechnology, 3, 294-299.

Fornal, J., Jelinski, T., Sadowska, J., Stanislaw, G., Nawrot, J., Niewiada, A., et al. (2007). Detection of granary weevil Sitophilus granarius (L.) eggs and internal stages in wheat grain using soft X-ray and image analysis. Journal of Stored Products Research, 43, 142-148.

Hagstrum, D. W., Reed, C., & Kenkel, P. (1999). Management of stored wheat insect pests in the USA. Integrated Pest Management Reviews, 4, 127-143.

Hagstrum, D. W., & Subramanyam, B. (2014). A review of stored-product entomology information sources. American Entomologist, 55, 174-183.

Hidayat, P., Phillips, T. W., & FrenchConstant, R. H. (1996). Molecular and morphological characters discriminate Sitophilus oryzae and S-zeamais (Coleoptera: Curculionidae) and confirm

reproductive isolation. Annals of the Entomological Society of America, 89, 645-652.

Hubert, J., Nesvorna, M., & Stejskal, V. (2009). The efficacy of sieving, filth flotation and Tullgren heat extraction for detecting various developmental stages of Tribolium castaneum and Ephestia kuehniella in samples of wheat grain, flour and semolina. Journal of Stored Product Research, 45, 279-288.

Jayas, D. S., White, N. D. G., & Muir, W. E. (1995). Stored-grain ecosystems. New York: Marcel Dekker Inc.

Jian, F., Jayas, D. S., & White, N. D. G. (2014a). How many kilograms of grain per sample unit is big enough? Part I. Comparison of insect detection and density estimation between manual probe sampling and Insector (R) system. Journal of Stored Products Research, 56, 60-66.

Jian, F., Jayas, D. S., & White, N. D. G. (2014b). How many kilograms of grain per sample unit is big enough? Part II. Simulation of sampling from grain mass with different insect densities and distribution patterns. Journal of Stored Products Research, 56, 67-80.

Karunakaran, C., Jayas, D. S. J., & White, N. D. G. (2003). X-ray image analysis to detect infestations caused by insects in grain. Cereal Chemistry, 80, 553-557.

King, R. A., Moreno-Ripoll, R., Agusti, N., Shayler, S. P., Bell, J. R., Bohan, D. A., et al. (2011). Multiplex reactions for the molecular detection of predation on pest and nonpest invertebrates in agroecosystems. Molecular Ecology Resources, 11, 370-373.

Laube, I., Zagon, J., Spiegelberg, A., Butschke, A., Kroh, L. W., & Broll, H. (2007). Development and design of a 'ready-to-use' reaction plate for a PCR-based simultaneous detection of animal species used in foods. International Journal of Food Science and Technology, 42, 9-17.

Maghirang, E. B., Dowell, F. E., Baker, J. E., & Throne, J. E. (2003). Automated detection of single wheat kernels containing live or dead insects using nearinfrared reflectance spectroscopy. Transactions of the Asae, 46, 1277-1282.

Meusnier, I., Singer, G. A., Landry, C. J.-F., Hickey, D. A., Hebert, P. D. N., & Hajibabaei, M. (2008). A universal DNA mini-barcode for biodiversity analysis. Bmc Genomics, 9.

Neethirajan, S., Jayas, D. S., & White, N. D. G. (2007). Detection of sprouted wheat kernels using soft X-ray image analysis. Journal of Food Engineering, 81, 509-513.

Nopsa, J. F. H., Daglish, G. J., Hagstrum, D. W., Leslie, J. F., Phillips, T. W., Scoglio, C., et al. (2015). Ecological networks in stored grain: Key postharvest nodes for emerging pests, pathogens, and mycotoxins. Bioscience, 65, 985-1002.

Nowaczyk, K., Obrepalska-Steplowska, A., Gawlak, M., Throne, J. E., Olejarski, P., & Nawrot, J. (2009). Molecular techniques for detection of tribolium confusum infestations in stored products. Journal of Economic Entomology, 102, 1691-1695.

Obrepalska-Steplowska, A., Nowaczyk, K., Holysz, M., Gawlak, M., & Nawrot, J. (2008). Molecular techniques for the detection of granary weevil (Sitophilus granarius L.) in wheat and flour. Food Additives and Contaminants Part A. Chemistry Analysis Control Exposure & Risk Assessment, 25, 1179-1188.

Parkin, E. A. (1956). Stored product enomology (the assessment and reduction of losses caused by insects to stored foodstuffs). Annual Review of Entomology, 1, 223-240.

Peng, W. K., Lin, H. C., Chen, C. N., & Wang, C. H. (2002). DNA identification of two laboratory colonies of the weevils,

Sitophilus oryzae (L.) and S-zeamais Motschulsky (Coleoptera : Curculionidae) in Taiwan. Journal of Stored Products Research, 39, 225-235.

Perez-Mendoza, J., Throne, J. E., Maghirang, E. B., Dowell, F. E., & Baker, J. E. (2005). Insect fragments in flour: Relationship to lesser grain borer (Coleoptera : Bostrichidae) infestation level in wheat and rapid detection using near-infrared spectroscopy. Journal of Economic Entomology, 98, 2282-2291.

Phillips, T. W., & Throne, J. E. (2010). Biorational approaches to managing storedproduct insects, annual review of entomology. Annual Review of Entomology, 55, 375-397.

Pimentel, D., Wilson, C., McCullum, C., Huang, R., Dwen, P., Flack, J., et al. (1997). Economic and environmental benefits of biodiversity. BioScience, 47, 747-757.

Riudavets, J., Castañe, C., Alomar, O., Pons, M. J., & Gabarra, R. (2009). Modified atmosphere packaging (MAP) as an alternative measure for controlling nine pests that attack processed food products. Journal of Stored Products Research, 45(2), 91-96.

Shah, M. A., & Khan, A. A. (2014). Imaging techniques for the detection of stored product pests. Applied Entomology and Zoology, 49, 201-212.

Solà, M., Agusti, N., & Riudavets, J. (2015). Molecular approaches based on PCR to identify and quantify arthropod internal feeders in grain. Integrated Protection of Stored Products IOBC-WPRS Bulletin, 111, 387-393.

Solà, M., Lundgren, J. G., Agusti, N., & Riudavets, J. (2017). Detection and quantification of the insect pest Rhyzopertha Dominica (F.) (Coleoptera: Bostrichidae) in rice by qPCR. Journal of Stored Product Pests, 71, 106-111.

Stejskal, V., Aulicky, R., & Kucerova, Z. (2014). Pest control strategies and damage potential of seed-infesting pests in the Czech stores: A review. Plant Protection Science, 50, 165-173.

Stejskal, V., Hubert, J., Aulicky, R., & Kucerova, Z. (2015). Overview of present and past and pest-associated risks in stored food and feed products: European perspective. Journal of Stored Products Research, 64, 122-132.

Storey, C. L., Sauer, D. B., Ecker, O., & Fulk, D. W. (1982). Insect infestations in wheat and corn exported from the United-States. Journal of Economic Entomology, 75, 827-832.

Toews, M. D., Campbell, J. F., Arthur, F. H., & Ramaswamy, S. B. (2006). Outdoor flight activity and immigration of Rhyzopertha

Dominica into seed wheat warehouses. Entomologia Experimentalis et Applicata, 121, 73-85.

Toews, M. D., Perez-Mendoza, J., Throne, J. E., Dowell, F. E., Maghirang, E., Arthur, F. H., et al. (2007). Rapid assessment of insect fragments in flour milled from wheat infested with known densities of immature and adult Sitophilus oryzae (Coleoptera: Curculionidae). Journal of Economic Entomology, 100, 1714-1723.

Trematerra, P. (2013). Aspects related to decision support tools and integrated pest management in food chains. Food Control, 34, 733-742.

Trematerra, P., Ianiro, R., Athanassiou, C. G., & Kavallieratos, N. G. (2015). Behavioral interactions between Sitophilus zeamais and Tribolium castaneum: The first colonizer matters. Journal of Pest Science, 88, 573-581.

Trematerra, P., Stejskal, V., & Hubert, J. (2011). The monitoring of semolina contamination by insect fragments using the light filth method in an Italian mill. Food Control, 22, 1021-1026.

Velebit, B., Markovic, B., Jankovic, V., & Borovic, B. (2009). Identification of animal species in heat treated muscle

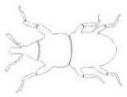
homogenates using the polymerase chain reaction. Acta Veterinaria (Belgrad), 59(2-3), 303-308.

Zeale, M. R. K., Butlin, R. K., Barker, G. L. A., Lees, D. C., & Jones, G. (2011). Taxonspecific PCR for DNA barcoding arthropod prey in bat faeces. Molecular Ecology Resources, 11, 236-244.

Zhang, T., Wang, Y.-J., Guo, W., Luo, D., Wu, Y., Kucerova, Z., et al. (2016). DNA barcoding, species-specific PCR and real-time PCR techniques for the identification of Six Tribolium pests of stored products. Scientific Reports, 6. article number: 28494.

3

Control of *Rhyzopertha dominica* and *Sitophilus zeamais* with *Anisopteromalus calandrae* in rice at two risk temperatures



BIOLOGICAL CONTROL OF WEEVILS

Introduction

Rice, with more than 700 million tons produced in 2014, is among the dominant grain crops grown worldwide (FAOSTAT, 2017). After harvest, rough rice is generally dried and stored under appropriate temperature and moisture content to preserve its quality. However, when stored, it is frequently attacked by a great diversity of insect pests. Chanbang et al. (2008) reported *Rhyzopertha dominica* (F.) (Coleoptera: Bostrichidae), *Sitophilus* spp. (*S*. *granarius* (L.), *S*. *oryzae* (L.) and *S*. *zeamais* (Motschulsky)) (Coleoptera: Curculionidae) and *Sitotroga cerealella* (Olivier), among the most concerning species in stored rice. In warmer zones, such as the Mediterranean region, and despite of his common name referred as a maize pest, *S*. *zeamais* is generally more abundant in rice than the other two *Sitophilus* species (Athanassiou et al., 2017; Smith, 1992). The attack of the rice by these pests leads to important quantitative and qualitative losses. Their voracious feeding causes weight loss through damaged kernels and frass production and facilitates the grain invasion by secondary pests (Hagstrum and Milliken,

1988; Trematerra and Savoldelli, 2014; Trematerra and Colacci, 2015). Additionally, these pests may be vectors of bacteria and molds that can produce harmful mycotoxines that threats the human's health (Beti et al., 1995; Fourar and Fleurat-Lessard, 1997; Gorham, 1979; Maier et al., 1996; Sinha and Sinha, 1992).

The introduction of natural enemies is one of the most encouraging tools for the control of the internal feeders, especially in closed environments where the grain is stored (Phillips and Throne, 2010; Steidle and Scholler, 2002). Among the natural enemies available in the stored product amenities, one of the most promising candidates as control agent for weevils is A*nisopteromalus calandrae* (Howard) (Hymenoptera: Pteromalidae) (Menon et al., 2002; Wen and Brower, 1994). This species is a generalist solitary ectoparasitoid that attack late instar coleopteran species that develop concealed inside the grain kernels such as *R*. *dominica* or *Sitophilus* species (Ahmed, 1996; Belda and Riudavets, 2012; Lucas and Riudavets, 2002; Menon et al., 2002; Schmale et al., 2001).

There are several studies focused on the assessment of the effectiveness of *A*. *calandrae* to control weevil pests, especially in stored wheat. Most of the works are based on laboratory observations that report the high potential of the parasitoid to suppress the weevils' population

(Adarkwah 2014; Belda and Riudavets, 2012; Chaisaeng, 2010; Cline, 1985; Khalafalla, 1996; Press and Mullen, 1992; Wen and Brower, 1994; Yoon et al., 2009). However, only few of them mention the capacity of the parasitoid to reduce the grain damage or the mold presence. Some studies report the numerical response of the parasitoid to the host density (Arbogast 1990, Khalafalla, 1996, Mahal et al. 2005). For example, studies with multiple parasitoid releases were it has been observed that the number of weevils decreases as the parasitoid density increases. Some studies have also reported lower parasitoid emergence with higher parasitoid density (Chianseng 2010, Mahal et al. 2005, Wen and Brower, 1994). Other studies have focused on the effect of the temperature on the parasitoid ability to supress the pest. Within a range of 20 to 30ºC, higher temperatures have been reported to offer better conditions for pest control. With this optimal temperaures, the parasitoid offspring number is increased, the intrinsic rate of parasitoid population increase is reduced and the host searching and handling time is also reduced (Menon et al., 2002, El-Aw, 2016; Smith; 1994).

Parasitoid fitness and sex allocation of *A. calandrae* have also been subject of many studies (Baker, 1998; Choi, 2001; Ghimire and Phillips, 2007; Ji et al., 2004; Van Den Assem, 1971). As an example,

the study of Ji et al. (2004) showed that the reproduction rate of large females was twice as high as for small females.

Despite the panoply of studies based on *A*. *calandrae*, there is still a lack of works where all the following factors are contemplated. First, the assessment of the optimal parasitoid density to control different pest populations. Second, the estimation of the grain damage reduction by the parasitoid under different conditions. Third, the consideration of different parasitoid parameters to predict if further parasitoid releases may be needed. For this reason, the aim of the present study was to evaluate the capacity of the parasitoid *A*. *calandrae* released in three different densities to control *R*. *dominica* or *S*. *zeamais* populations and to reduce their associated rice damage under two risk storage temperatures: 23ºC or 28ºC. The first temperature (23ºC) was reported as the minimal threshold temperature for the development of *R*. *dominica* (Navarro and Calderon;1980) while the second, 28ºC, is a temperature that could harbor elevated pest populations.

Material & methods

R. *dominica* and *S*. *zeamais* were reared at 28±2ºC; 70±10%RH; 16:8L on brown rice (Bahia variety). The parasitoid *A*. *calandrae* was

reared on 3 weeks old *R*. *dominica* or *S*. *zeamais* populations at 25±2ºC; 70±10% RH; 16:8L: D. To increment parasitoid's survival, sugary water was included in the rearing and in the trials. All weevils and parasitoid colonies were started with adults collected from several stored product warehouses in the north-east of Spain.

To assess the effectiveness of different *A*. *calandrae* release densities to control the two weevil species, tests were carried out under controlled conditions (23±2ºC and 28±2ºC at 70±10% RH and 16:8L:D) during two pests generations. Pest generation time of each weevil species was calculated according to their developing time in our rearing colonies. Before starting the experiment, rice was freeze at -20ºC for 96h to prevent any insect development and then kept at 25ºC for at least 24h for acclimation. Cylindrical plexi-glass containers of 2 L with 2 kg of rice were used in the experiments. These containers were ventilated by a hole on the lid that was covered with a mesh. Then, 34 *R*. *dominica* or 36 *S*. *zeamais* unsexed adults were released in each container. This represented approximately 10♀♀ weevils per kilo of rice according to the sex ratio observed when dissecting 100 adults from the respective rearing colonies. Once the pest species were estimated to be on 3rd to 4th instar larvae, three different parasitoid densities were released twice. For *R*. *dominica*, the first release was accomplished after 51 and 23 days at 23ºC

and 28ºC, respectively, while the second was completed after 63 and 28 days at 23ºC and 28ºC, respectively. For *S*. *zeamais*, the first parasitoid release was carried out after 28 days at 23ºC or after 20 days at 28ºC and the second after 34 days and 27 days at 23ºC and 28ºC, respectively. In each of both releases, 5, 10 or 20♀♀ parasitoids were introduced. These females were 1-4 days old and to assure that they had mated, they were maintained with males for 24h. For *R*. *dominica*, tests lasted 193 days at 23ºC and 84 days at 28ºC and for *S*. *zeamais* tests lasted 95 days at 23ºC and 71 days at 28ºC. According to the development time of the parasitoid estimated from our rearing colonies at the end of the experiment, *A*. *calandrae* had approximately nine generations at 23ºC and six at 28ºC when the host was *R*. *dominica*. Alternatively, when the host was *S*. *zeamais*, this time corresponded to approximately five parasitoid generations at both temperatures. Five replicates per each pest species, temperature, insect density and the respective control (parasitoid free containers) were carried out. Temperature (ºC) of the grain was weekly measured with a sounding line introduced in the center of the rice bulks (Testostor 171, Testo, Spain).

Once the estimated period to obtain two pest generations had passed, the rice containers were frozen at -20º C for at least 96 h to stop insect development. Afterwards, rice, frass, weevils and parasitoids were

sieved-separated and the number of weevils and *A*. *calandrae* were counted. In the case of *A*. *calandrae*, adults were also sexed. Rice and frass were weighted and the number of damaged and undamaged kernels of two random samples of 5 ml of rice per each container were visually inspected. The water activity (Aw) of the rice kernels was measured with an Aqualab Lite water activity meter (Decagon Devices, Inc.). Mold presence was evidenced by the presence of band of brownish colored rice, stuck to the bottom of the container. The quantity of mold in the rice containers was estimated by measuring (cm) the size of this band of rice that presented mold symptoms. In order to only assess the changes in temperature and water activity due to insect presence, measures of water activity and temperature from insect free rice maintained at 23ºC or 28ºC were used to correct the values in the treatments.

The following variables were assessed: percentage of host survival (number of pest adults emerged in the treatments divided by the average number of adults in the corresponding control), percentage of emergence of the parasitoid (number of emerged parasitoids in the treatment divided by the average number of pest in the corresponding control) and percentage of host mortality induced by the parasitoid (% of host induced mortality=100-(% of host survival+ % of successful

emergence of the parasitoid)). The body length and hind tibia length of 250 parasitoids were measured using ImageJ 1.x (Schneider et al., 2012).

The percentage of rice weight loss was calculated by resting to the initial rice weight (2000g) the value obtained from the division of final rice weight by the initial rice weight. The percentage of frass produced was calculated by dividing the weight of the frass at the end of the experiment by the final rice weight. On the other side, the percentage of insect damaged kernels was determined by dividing the number of damaged kernels by the total number of kernels.

1. Statistical analysis

Numerical data was log (x+1) transformed when needed while percentage data was arcsine transformed to comply with the requirements of parametric tests. A one way Anova analysis or a Kruskal–Wallis analysis (K-W) when the data did not comply with the requirements of parametric test, were used for the following parameters. First, to assess the effect of the parasitoid on each weevil adult emergence. Second, to assess the effect of the temperature on the number of weevil's offspring. Third, for the effect of the parasitoid density released on the percentage of host survival. Forth, for the comparison of female's body length emerged from both host species. The difference in rice temperature across time per each species and tested temperature and

the differences in mold presence among weevil species were also assessed with a one way Anova analysis. When needed, means where separated by the Tukey test (P<0.05). Alternatively, when K-W was significant, this test was followed by a pairwise Mann–Whitney *U*-tests and the *p*-values were corrected for multiple comparisons using the Bonferroni technique. A two way Anova to assess the effect of the temperature and parasitoid densities per each weevil species was applied to the number of parasitoid offspring and most of the rice quality parameters (percentage of weight loss, percentage of frass production, percentage of insect damaged kernels, water activity and mold presence). Also, a two way Anova was used to assess the effect of the sex and temperature on the parasitoid body and tibia length. A chi-square test was used for testing the proportion of *A*. *calandrae* females emerged. Finally, Pearson correlation analysis was used to relate body and tibia length measures. The relationship among the pest population and the rice damage (percentage of weight loss, percentage of frass production and percentage of insect damaged kernels) and the relationship between water activity and mold presence were also assessed with Pearson correlation analysis. For all the analyses, the statistical package JMP was used (SAS Institute Inc., 2009).

Results

The introduction of the parasitoid drastically reduced both weevils' adult emergence (K-W test for *R*. *dominica*: χ^2= 18.9, *P*<0.001 and one way Anova for *S*. *zeamais*: $F_{1,38}$=139.3, *P*<0.001) (Table 8). This reduction was higher at 28ºC than at 23ºC for both species (K-W test for *R*. *dominica*: χ^2=11.9, *P*<0.001 and for *S*. *zeamais*: χ^2=5.3, *P*=0.021). At 28ºC, the introduction of the lower parasitoid density (10♀♀) reduced both species more than 30 fold the weevil's population compared to the controls while at 23ºC the reduction was less than 7 fold. Moreover, *A*. *calandrae* reduced 5 fold more *S*. *zeamais* than *R*. *dominica* at the lower temperature and with the minimal density, whereas at 28ºC the reduction was similar in both species.

Table 8. Number (mean ± SE) of hosts produced at the two temperatures and parasitoid (Ac) densities tested.

T°	*R. dominica*				*S. zeamais*			
	0 ♀♀***Ac***	**10** ♀♀***Ac***	**20** ♀♀***Ac***	**40** ♀♀***Ac***	**0** ♀♀***Ac***	**10** ♀♀***Ac***	**20** ♀♀***Ac***	**40** ♀♀***Ac***
23ºC	4163± 685	2233±879	525±233	537±408	5389±153	716±188	250±86	165±62
28ºC	4064±556	108±15	83±31	44±3	6354±400	190±69	131±16	90±6

At 23ºC, the emergence of *R*. *dominica* adults in comparison with the emergence of adults in the control treatment was reduced to 26%. Parasitoid density did not significantly affected the percentage of host adult emergence (χ^2=3, df=2, *P*=0.221) although a tendency to decrease was observed: host adult emergence was fourfold lower when 40♀♀ *A*. *calandrae* where released (13%) than when 10♀♀ were released (53%) (Fig. 15. A). The main host mortality cause was host induced mortality (52%) while approximately 22% of the mortality was due to the successful emergence of the parasitoid. At 28ºC the overall adult emergence of *R*. *dominica* was on average 2% of that in the control and parasitoid density significantly affected this emergence (χ^2=8.3, df=2, *P*=0.016). When 10♀♀ *A*. *calandrae* were released, host adult emergence was three fold higher (3%) than with the release of 40♀♀ parasitoids (1%). At this temperature, host induced mortality was higher (85%) than at 23ºC while successful emergence of the parasitoid was lower (13%) (Fig. 15. B).

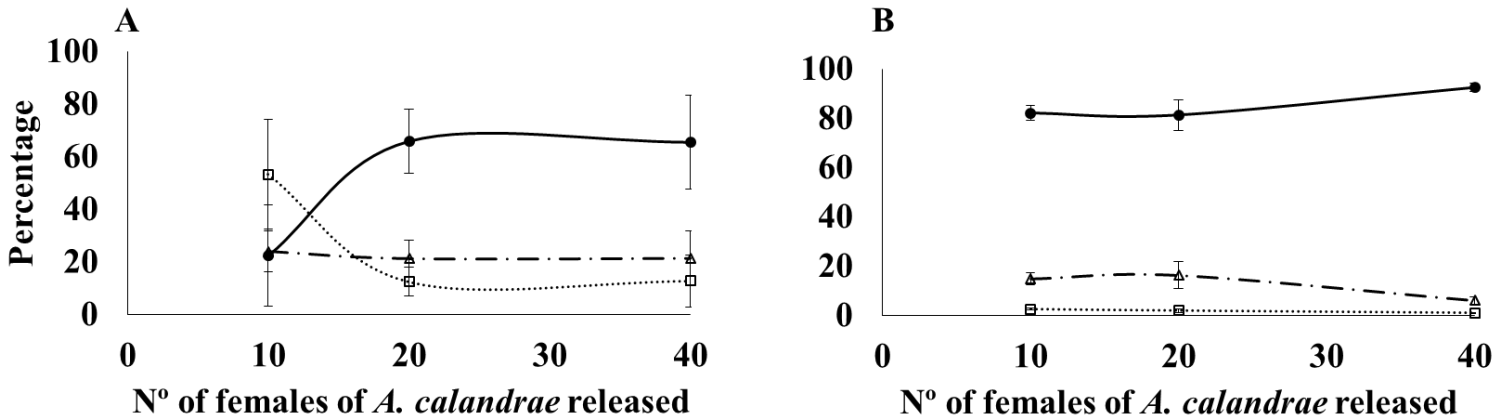


Figure 15. Percentage (mean ± SE) of emergence of *R*. *dominica* adults (··□··), of emergence of *A*. *calandrae* adults (·-Δ-·) and of parasitoid induced mortality (-●-) when three parasitoid densities (10, 20 and 40 ♀♀) were released at 23ºC (**A**) and 28ºC (**B**).

At 23ºC, the emergence of *S*. *zeamais* adults was 7% of that of the control and was significantly affected by the parasitoid density ($F_{2,12}$=5.6, *P*=0.019). The percentage of host adult emergence was lower when 40♀♀ were released (3%) than when 10♀♀ were released (13%). The main mortality cause was host induced mortality (60%) while 33% of mortality was due to successful emergence of the parasitoid (Fig.16. A). At 28ºC, the emergence of *S*. *zeamais* adults was 2% of that of the control and parasitoid density did not significantly affected the adult host emergence (K-W test: χ^2=6, df=2, *P*=0.05) although a tendency to decrease was observed: host adult emergence was threefold lower when 40♀♀ *A*. *calandrae* where released (1%) than when 10♀♀ were released (3%). The main mortality cause under these circumstances was also host induced mortality (81%) while successful emergence of the parasitoid represented 17% of the mortality (Fig. 16. B).

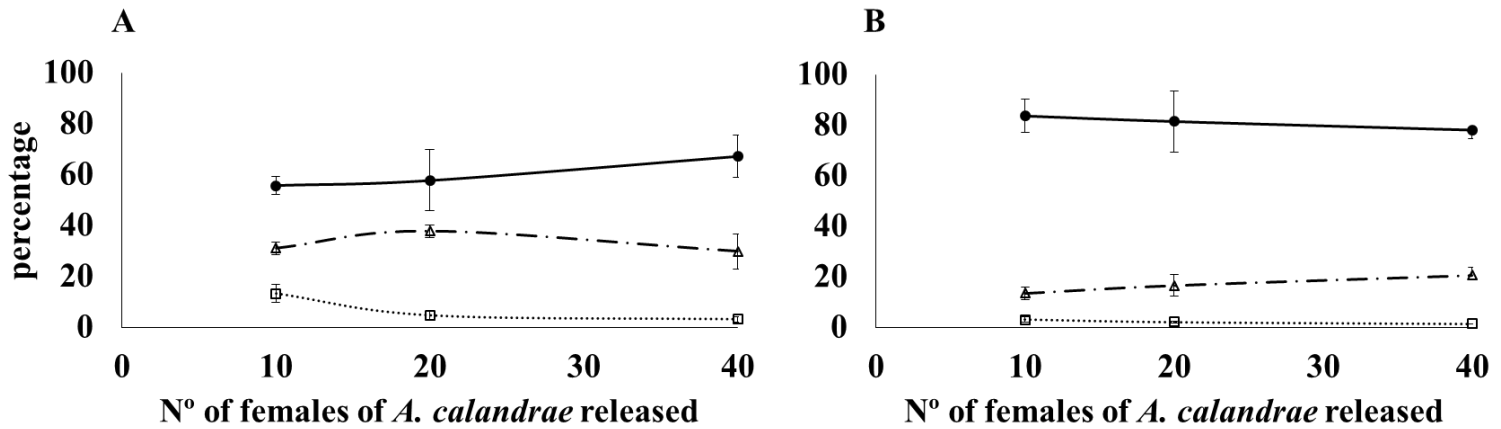


Figure 16. Percentage (mean ± SE) of emergence of *S*. *zeamais* adults (··□··), of emergence of *A*. *calandrae* adults (·-Δ-·) and of parasitoid induced mortality (-●-) when three parasitoid densities (10, 20 and 40 ♀♀) were released at 23ºC (**A**) and 28ºC (**B**).

The offspring produced by the parasitoid in both host species was neither affected by the temperature nor by the parasitoid density, although a tendency to have more progeny at the lower temperature and lower parasitoid density released, especially for *R*. *dominica*, was observed. When the host was *R*. *dominica*, parasitoid offspring was male biased at the higher parasitoid density (40♀♀) at both temperatures and also at medium density (20♀♀) at 28ºC. However, parasitoids produced on *S*. *zeamais* presented no bias in sex ratio under all tested conditions (Table 9).

Table 9. Number (mean ±SE) of parasitoid's offspring produced in each host species according to temperature and parasitoid density released. Percentage (mean ±SE) of females (in brackets) is shown only when significantly different from 50%.

	A. calandrae* on *R. dominica			***A. calandrae* on *S. zeamais***		
T°C	**10♀♀**	**20♀♀**	**40♀♀**	**10♀♀**	**20♀♀**	**40♀♀**
23°C	1002±319	892±282	895±182 (42±2)	1672±133	2032±261	1607±376
28°C	611±103	668±223 (38±2)	254±59 (37±5)	854±164	1314±104	1309±195

A. calandrae offspring from *R. dominica*: temperature ($F_{1,24}$=1.9, P=0.176), density ($F_{2,24}$=1.6, P=0.226). *S. zeamais* offspring: temperature ($F_{1,24}$=3.4, P=0.079), density $F_{2,24}$=0.4, P=0.68). % females of *A. calandrae* on *R. dominica*: 40♀♀ at 23ºC (t_4=-3.4, P=0.07), 20♀♀ at 28ºC (t_4=-5.2, df=4, P=0.01) and 40♀♀ at 28ºC (t_4=-2.8 df=4, P=0.01).

Female parasitoids developed on *S. zeamais* were significantly bigger than the ones developed on *R. dominica*, in terms of body and tibia length (K-W test for body length: χ^2=51.3, P<0.001 and for tibia length: χ^2=56.1, P<0.001). When the host was *R. dominica*, female and male parasitoids developed at 23ºC were significantly larger than those developed at 28ºC. However, only male parasitoids developed on *S. zeamais* at 23ºC were larger than those developed at 28ºC, although statistically significant only for body length (Table 10). Body and tibia length were positively correlated (r=0.9, df= 238, P<0.001), and females were bigger than males, independently of the host species in which they developed.

Table 10. Mean (±SE) body length and hind tibia length of females and males of *A. calandrae* that developed on *R. dominica* or on *S. zeamais* at two temperatures.

		A. calandrae* on *R. dominica		***A. calandrae* on *S. zeamais***	
Parameter	Tº	♀♀	♂♂	♀♀	♂♂
Body length (*μ*m)	23º	2099±40Aa	1754±35Ab	2384±76Aa	2024±51Ab
	28º	1783±56Ba	1347±51Bb	2485±27Aa	1806±56Bb
Tibia length (*μ*m)	23º	530±15Aa	464±10Ab	569±13Aa	508±14Ab
	28º	432±13Ba	324±8Bb	577±6Aa	476±13Ab

Values followed by the same upper case letter in the same column for each host species, sex and parameter are not significantly different between temperatures. Values followed by the same lower case letter in the same row for each temperature, host species and parameter are not significantly different for each sex. *A. calandrae* on *R. dominica* body length (Tº: $F_{1,16}$=66.4, P<0.001; sex: $F_{1,16}$=77.2, P<0.001), tibia length (Tº x sex: $F_{1,16}$=8.5, P<0.05). *A. calandrae* on *S. zeamais* body length (Tºx sex $F_{1,16}$=12.3, P<0.001) tibia length, (Tº: $F_{1,16}$=1.1, P=0.3; sex: $F_{1,16}$=47.6, P<0.001).

The number of weevils produced in each treatment was positively correlated with the grain damage calculated as percentage of weight loss (r= 0.9, df= 75, P<0.001), frass production (r=0.8, df=75, P<0.001) and insect damaged kernels (r=0.7, df=75, P<0.001).

When no parasitoids were released (control treatment), *R. dominica* caused a significantly lower percentage of rice weight loss, a higher percentage of frass production and a higher percentage of insect damaged rice kernels than *S. zeamais,* after two pest generations ($F_{1,18}$=11.82, P<0.01; $F_{1,18}$=49.8, P=0.001 and, $F_{1,18}$=18.2, P=0.001 for percentage of weight loss, percentage of frass and percentage of insect damaged kernels respectively). This high percentage of rice damage in the control treatments corresponded to an average of 18±4g and 48±7g of rice weight loss for *R. dominica* and *S. zeamais*, respectively and, 29±5g and 9±1g of frass produced for *R. dominica* and *S. zeamais*, respectively.

Table 11. Percentage (mean ± SE) of rice weight loss, of frass production and of insect damaged rice kernels at the end of the experiment caused by *R. dominica* or *S. zeamais* when developed under different *A. calandrae* (*Ac*) densities at 23ºC or 28ºC and their corresponding Anova parameters.

Rice damage parameters	T°C	*R. dominica* 0♀♀ *Ac*	10♀♀ *Ac*	20♀♀ *Ac*	40♀♀ *Ac*	Density Anova	*S. zeamais* 0♀♀ *Ac*	10♀♀ *Ac*	20♀♀ *Ac*	40♀♀ *Ac*	Density Anova
% of weight loss	23°C	8.6±0.6	4.6±1.5	2.2±0.5	1.9±0.9	$F_{3,32}$=41.9	11.6±2.6	2.8±0.6	1.1±0.8	0.9±0.4	$F_{3,32}$=53.5
	28°C	8.1±0.8	0.9±0.3	0.6±0.3	0.2±0.1	*P*<0.001	16.6±1.3	1.4±0.5	2.1±0.4	2.3±1.1	*P*<0.001
	Tº Anova	$F_{1,32}$=13.1, *P*<0.001					$F_{1,32}$= 3, *P*=0.092				
% of frass production	23°C	5.9±0.4	3.1±1.2	1±0.3	0.9±0.6	$F_{3,32}$=40.4	2.1±0.6	0.5±0.1	0.3±1	0.3±0.1	$F_{3,32}$=39.7
	28°C	6.1±0.7	0.3	0.2±0.1	0.1	*P*<0.001	2.7±0.3	0.1	0.1	0.1	P<0.001
	Tº Anova	$F_{1,32}$=6.5, *P*<0.05					$F_{1,32}$=0.2, *P*=0.697				
% of insect damaged kernels	23°C	79±5.2	43.8±13	17.1±2.8	19.5±10.5	$F_{3,32}$=21.4	32.7±6.7	7.9±1.8	1.4±0.2	2.6±0.5	$F_{3,32}$=55.1
	28°C	60.2±9.9	6.7±1.9	8.6±4.8	14.1±6.5	*P*<0.001	40.5±4.5	7.3±1.6	3.2±0.4	3.4±1	*P*<0.001
	Tº Anova	$F_{1,32}$=8.6, *P*<0.01					$F_{1,32}$=1.2, *P*=0.272				

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At the end of the experiment, rice damage (percentage of weight loss, percentage of frass production and percentage of insect damaged kernels) was significantly reduced by the introduction of *A*. *calandrae* in both species (Table 11). However, there were no significant differences among parasitoid densities on the rice damage produced by the two hosts. When the rice was infested with *R*. *dominica*, significantly more rice damage (in the three estimated percentage parameters) was observed at 23ºC than at 28ºC. However, when the rice was infested with *S*. *zeamais*, there were no differences in grain damage (in none of the three estimated percentage parameters) between temperatures (Table 11).

Rice temperature remained constant at 23±0.5ºC or 28±0.5ºC along the experiment in both host species and in all parasitoid densities tested. Only *S*. *zeamais* at 28ºC had a tendency to raise the temperature of the rice across time when no parasitoid was released ($F_{3,18}$=8.3, P=0.001). In this situation, average temperature was 30±1ºC, with a maximum of 34ºC.

When the rice was infested with *R*. *dominica*, the water activity was significantly higher at 23ºC than at 28ºC at the end of the experiment. The introduction of 10♀♀ *A*. *calandrae* significantly reduced its value. With this weevil species, molds were only present at 23ºC and the release of 20♀♀ *A*. *calandrae* significantly controlled its

presence (Table 12). When the rice was infested with *S*. *zeamais*, the water activity of the rice after two pest generations was significantly higher at 28ºC than at 23ºC except when 20♀♀ where released that the opposite situation was recorded. Nevertheless, the introduction of 20♀♀ *A*. *calandrae* at 23ºC and 10♀♀ at 28ºC significantly reduced its value. With *S*. *zeamais*, molds were present at both temperatures at the end of the experiment. At 23ºC, the introduction of 20♀♀ *A*. *calandrae* controlled its presence while at 28ºC the addition of 10♀♀ was enough for controlling the presence of molds. Moreover, in the rice containers free of parasitoids (control treatment), *S*. *zeamais* presented fourfold more molds than when the rice was infested with *R*. *dominica* (Table 12). In all treatments there was a significant and positive correlation between water activity and mold presence (r=1, n=48, P<0.0001).

Table 12. Mean (± SEM) water activity (Aw) and presence of molds (M) (cm) according to the temperature and *A. calandrae* (*Ac*) density tested in the rice containers infested with *R. dominica* or with *S. zeamais*.

		R. dominica				***S. zeamais***			
Parameter	**TºC**	**0♀♀ *Ac***	**10♀♀ *Acc***	**20♀♀ *Ac***	**40♀♀ *Ac***	**0♀♀ *Ac***	**10♀♀ *Ac***	**20♀♀ *Ac***	**40♀♀ *Ac***
Aw	**23º C**	0.76±0.017	0.70±0.004	0.70±0.002	0.69±0.004	0.69±0.027	0.64±0.011	0.58±0.007	0.58±0.004
	28º C	0.71±0.018	0.58±0.003	0.58±0.001	0.59±0.003	0.82±0.018	0.62±0.004	0.63±0.003	0.62
M	**23º C**	1.5±0.624	0.9±0.538	0	0	5.5±1.434	0.9±0.88	0	0
	28º C	0	0	0	0	7.4±1.213	0	0	0

For *R. dominica*, water activity (Tº x density: $F_{3,16}$=7.8, P=0.002) and mold presence (Tº x density: $F_{3,32}$=3, P=0.044). For *S. zeamais* water activity ($F_{3,16}$=12.2, P<0.001) and mold presence (Tº: $F_{1,32}$=0.02, P=0.848) and density ($F_{3,32}$=39.8, P<0.001).

Discussion

The introduction of the parasitoid highly reduced both pest populations and their associated rice damage (Tables 8, 11, 12 and Figures15, 16). This reduction was affected by three factors and their interaction: the host species, the temperature and the parasitoid density released.

Results show that 28ºC was the best tested temperature for the reduction of both pest adult emergence (Table 8). This result was not surprising when considering *A*. *calandrae* thermal preferences: optimal at higher temperatures (El-Aw et al., 2016; Ileleji et al., 2007; Smith, 1994). Menon et al. (2002) observed the same behaviour when assessing the functional response of this parasitoid attacking *R*. *dominica* on wheat over a range of temperatures. Additionally, our results also show that *A*. *calandrae* was more efficient controlling *S*. *zeamais* than *R*. *dominica*, especially at 23ºC (Table 8, Figures 15 & 16). This difference in pest control observed at 23ºC may be due to the reduced avaiability of *R*. *dominica* larvae for the parasitoid under these conditions, as a consequence of the long development period of *R*. *dominica* at lower temperatures. In fact, at 23ºC, *R*. *dominica*, was estimated to need 112 more days to complete two generations than at 28ºC.

For both species, the percentage of emergence of host adults was reduced along with the increase of the number of female parasitoids released (Figure 15 & 16). More specifically, for both pest species, the host emergence reduction was at least threefold higher with the release of 40♀♀ than with the release of 10♀♀. Then, for a better pest reduction, the release of the higher of parasitoid density may be the most effective parasitoid density. Especially at 23º C, where this relationship among host reduction and parasitoid density was more evident (Figure 15, A & 16, A). The high pest reduction observed was mainly due to parasitoid induced mortality while successful emergence of the parasitoid was low (Table 8, Figure 15 & 16). Parasitoid induced mortality has also been reported in other parasitoid species as the main source of pest control (Kasamatsu and Abe, 2015; Kidd and Jervis, 1989). In our experiment, parasitoid induced mortality, which was higher at 28ºC, involved host killing due to host feeding but also unsuccessful or incomplete parasitoid development and super- parasitism. Whether to use a host for host feeding or oviposition represents a reproductive tradeoff. According to Jervis et al. (2008), it is expected that host feeding decreases as the pest population increases (Heimpel and Collier, 1996). However, in our results, we observed that the parasitoid induced mortality (which involves host feeding) increased along with the host reduction (Fig.15 &

16). Then, as reported by Mahal et al. (2007) and Chaisaeng et al. (2010), we presume that this increase of parasitoid induced mortality at the higher parasitoid densities, when less host were present, was mainly due to super- parasitism or incomplete parasitoid development rather than to host feeding (Table 8, Figure 15 & 16). As mentioned, the experiment was designed to last two pest generations. This implicated the development of 5 or more parasitoid generations. However, by the time of stopping the experiment, a portion of the parasitoid population, probably, was still at immature stages. Then, we may have accounted them as parasitoid induced mortality rather than successful parasitoid reproduction. Furthermore, at 28ºC, insect species developed faster. Then, due to the increase in nutrient requirements, higher host feeding was expected. This, increased the parasitoid induced mortality numbers.

The introduction of the parasitoid represented a high reduction of rice damage in terms of weight loss, frass production and insect damaged kernels. As expected, this damage reduction was in accordance with the pest emergence reduction: the reduction of rice damage was higher at 28ºC (Tables 8 & 11). Other authors have also reported the relationship among pest population and grain damage. They have added a significant negative relationship among the number of weevils and the percentage of rice germination: The feeding of weevil's deplets the kernel nutritional

reserves needed for the germination (Abebe et al., 2009; Fourar and Fleurat-Lessard; 1997, Masasa et al., 2013).

When no parasitoid was introduced, frass production and insect damaged kernels were higher with *R*. *dominca* than with *S*. *zeamais*. However, *S*. *zeamais* produced more percentage of rice weight loss although less frass production and insect damaged kernels. The main explanation for that unexpected result is that the rice infested by *S*. *zeamais* presented higher mold presence than the rice infested by *R*. *dominca*. This mold presence increased the rice weight. However, the presence of molds produced big masses of sticky rice that embedded high part of the frass produced and the insect damaged kernels. Then, both values may have been underestimated when no parasitoid was released (Table 12).

The rice temperature, the moisture content and the mold presence can facilitate the development of insect populations while the inverse situation (the development of insect population increases the rice temperature, the moisture content and the mold presence) is also common. As a result, the rice quality may be alterated (Jian and Jayas, 2012, Pedersen, 1992) (Table 12). Our results show that only *S*. *zeamais* at 28ºC raised the rice temperature along with the experiment and this species presented higher mold presence than *R*. *dominica* when no

parasitoid was released. According to that, *S*. *zeamais* represents a major threat to the rice than *R*. *dominica*. This result is supported by Birch (1947) who found that *S*. *oryzae*, a close species of *S*. *zeamais*, consumed oxygen more rapidly than *R*. *dominica,* heating the stored grain and also by Potter (1935) who reported *S*. *zeamais* as better mold disperser than *R*. *dominica*. In fact, Beti et al. (1995) had also described the positive correlation of *S*. *zeamais* and aflatoxin content in grain as a result of the weevil metabolic activity but also because of carrying mold spores internally and externally.

It is generally considered that fungal growth on cereals is not significant at water activity inferior to 0.62±0.70 (Abdullah et al., 2000). This statement was confirmed in all of our trials except for *R*. *dominica* at 28ºC that overpassed this moisture threshold without presenting molds. Nevertheless, the introduction of *A*. *calandrae* controlled the raise of these three parameters (rice temperature, water activity of rice and mold presence).

At 23ºC, the release of at least 20♀♀ was needed to control the mold presence for the rice infested by the two species. Alternatively, at 28ºC the introduction of 10♀♀was enough to suppress the mold presence in the rice (which was only present when the rice was infested with *S*. *zeamais*).

The parasitoid fitness, estimated as number of parasitoid offspring, percentage of females and body and tibia length, is an indicator of the status of *A*. *calandrae* population. The consideration of these parameters for a biocontrol management plan may help to reveal if the parasitoid is under stress and in consequence, may facilitate the prediction of the need of further releases for a long term storage situations (Arbogast 1990; Chianseng 2010, Mahal et al. 2005, Wen and Brower, 1994). A good parasitoid fitness is indicated by a high number of parasitoid offspring that are large and female biased (Charnov et al., 1981; Choi et al., 2001; Kasamatsu and Abe, 2015; Lebreton et al., 2009; Ji et al., 2004). The higher reduction of *S*. *zeamais* emergence compared to *R*. *dominica* (Table 8 and Figure 15 & 16) as well as the higher *A*. *calandrae* offspring of balanced sex ratio (Table 9) and larger body size (Table 10) obtained with *S*. *zeamais* compared to *R*. *dominica* suggested that *S*. *zeamais* is a more suitable host for *A*. *calandrae* than *R*. *dominica*. This better suitability of *Sitophilus sp*. for *A*. *caladrae* observed has been also demonstrated in other studies when comparing the close species *S*. *oryzae* to *R*. *dominica* (Belda and Riudavets, 2012; Ghimire and Phillips, 2007).

The emerged parasitoids were bigger at 23ºC that at 28ºC for both species (Table 10). This observation was also supported by Smith (1992)

when assessing the effect of temperature on life history characteristics of *A. calandrae* parasiting *S. zeamais* in corn kernels. It may been plausible that when the development was slow, as it happened at 23ºC, the parasitoids had more time to grow, developing larger body and hind tibia (Table 10). In fact, it has been reported that larger parasitoid proportionate better reproduction rate (Ji, 1997). Our results supports this statement when a tendency of higher number of parasitoid offspring was observed at 23ºC (Table 9 & Table 10). On the other side, lower parasitoid fitness (less parasitoid offspring) was observed with the highest parasitoid density released as a consequence of the deficiency of larval hosts and high parasitoid competence (Table 9) (Chaisaeng et al., 2010; Wen and Brower, 1994). Moreover, the male offspring bias observed in the rice infested by *R. dominica* at 28ºC and higher parasitoid density suggested that the parasitoid may be stressed under this circumstances.

Given that the host-parasitoid ratio as well as stored temperature greatly affects the biological control success, for a proper pest management control, before the parasitoid release, it is important to reveal the species affecting the grain and estimate the weevil population size in order to calculate the optimum number of parasitoids that should be released (Chianseng, 2010). Moreover, for products destined to long

storage it may be interesting to also consider the conditions that proportionate an optimal parasitoid fitness.

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References

Abdullah, N., Nawawi, A., Othman, I., 2000. Fungal spoilage of starch-based foods in relation to its water activity (aw). Journal of Stored Products Research 36**,** 47-54.

Abebe, F., Tefera, T., Mugo, S., Beyene, Y., Vidal, S., 2009. Resistance of maize varieties to the maize weevil Sitophilus zeamais (Motsch.) (Coleoptera: Curculionidae). African Journal of Biotechnology 8**,** 5937-5943.

Ahmed, K.S., 1996. Studies on the ectoparasitoid, Anisopteromalus calandrae (How.) (Hymenoptera: Pteromalidae) as a biocontrol agent against the lesser grain borer, Rhyzopertha dominica (Fab.) in Saudi Arabia. Journal of Stored Products Research 32**,** 137-140.

Alonso- Amelot, M.E, Avila-Nuñez, J.L, 2011. Comparison of seven methods for stored cereal losses to insects for their application in rural conditions. Journal of Stored Product Research 47:82-87.

Athanassiou, C.G., Kavallieratos, N.G., Campbell, J.F., 2017. Competition of three species of Sitophilus on rice and maize. Plos One 12, 3: e0173377.

Belda, C., Riudavets, J., 2012. Reproduction of the parasitoids Anisopteromalus calandrae (Howard) and Lariophagus distinguendus (Forster) on arenas containing a mixed population of the coleopteran pests Sitophilus oryzae and Rhyzopertha dominica. Journal of Pest Science 85**,** 381-385.

Beti, J.A., Phillips, T.W., Smalley, E.B., 1995. Effects of maize weevils (Coleoptera, Curculionidae) on production of Aflatoxin B-1 by Aspergillus-Flavus in stored corn. Journal of Economic Entomology 88**,** 1776-1782.

Birch, L.C., 1947. The oxygen consumption of the small strain of Calandra Oryzae L. and Rhyzopertha Dominica Fab. as affected by temperature and humidity. Ecology 28**,** 17-25.

Chaisaeng, P., Chongrattanameteekul, W., Visarathanonth, P., Vajarasathiara, B., 2010. Laboratory studies on control of the maize weevil Sitophilus zeamais by the parasitoid Anisopteromalus calandrae. Scienceasia 36**,** 6-11.

Chanbang, Y., Arthur, F.H., Wilde, G.E., Throne, J.E., Subramanyam, B.H., 2008. Methodology for assessing rice varieties for resistance to the Lesser Grain Borer, Rhyzopertha dominica. Journal of Insect Science 8**,** 16.

Charnov, E.L., Los-den Hartogh, R.L., Jones, W.T., Van Den Assem, J., 1981. Sex ratio evolution in a variable environment. Nature 289**,** 27-33.

Choi, W.I., Yoon, T.J., Ryoo, M.I., 2001. Host-size-dependent feeding behaviour and progeny sex ratio of Anisopteromalus calandrae (Hym., Pteromalidae). Journal of Applied Entomology-Zeitschrift Fur Angewandte Entomologie 125**,** 71-77.

El-Aw M. A, Askar, S. I. S., El-Latif, A. M. Abd, Al-Assaal, M. S., 2016. Effect of different temperatures on some biological parameters of Anisopteromalus Calandrae (Howard) (Hymenoptera:

Pteromalidae) and population fluctuation of the parasitoid and their insect hosts of the genus Sitophilus (Coleoptera: Curculionidae). International Journal of Entomology and Nematology Research, 1, 1-12.

[FAOSTAT] Food and Agriculture Organization of the United Nations. (2012). FAOSTAT Database. Rome, Italy: FAO. Retrieved June 2017 from http://www.fao.org/faostat/en/#data

Fourar, R., Fleurat-Lessard, F., 1997. Effects of damage by wheat bug, Aelia germari Hemiptera: Pentatomidae, on grain quality and on reproductive performance of the rice weevil, Sitophilus oryzae Coleoptera: Curculionidae, on harvested grain. Phytoprotection 78**,** 105-116.

Ghimire, M.N., Phillips, T.W., 2007. Suitability of five species of stored-product insects as hosts for development and reproduction of the parasitoid Anisopteromalus calandrae (Hymenoptera: Pteromalidae). Journal of Economic Entomology 100**,** 1732-1739.

Gorham, J.R., 1979. Significance for human health of insects in food. Annual Review of Entomology 24**,** 209-224.

Hagstrum, D.W., Milliken, G.A., 1988. Quantitative analysis of temperature, moisture, and diet factors affecting insect

development. Annuals of the Entomological Society of America 81, 539-546.

Heimpel, G.E., Collier, T.R., 1996. The evolution of host-feeding behaviour in insect parasitoids. Biological Reviews of the Cambridge Philosophical Society 71, 373-400.

Ileleji, K.E., Maier, D.E., Woloshuk, C.P., 2007. Evaluation of different temperature management strategies for suppression of Sitophilus zeamais (Motschulsky) in stored maize. Journal of Stored Products Research 43, 480-488.

Jervis, M.A., Ellers, J., Harvey, J.A., 2008. Resource acquisition, allocation, and utilization in parasitoid reproductive strategies. Annual Review of Entomology 6. 361-385.

Ji, J., Choi, W.I., Ryoo, M.I., 2004. Fitness and sex allocation of Anisopteromalus calandrae (Hymenoptera: Pteromalidae): Relative fitness of large females and males in a multi-patch system. Annuals of the Entomological Society of America 97, 825-830.

Jian, F., Jayas, D.S., 2012. The ecosystem approach to grain storage. Agricultural Research 1, 148-156.

Kasamatsu, E., Abe, J., 2015. Influence of body size on fecundity and sperm management in the parasitoid wasp Anisopteromalus calandrae. Physiological Entomology 40, 223-231.

Kidd, N.A.C., Jervis, M.A., 1989. The effects of host-feeding behaviour on the dynamics of parasitoid-host interactions, and the implications for biological control. Researches on population ecology 31, 235-274.

Lebreton, S., Darrouzet, E., Chevrier, C., 2009. Could hosts considered as low quality for egg-laying be considered as high quality for host-feeding? Journal of Insect Physiology 55, 694-699.

Lucas, E., Riudavets, J., 2002. Biological and mechanical control of Sitophilus oryzae (Coleoptera : Curculionidae) in rice. Journal of Stored Products Research 38, 293-304.

Mahal, N., Islam, W., Parween, S., Mondal, K.A.M.S.H., 2007. Effect of Anisopteromalus calandrae (Hymenoptera: Pteromalidae) in controlling residual populations of Rhyzopertha dominica (Coleoptera: Bostrichidae) in wheat stores. International Journal of Tropical Insect Science 25, 245-250.

Maier, D.E., Adams, W.H., Throne, J.E., Mason L.J, 1996. Temperature management of the Maize weevil Sitophilus zeamais

(Coleoptera: Curculionidae) in three locations in the United States. Journal of Stored Product Pests Research 32:3;255-273.

Masasa, R.T., Setimela, P.S., Chiteka, Z.A., 2013. Evaluation of open pollinated varieties of maize for resistance to the maize weevil in a controlled temperature and humidity laboratory in Zimbabwe. Euphytica 193**,** 293-302.

Menon, A., Flinn, P.W., Dover, B.A., 2002. Influence of temperature on the functional response of Anisopteromalus calandrae (Hymenoptera: Pteromalidae), a parasitoid of Rhyzopertha dominica (Coleoptera: Bostrichidae). Journal of Stored Products Research 38**,** 463-469.

Navarro, S., Calderon, M., 1980. Integrated approach to the use of controlled atmospheres for insect control in grain storage. Amsterdam, Elsevier**,** 73-78.

Oerke, E.C., 2005. Crop losses to pests. The Journal of Agricultural Science 144**,** 31-43.

Phillips, T.W., Throne, J.E., 2010. Biorational approaches to managing stored-product insects. Annual Review of Entomology 55, 375-397.

Pimentel, D., Wilson, C., McCullum, C., Huang, R., Dwen, P., Flack, J., Tran, Q., Saltman, T., Cliff, B., 1997. Economic and Environmental Benefits of Biodiversity. BioScience 47, 747-757.

Potter, C., 1935. The biology and distribution of Rhizopertha dominica (Fab.). Ecological Entomology 83, 449-482.

Schmale, I., Wäckers, F.L., Cardona, C., Dorn, S., 2001. Control potential of three Hymenopteran parasitoid species against the Bean weevil in stored beans: The effect of adult parasitoid nutrition on longevity and progeny production. Biological Control 21, 134-139.

Schneider, C.A., Rasband, W.S., Eliceiri, K.W., 2012. NIH Image to ImageJ: 25 years of image analysis. Nature methods 9, 671-675.

Sinha, K.K., Sinha, A.K., 1992. Impact of stored grain pests on seed deterioration and aflatoxin contamination in maize. Journal of Stored Products Research 28, 211-219.

Smith, L., 1992. Effect of temperature on life history characteristics of Anisopteromalus calandrae (Hymenoptera, Pteromalidae) parasiting maize weeil larvae in corn kernels. Environmental Entomology 21, 877-887.

Smith, L., 1994. Temperature influences funcional response of Anisopteromalus calandrae (Hymenoptera, Pteromalidae)

parasiting maize weevil larvae in shelled corn. Annuals of the Entomological Society of America 87**,** 849-855.

Steidle, J.L.M., Scholler, M., 2002. Fecundity and ability of the parasitoid Lariophagus distinguendus (Hymenoptera: Pteromalidae) to find larvae of the granary weevil Sitophilus granarius (Coleoptera: Curculionidae) in bulk grain. Journal of Stored Products Research 38**,** 43-53.

Throne, J.E., 1994. Life history of immature maize weevils (Coleoptera: Curculionidae) on corn stored at constant temperatures and relative humidities in the laboratory. Environmental Entomology 23**,** 1459-1471.

Trematerra, P., Colacci, M., 2015. Preliminary results on impact of nitrogen fertilisation on Sitophilus zeamais wheat-food preferences and progeny production. Bulletin of Insectology 68**,** 281-286.

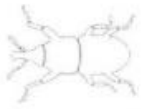
Trematerra, P., Savoldelli, S., 2014. Pasta preference and ability to penetrate through packaging of Sitophilus zeamais Motschulsky (Coleoptera: Dryophthoridae). Journal of Stored Products Research 59**,** 126-132.

Van Den Assem, J., 1971. Some experiments on the sex ratio and sex regulation in the Pteromalid Lariophagus distinguendus. Netherlands Journal of Zoology 21**,** 373-402.

Wen, B., Brower, J.H., 1994. Suppression of Maize Weevil, Sitophilus zeamais (Coleoptera: Curculionidae), populations in drums of corn by single and multiple releases of the parasitoid Anisopteromalus calandrae (Hymenoptera: Pteromalidae). Journal of the Kansas Entomological Society 67**,** 331-339.

4

Optimisation of a bankerbox system with *Galleria mellonella* as an alternative host to rear and release the parasitoid *Habrobracon hebetor* for the control of stored product moths



BIOLOGICAL CONTROL OF MOTHS

Introduction

Pyralid moths are among the most destructive pests in mills and food-processing facilities (Ghimire and Phillips, 2010b; Grieshop et al., 2006). In Europe, the Mediterranean flour moth, *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae) is one of the most abundant and concerning pest encountered in these environments (Belda et al., 2011; Eliopoulos et al., 2002; Trematerra and Gentile, 2010). *Ephestia kuehniella* eggs are laid on the flour and grain surface, and larvae are often burrowed within their produced silk (Williams, 1951). They breed hidden inside the machinery and tubing systems of mills and food-processing facilities, which causes obstructions due to the accumulation of silks, exuviae, faeces and dust (Belda et al., 2011). Moreover, their metabolic activity increases moisture and temperature of the stored products, providing favourable environmental conditions for mould development, which decreases food quality and may be harmful to human health (Gorham, 1979; Nopsa et al., 2015).

In order to control insect pests, manufacturers and farmers have often relied on the use of pesticides. However, due to the hazards related to their use, legislative restrictions over the last decade have limited the application of contact insecticides and fumigants in the food industry (Hagstrum and Subramanyam, 2009; Phillips and Throne, 2010; Schöller et al., 1997). In addition, consumers are more conscious on the effects of pesticides on their health and on the environment, and they are searching more frequently for non-chemical alternatives (Arbogast et al., 1998; Fields and White, 2002; Phillips and Throne, 2010; Pimentel et al., 1997). One option to control stored pests is to use biological control through the release of natural enemies (predators, parasitoids or enthomopathogenic fungi), a strategy well developed in Integrated Pest management programs (IPM) against plant pests in greenhouse crops (Grieshop et al., 2006; King et al., 1985).

The ectoparasitoid *Habrobracon hebetor* (Say) (Hymenoptera: Braconidae) is a gregarious idiobiont that is considered to be one of the best potential biological control agents for stored product Lepidoptera pests (Darwish et al., 2003; Ghimire and Phillips, 2010a; Keever et al., 1986; Press et al., 1982). This cosmopolitan parasitoid is naturally encountered in mills and alimentary industries worldwide (Alam et al., 2014; Belda and Riudavets, 2013; Keever et al., 1986; Suma et al., 2014),

and it parasitises species of the family Pyralidae, such as *Plodia interpunctella* (Hübner) (Milonas, 2005) or *Ephestia kuehniella* (Zeller) (Darwish et al., 2003; Eliopoulos and Stathas, 2008; Press et al., 1982). It is also known as a parasitoid of other lepidopteran species, independent of the stored product environment, such as *Galleria mellonella* (L.) (Lepidoptera, Pyralidae) (Amir-maafi and Chi, 2006; Ghimire and Phillips, 2008; Golizadeh et al., 2017; Grieshop et al., 2006; Saadat et al., 2014). *Habrobracon hebetor* females preferentially attack last instar larvae (Aamer et al., 2015; Akinkurolere et al., 2009; Alam et al., 2014) by paralysing them with venom before laying a variable number of eggs on or near the surface of the immobilised larvae (Alam et al., 2014; Baker et al., 1998; Ghimire and Phillips, 2014; Kryukov et al., 2017; Yu et al., 2003). Paralysed host larvae are then used as a food source for both developing wasps and for adult females (Alam et al., 2014; Heimpel and Collier, 1996; Isitan et al., 2011).

Habrobracon hebetor is commercially available and commonly used as an adult in augmentative releases. However, the short life span of the parasitoid adults, the possible damage to the agent caused during transportation and the difficulty of application in industries highlights the need to optimise biological control methodologies (Hodges, 1998; Kehrli et al., 2005). For this reason, Lucas et al. (2015) designed a banker

box system to progressively release *H*. *hebetor* for the control of moth pests in stored products. This technology, which is very easy to use, reduces the transportation risk and optimises the parasitoid quality by introducing newly born natural enemies that are pre-adapted to the environment. The authors tested different host–parasitoid ratios with *E*. *kuehniella*. Their results showed that low infestation rates (3 females and 2 males per 20 *E*. *kuehniella* larvae) were optimal for parasitoid and, specifically, female production. In their study, parasitoids were released during a span of approximately 22 days. Alternatively, Niedermayer and Steidle (2013) also developed a rearing and releasing box to control the granary weevil *Sitophilus granarius* (L.) (Coleoptera: Curculionidae) with the parasitoid *Lariophagus distinguendus* (Forster) (Hymenoptera: Pteromalidae). They were able to progressively extend the release of parasitoids over several months.

The aim of the present study was to optimise the banker box that was designed by Lucas et al. (2015). Because they used *E*. *kuehniella* as the host species to rear the parasitoid, their device presented a certain risk of contamination in food processing facilities. We then tested an alternative host: *G*. *mellonella*. This species is a major pest of honey bee colonies and is bigger than *E*. *kuehniella*. Therefore, with the new

species, we expected to reduce the risk of contamination, increase the parasitoid production and extend the parasitoid emergence period.

Materials and methods

1. Insects

Colonies of *E. kuehniella* and *H. hebetor* were started with individuals from samples collected in stored-product facilities and mills located in Northeastern Spain. The *G. mellonella* colony was started with individuals provided by Dr. F. García del Pino (Universitat Autònoma de Barcelona). *Ephestia kuehniella* was reared on wheat bran, 7% yeast and glycerine. *Galleria mellonella* (L.) was reared in a mixture consisting of 70 g baby cereal, 5 ml vitamin, 30 g sugar, 30 ml glycerin, 35 ml water, 30 g wheat germ and 5g yeast. *Habrobracon hebetor* was reared on third to fourth instar larvae of *P. interpunctella*. To increase their egg loads, absorbent paper soaked in honey was given to the parasitoids. Colonies were maintained and experiments performed in a climate-controlled room at 28 ± 2ºC, 70 ± 5% Relative Humidity (RH) and 16:8 h of a light: dark photoperiod.

2. Banker box system

The banker box system was designed according to Lucas et al. (2015). It consisted of a Plexiglas cage (15 cm high x 15.5 cm wide x 22 cm long with a hole on the lid covered with mesh for ventilation) that contained two smaller boxes (11 cm high x 11 cm diameter); one box was for rearing the parasitoid and the other box contained pest larvae for attracting the emerged parasitoids out of the rearing box. In the parasitoid rearing box, 20 lepidopteran larvae (*E. kuehniella* or *G. mellonella*) with their respective diets were offered to three females and two males of *H. hebetor* (0–48 h old). A paper strip moistened in a honey solution was also included for parasitoid feeding. The rearing box was covered tightly using a rubber band and a thin mesh covering on the top to prevent moth larvae and parasitoid adults from escaping and to allow for ventilation. The pest box contained 10 fourth instar larvae of *E. kuehniella* and some of its rearing diet. This box was open to allow the parasitoids to enter; however, to prevent the escape of *E. kuehniella* larvae, a thin layer of tanglefoot was painted on the top of the opening.

3. Bioassays

Three different host treatments were considered. First, a treatment with larvae of *E. kuehniella* with fourth instar larvae (EK). Second, a treatment with *Galleria mellonella* with fourth instar larvae

(GA). Third, a treatment of *G. mellonella* with a mixture of the same proportion of second and fourth instar larvae (GA2). A rearing box with each host treatment but without parasitoids was used as a control treatment. Fifteen replicates where carried out per parasitoid treatment and five for the controls.

Eleven days after the introduction of *H. hebetor* adults (which is the time needed for the emergence of adults of the next generation), a lid replaced the thin mesh of the rearing boxes. This lid had 50 holes to allow the exit of the emerged parasitoids and to prevent the escape of pyralid larvae. At this same date, the pest box was added to the Plexiglas cage. This was designed as time 0 for the evaluation of the production of the banker box system. Four days later, the first count was done. For that purpose, all parasitoids outside the rearing box were counted, sexed and retired from the system, and the pest box was replaced by a new one. Old pest boxes were covered with a thin mesh and maintained until emergence of host or parasitoid. Four more samplings with the same procedure were performed after 7, 14, 21 and 25 days. At the last sampling date, the rearing box was opened and the number of remaining *H. hebetor* adults and the number of lepidopteran adults that emerged were counted.

4. Data analysis

The following variables were evaluated: total number of emerged *H*. *hebetor* adults and their sex ratios; percentage of emergence of *H*. *hebetor* adults and females from the total emerged females at each sampling date; percentage of *H*. *hebetor* adults found outside of the rearing cage; percentage of mortality in the absence of the parasitoid (control); and percentage of mortality of *E*. *kuehniella* in the pest box. To assure that the percentage of mortality of *E*. *kiehniella* was due to the parasitoid attack, this value was previously corrected by the percentage of *E*. *kuehniella* mortality observed in the control. The percentage of 'host profitability' as the host-induced mortality in the rearing box was also was corrected by the specific host mortality in the control.

Data of total number of emerged parasitoids did not comply with the requirements of parametric tests, and the Kruskal–Wallis analysis of variance, a non-parametric equivalent of analysis of variance (ANOVA), was used to compare the treatments; when significant, this test was followed by a pairwise Mann–Witney *U*-tests. The *p*-values were corrected for multiple comparisons using the Bonferroni technique. The proportion of emerged *H*. *hebetor* females was evaluated by a chi-square test. The percentage data were arcsine transformed, and the analysis of variance was used to compare treatments. When significant, means were

compared by the Tukey test ($p > 0.05$). Statistical analysis was performed using the statistical package JMP (SAS Institute Inc., 2009).

Results

The designed banker box successfully produced parasitoids in all of the rearing conditions tested. However, the number of *H. hebetor* produced was dependent on the treatment applied ($\chi^2 = 15.756$, degree of freedom [df] = 2, $p < 0.001$). The treatment that produced significantly more parasitoids was the one with two larval sizes of *G. mellonella* (GA2), while statistically similar *H. hebetor* production was obtained in the treatments where the host larvae that was offered corresponded to a only one size, independent of the species (*E. kuehniella* or *G. mellonella)* (Fig. 17).

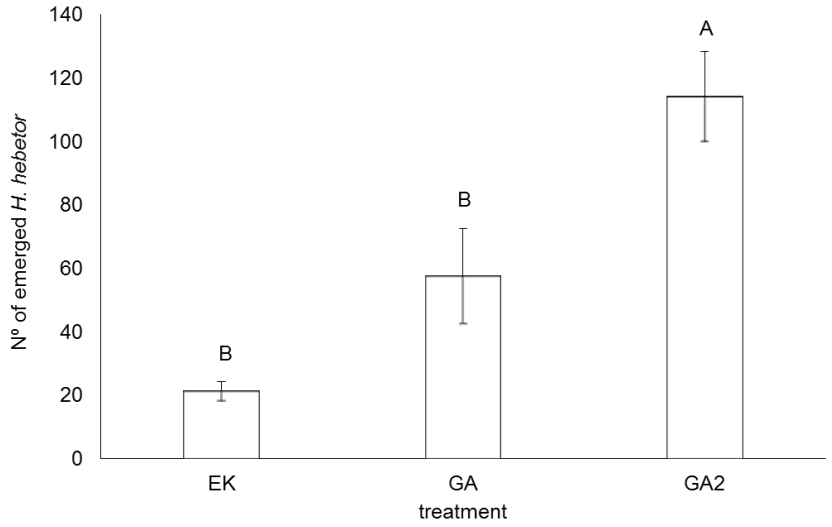


Fig. 17. The number (mean ± standard error [SE]) of *Habrobracon hebetor* adults emerged from each treatment: *E. kuehniella* fourth instar larvae (EK), *G. mellonella* fourth instar larvae (GA) and *G. mellonella* of fourth and second instars (GA2). Different letters above error bars denote significant statistical differences ($p > 0.05$).

The banker box system released parasitoids during approximately 25 days in all treatments. However, the percentage of emerged parasitoids decreased significantly across time ($F_{4,210}$= 100.727, $p <$ 0.001). More than 98% of the parasitoids were released during the first 14 days (Table 13).

Table 13. Percentage (mean ± standard error [SE]) of emerged *H. hebetor* adults at each sampling date and treatment.

	Sampling day (% *H. hebetor*)				
Treatment	**4**	**7**	**14**	**21**	**25**
EK	55 ± 6	24 ± 5	20 ± 6	0.2	0.4
GA	42 ± 6	37 ± 4	19 ± 7	1	1 ± 1
GA2	52 ± 6	34 ± 6	12 ± 3	1 ± 1	0.3
Mean percentage	50 ± 3a	32 ± 3ab	17 ± 3b	1c	1c

Abbreviations: EK, *E. kuehniella* fourth instar larvae; GA, *G. mellonella* fourth instar larvae; GA2, *G. mellonella* of fourth and second instars.

Note: Mean percentage values followed by the same letter are not significantly different ($p < 0.05$).

At the end of the experiment, the percentage of *H. hebetor* adults that had left the rearing box differed between treatments ($F_{2,42}= 48.44$, $p < 0.0001$): more than 98% of parasitoids were found outside of the rearing box in treatments with *G. mellonella*, while only 81% of parasitoids left the rearing box in the treatment with *E. kuehniella* (Fig.18).

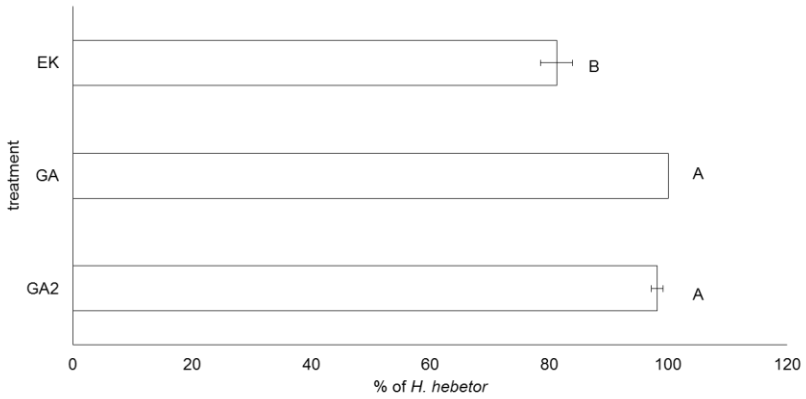


Fig. 18. Percentage of *H*. *hebetor* adults (mean ± standard error [SE]) that left the rearing box in each treatment: *E*. *kuehniella* fourth instar larvae (EK), *G*. *mellonella* fourth instar larvae (GA) and *G*. *mellonella* of fourth and second instars (GA2). Different letters next to error bars denote statistical differences ($p > 0.05$).

Emerged parasitoids that left the rearing box generated a high host mortality in the pest box. As expected, host mortality in the pest box was significantly correlated with 2the parasitoid production (R=0.373, $p < 0.00001$). Thus, after 4 days, 100% of pest mortality was observed, 98.3 ± 0.7% after one week and 57.8 ± 5% after fourteen days.

The sex ratio of the emerged *H*. *hebetor* adults was biased toward females (58.5% of females) in the treatment with *G*. *mellonella* second instar larvae (GA2) ($t = 2.03$, df = 14, p = 0.031), while no differences

between sexes were observed in the other two treatments (55.9% and 57.1% of emerged females for EK & GA, respectively) (t = 1.08, df = 14, p = 0.150 for *E. kuehniella* and t = 1.33, df = 14, p = 0.0.102 for *G. mellonella* larvae with first instar larvae [GA]).

The proportion of females that left the rearing box from the total female offspring decreased in successive counts over time. The pace of emerging females was different in the two host species. In the treatment with *E. kuehniella* this decrease was sharp after three days ($F_{2,37}$ = 6.49, p = 0.004). On the contrary, when the host was *G. mellonella*, the percentage of females produced was maintained during the first week, independent of the larval size ($F_{2,36}$ = 3.525, p = 0.04 and $F_{2,40}$ = 8.857, p < 0.001 for GA and GA2, respectively) (Fig. 19).

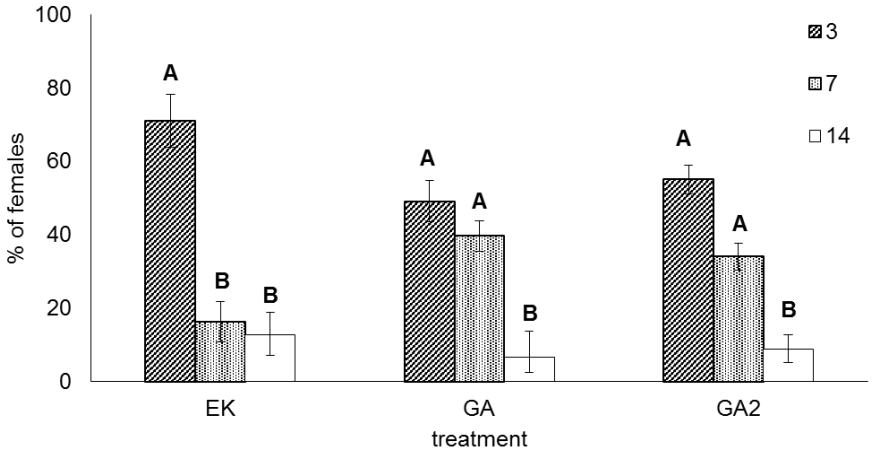


Fig. 19. Percentage of females (from the total emerged females) (mean ± standard error [SE]) leaving the rearing box at each sampling date (3, 7 and 14 days after starting the emergence of adults) per treatment: *E. kuehniella* fourth instar larvae (EK), *G. mellonella* fourth instar larvae (GA) and *G. mellonella* of fourth and second instars (GA2). Different letters above error bars denote statistical differences within each treatment ($p > 0.05$).

Host survival in absence of parasitoids varied between treatments. The percentage of hosts that developed to adult stage was 64 ± 1% for *E. kuehniella* larvae, 67 ± 1% for of *G. mellonella* treatment with second instar larvae (GA2) and 97 ± 1% for *G. mellonella* treatment with first instar larvae (GA).

The percentage of host killed in the rearing box (host profitability), corrected by the specific larvae mortality observed in the absence of the parasitoid, differed among treatments ($F_{2,42} = 15.27$, $p < 0.0001$). When *E. kuehniella* larvae was offered as the host, a higher percentage of larvae were killed (85%) compared to when the host was *G. mellonella* (47% and 62% of GA and GA2, respectively) (Fig. 20).

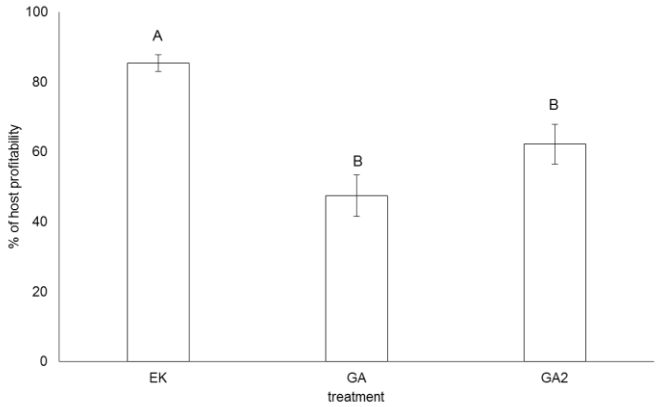


Fig. 20. Percentage (mean ± standard error [SE]) of host mortality in the rearing box (host profitability) in each treatment: *E. kuehniella* fourth instar larvae (EK), *G. mellonella* fourth instar larvae (GA) and, *G. mellonella* of fourth and second instars (GA2). Different letters above error bars denote statistical differences ($p > 0.05$).

Discussion

In the present research, we successfully optimised the banker box system designed by Lucas et al. (2015) by modifying the host conditions in the rearing box. As expected, results pointed at *G. mellonella* with larvae at mixed ages as better host condition.

We selected *G. mellonella* as a host to improve the banker box for two main reasons: (1) this species is of no risk for the contamination of mill and grain industries, and (2) this species has a larger larvae, which has been suggested to be qualitatively superior for parasitoid fitness (Akinkurolere et al., 2009; Charnov et al., 1981; Ghimire and Phillips, 2010a; Godfray and Shimada, 1999). This is the case especially with gregarious species such as *H. hebetor*, where large larval competition is common (Boivin and Martel, 2012; Rasool et al., 2017; Taylor, 1988). Additionally, because larger larvae have less refuge opportunities compared to small larvae that feed deep within the food medium (Akinkurolere et al., 2009), they may be attacked earlier, leading us to expect a higher oviposition rate. In fact, Amir-maafi and Chi (2006) observed a higher fecundity of *H. hebetor* in *G. mellonella* compared to *E. kuehniella* (78.3 eggs/female and 66.3 eggs/female, respectively).

Interestingly, when Ghimire and Phillips (2010b) compared the performance of *H. hebetor* on twelve different lepidopteran species

(among them, our two host species *E. kuehniella* and *G. mellonella*), they observed that despite having greater oviposition in response to large host larvae, there was a lower parasitoid survival rate in the larger hosts. Similarly, despite the fact that our results showed a trend in which the parasitoid production was higher in the treatments with *G. mellonella*, the only significant difference was observed when *G. mellonella* was offered in mixed ages (Fig 17). The most plausible explication for this situation is that larger larvae, although inducing a higher oviposition rate, exhibit greater defensive behaviour, presumably increasing mortality of the immature parasitoids and elongating the developmental time (Milonas, 2005). The former theory is supported by the results obtained by Rasool et al. (2017) when comparing the effect of the host size on *H. hebetor*. They observed that the number of parasitoid offspring was reduced drastically when reared on the larger host *Spodoptera litura* (Fabricius) (Lepidoptera: Noctuidae) compared to when reared on the smaller *G. mellonella* larvae. In fact, in our study, like in Ghimire (2010a), a high number of *G. mellonella* larvae were observed with melanisation rings and rapid decomposition. This is a typical consequence from cellular encapsulation, which is an innate defensive immune response in some Lepidoptera species (Kryukova et al., 2011).

The designed banker box successfully released parasitoids during at least 25 days in all the treatments. It seems that the females paralysed the host larvae in the first days of exposure, which is based on the observation of a peak in the number of offspring observed after the second week of introducing parental adults (corresponding to the first sampling date). There was a small decrease in the peak by the third week, and then a sharp decrease later (Table 13). Thereby, although we successfully released parasitoids for quite a long period of time, we did not succeed in obtaining a second generation of parasitoids, even when offering larvae of different sizes (GA2).

Another improvement of the rearing box with the introduction of *G. mellonella* as the host was that more than 98% of the parasitoids left the rearing box, compared to the 81% observed with *E. kuehniella*. One of the reasons for this behaviour could be that, as previously suggested, *G. mellonella* may be a suboptimal host, forcing the parasitoid to leave the rearing box because of its attraction to the odors of *E. kuehniella* larvae in the pest box. Moreover, results showed a high rate of mortality of the *E. kuehniella* offered in the pest box, even when the parasitoids were reared on the alternative host *G. mellonella*. Keeping in mind that the idea of the present banker box is to use it for the control of real moth infestations in industries, the latest finding is very promising.

Ryoo (1991) suggested that *H*. *hebetor* females can vary clutch size and the resulting progeny sex ratio to optimise the host, so that the overall sex ratio could be stabilised. Like many parasitic Hymenoptera, female *H*. *hebetor* develop from fertilised eggs and males from unfertilised eggs (Benson, 1973a). Some theoretical models proposed that the changes in the progeny sex ratio of gregarious parasitoids that generate a female bias are based on host quality (Charnov et al., 1981; Ghimire and Phillips, 2014). In our study, only the treatment with mixed-age larvae presented a female bias, suggesting GA2 to be the most optimal treatment (and this same treatment significantly produced more progeny) (Fig. 17).

The proportion of female offspring from the total number of emerged females in our study decreased with time, which suggests the proportion might be dependent on the age of the *H*. *hebetor* parental female (Fig 19). Because *H*. *hebetor* females are obtained from fertilised eggs, the observed shift in sex ratio can be explained because of sperm depletion from the reserves of the initial mating (Ghimire and Phillips, 2014). Surprisingly, although more eggs are probably laid on *G*. *mellonella* than on *E*. *kuehniella*, the shift in sex ratio for larger larvae is observed later than for *E*. *kuehniella*, independent of the larval size offered. (Benson; 1973b, cited in Eliopoulos and Stathas, 2008) found three to four times as many males than females when there were 18 eggs of *H*. *hebetor* per

host. They attributed this change to differential mortality acting more severely on females than on males. This result contradicts the results obtained by Ghimire and Phillips (2014), who observed an earlier shift of the sex ratio on *E. kuehniella* than on *G. mellonella*.

G. mellonella presented a higher percentage of survivorship in the control treatments, where no parasitoid was introduced, compared to *E. kuehniella,* but only when offered as a unique instar. When offered in mixed ages, a higher mortality was observed, presumably from the smaller larvae. When the parasitoid was introduced in the rearing box, the highest percentage of host profitability (host-induced mortality) was observed with *E. kuehniella* larvae (Fig. 20), although less parasitoids were produced (Fig.17). Therefore, *H. hebetor* left more than 15% of *G. mellonella* alive in the rearing box. This could be due to the presence of refuges offered by the diets (*E. kuehniella* diet offers less refuge opportunities than the diet of *G. mellonella)*, the host size (*H. hebetor* may need less large larvae to oviposit the same amount of eggs compared to smaller larvae) and the host preference of the parasitoid (Ghimire 2010b). Finally, the parasitoid venom may have been depleted more quickly when females attempted to subdue the larger prey *G. mellonella*, resulting in less effective attacks, as stated by Ghimire and Phillips (2010a).

In summary, the results obtained in this study revealed that *G. mellonella* could be a suitable host for the rearing of *H. hebetor* in the conditions of a banker box system. This species, when offered in mixed ages, provided a higher parasitoid production after 25 days that was biased toward females, and a significantly higher number of parasitoids left the bankerbox compared to *E. kuehniella*. Furthermore, by using *G. mellonella* as a host instead of *E. kuehniella,* we eliminated the risk of contamination.

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References

Aamer, N., Hegazi, E., Khafagi, W., 2015. Reproductive Capacity, Sex Ratio and Longevity of the Parasitoid, Bracon hebetor (Say) Parasitizing the Wax Moth Larvae, Galleria mellonella (L.). Egyptian Journal of Biological Pest Control 25, 433-437.

Akinkurolere, R.O., Boyer, S., Chen, H., Zhang, H., 2009. Parasitism and host-location preference in Habrobracon hebetor (Hymenoptera: Braconidae): role of refuge, choice, and host instar. J. Econ. Entom. 102, 610–615.

Alam, M.S., Alam, M.Z., Alam, S.N., Miah, M.R.U., Mian, M.I.H., Hossain, M.M., 2014. Biology of Bracon Hebetor reared on wax moth (Galleria mellonella) larvae. Pers. Gulf Crop Protect. 3, 54–62.

Amir-maafi, M., Chi, H., 2006. Demography of Habrobracon hebetor (Hymenoptera: Braconidae) on Two Pyralid Hosts (Lepidoptera: Pyralidae). Annals of the Entomological Society of America 99, 84-90.

Arbogast, R.T., Weaver, D.K., Kendra, P.E., Brenner, R.J., 1998. Implications of spatial distribution of insect populations in storage ecosystems. Environ. Entom. 27, 202–216.

Baker, J.E., Mendoza, P.J., Beeman, R.W., Throne, J.E., 1998. Fitness of a Malathion-resistant strain of the parasitoid Anisopteromalus calandrae (Hymenoptera: Pteromalidae). J. Econ. Entomol. 91**,** 50–55.

Belda, C., Ribes-Dasi, M., Riudavets, J., 2011. Improving pest management in pet food mills using accurate monitoring and spatial analysis. J. Stor. Prod. Res. 47**,** 385–392.

Belda, C., Riudavets, J., 2013. Natural enemies associated with lepidopteran pests in food and feed processing companies. J. Stor. Prod. Res. 53**,** 54–60.

Benson, J.F., 1973a. Intraspecific competition in the population dynamics of Bracon hebetor Say (Hymenoptera: Braconidae). Journal of Animal Ecology 42**,** 105–124.

Benson, J.F., 1973b. The biology of lepidoptera infesting stored products, with special reference to population dynamics. Biol. Rev. 48, 1–26.

Boivin, G., Martel, V., 2012. Size-induced reproductive constraints in an egg parasitoid. J. Insect Physiol. 58, 1694–1700.

Charnov, E.L., Los-den Hartogh, R.L., Jones, W.T., Van den Assem, J., 1981. Sex ratio evolution in a variable environment. Nature 289**,** 27–33.

Darwish, E., El-Shazly, M., El-Sherif, H., 2003. The choice of probing sites by Bracon hebetor Say (Hymenoptera: Braconidae) foraging for Ephestia kuehniella Zeller (Lepidoptera: Pyralidae). J. Stor. Prod. Res. 39, 265–276.

Eliopoulos, P.A., Athanaaiou, C.G., Buchelos, C.H., Adler, C., Navarro, S., Schöler, M., Stengard-Hansen, L., 2002. Occurrence of Hymeopterous parasitoids of stored product pests in Greece. Integrated Prot. Stored Prod. IOBC Bull. 25, 127–139.

Eliopoulos, P.A., Stathas, G.J., 2008. Life tables of Habrobracon hebetor (Hymenoptera: Braconidae) parasitizing Anagasta kuehniella and Plodia interpunctella (Lepidoptera: Pyralidae): effect of host density. J. Econ. Entomol. 101, 982–988.

Fields, P.G., White, N.D., 2002. Alternatives to methyl bromide treatments for stored-product and quarantine insects. Annu. Rev. Entomol. 47, 331–359.

Ghimire, M.N., Phillips, T.W., 2008. Effects of prior experience on host selection and host utilization by two populations of Anisopteromalus calandrae (Hymenoptera: Pteromalidae). Environ. Entomol. 37, 1300–1306.

Ghimire, M.N., Phillips, T.W., 2010a. Mass rearing of Habrobracon hebetor Say (Hymenoptera: Braconidae) on larvae of the Indian

meal moth, Plodia interpunctella (Lepidoptera: Pyralidae): effects of host density, parasitoid density, and rearing containers. J. Stor. Prod. Res. 46, 214–220.

Ghimire, M.N., Phillips, T.W., 2010b. Suitability of different lepidopteran host species for development of Bracon hebetor (Hymenoptera: Braconidae). Environ. Entomol. 39, 449–458.

Ghimire, M.N., Phillips, T.W., 2014. Oviposition and reproductive performance of Habrobracon hebetor (Hymenoptera: Braconidae) on six different pyralid host species. Annals Entomol. Soc. Am. 107, 809–817.

Godfray, H.C.J., Shimada, M., 1999. Parasitoids as model organisms for ecologists. Res. Pop. Eco. 41, 3–10.

Golizadeh, A., Alikhani, M., Hassanpour, M., Enkegaard, A., Rafiee-Dastjerdi, H., Razmjou, J., 2017. Comparative biology and life table of Habrobracon hebetor (Hymenoptera: Braconidae) on Anagasta kuehniella (Lepidoptera: Pyralidae) at five constant temperatures. Int. J. Pest Manag. 1–7.

Gorham, J.R., 1979. Significance for human health of insects in food. Annu. Rev. Entomol. 24, 209–224.

Grieshop, M.J., Flinn, P.W., Nechols, J.R., 2006. Biological control of indianmeal moth (Lepidoptera: Pyralidae) on finished stored

products using egg and larval parasitoids. J. Econ. Entomol. 99, 1080–1084.

Hagstrum, D.W., Smittle, B.J., 1978. Host utilization by Bracon Hebetor (Hymenoptera: Braconidae). Environ. Entomol. 7, 596–600.

Hagstrum, D.W., Subramanyam, B., 2009. A Review of Stored-Product Entomology Information Sources. Am. Entomol. 55, 174–183.

Heimpel, G.E., Collier, T.R., 1996. The evolution of host-feeding behaviour in insect parasitoids. Biol. Rev. Cambridge Philosoph. Soc. 71, 373–400.

Hodges, R. J., 1998. Biological control methods for insect pests of stored grain in the Tropics-constraints and prospects for developing countries. In Proceedings of the 7th International Working Conference on Stored Product Protection, Beijing, China (pp. 1193–1204).

Isitan, O.V., Gunduz, E.A., Gulel, A., 2011. Effect of host density on some biological characteristics and glycogen, total sugar and lipid levels of parasitoid Bracon hebetor Say, 1836 (Hymenoptera: Braconidae). Turk. J. Entomol. 35, 195–206.

Keever, D.W., Mullen, M.A., Press, J.W., Arbogast, R.T., 1986. Augmentation of natural enemies for suppressing two major

insect pests in stored farmers stock peanuts 1. Environ. Entomol. 15**,** 767–770.

Kehrli, P., Lehmann, M., Bacher, S., 2005. Mass-emergence devices: a biocontrol technique for conservation and augmentation of parasitoids. Biolog. Cont. 32, 191–199.

Kryukov, V.Y., Tyurin, M.V., Tomilova, O.G., Yaroslavtseva, O.N., Kryukova, N.A., Duisembekov, B.A., Tokarev, Y.S., Glupov, V.V., 2017. Immunosuppression of insects by the venom of Habrobracon hebetor increases the sensitivity of bait method for the isolation of entomopathogenic fungi from soils. Biol. Bullet. 44**,** 401–405.

Kryukova, N.A., Dubovskiy, I.M., Chertkova, E.A., Vorontsova, Y.L., Slepneva, I.A., Glupov, V.V., 2011. The effect of Habrobracon hebetor venom on the activity of the prophenoloxidase system, the generation of reactive oxygen species and encapsulation in the haemolymph of Galleria mellonella larvae. J. Insect Physiol. 57**,** 796–800.

Lucas, E., Riudavets, J., Castañe, C., 2015. A banker box to improve the impact of Habrobracon hebetor on stored products insects. Int. Prot. Stored Prod. IOBC-WPRS Bullet. 111, 403–407.

Milonas, P.G., 2005. Influence of initial egg density and host size on the development of the gregarious parasitoid Bracon hebetor on three different host species. BioCont. 50**,** 415.

Niedermayer, S., Steidle, J.L.M., 2013. The Hohenheimer Box - A new way to rear and release Lariophagus distinguendus to control stored product pest insects. Biolog. Cont. 64**,** 263–269.

Nopsa, J.F.H., Daglish, G.J., Hagstrum, D.W., Leslie, J.F., Phillips, T.W., Scoglio, C., Thomas-Sharma, S., Walter, G.H., Garrett, K.A., 2015. Ecological networks in stored grain: key postharvest nodes for emerging pests, pathogens, and mycotoxins. Biosci. 65**,** 985–1002.

Phillips, T.W., 1997. Semiochemicals of stored-product insects: research and applications. J. Stor. Prod. Res. 33**,** 17–30.

Phillips, T.W., Throne, J.E., 2010. Biorational approaches to managing stored-product insects. Annu. Rev. Entomol. 55, 375–397.

Pimentel, D., Wilson, C., McCullum, C., Huang, R., Dwen, P., Flack, J., Tran, Q., Saltman, T., Cliff, B., 1997. Economic and environmental benefits of biodiversity. BioSci. 47**,** 747–757.

Press, J.W., Cline, L.D., Flaherty, B.R., 1982. A comparison of two parasitoids, Bracon hebetor (Hymenoptera: Braconidae) and Venturia canescens (Hymenoptera: Ichnemonidae), and a

predator Xylocoris flavipes (Hemiptera: Anthocoridae) in supressing residual populations of the almond moth, Ephestia cautella (Lepidoptera: Pyralidae). J. Kansas Entomolog. Soc. 55, 725–728.

Rasool, S., ul Abdin, Z., Abbas, S.K., Ashraf, S., Anwer, M., Manzoor, A., Tahir, M., Shaina, H., 2017. Effect of host size on larval competition of the gregarious parasitoid Bracon hebetor (Say.)(Hymenoptera: Braconidae). Pakistan J. Zool. 49, 1085–1085.

Saadat, D., Seraj, A.A., Goldansaz, S.H., Karimzadeh, J., 2014. Environmental and maternal effects on host selection and parasitism success of Bracon hebetor. Biocont. 59, 297–306.

Schöller, M., Prozell, S., Al-Kirshi, A.G., Reichmuth, C., 1997. Towards biological control as a major component of integrated pest management in stored product protection. J. Stor. Prod. Res. 33, 81–97.

Suma, P., Amante, M., Bella, S., La Pergola, A., Russo, A., 2014. Stored-product insect natural enemies in wheat industry in Sicily. Integrated Prot. Stored Prod. IOBC-WPRS Bullet. 98, 227–233.

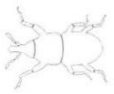
Taylor, A.D., 1988. Host effects on larval competition in the gregarious parasitoid Bracon hebetor. J. Anim. Ecol. 57, 163–172.

Trematerra, P., Gentile, P., 2010. Five years of mass trapping of Ephestia kuehniella Zeller: a component of IPM in a flour mill. J. Appl. Entomol. 134**,** 149–156.

Williams, J.R., 1951. The factors which promote and influence the oviposition of Nemeritis canescens Grav (Ichneumonidae, Ophioninae). Physiolog. Entomol. 26**,** 49–58.

Yu, S.H., Ryoo, M.I., Na, J.H., Choi, W.I., 2003. Effect of host density on egg dispersion and the sex ratio of progeny of Bracon hebetor (Hymenoptera: Braconidae). J. Stor. Prod. Res. 39**,** 385–393.

General discussion



GENERAL DISCUSSION

Preventing the presence of pests in the final product in a more sustainable manner requires appropriate pest management practices throughout the different stages of food production (Belda et al., 2011).

Unfortunatelly, one hundred years after the first successful use of biological control, fundamental questions regarding the use of natural enemies such as: how often, how many, and how and where they should be released are still not known for many of the damaging pest species.

Following the needs in research for stored product control, this thesis has been focused on the development of two principal lines of research: first, the study of two molecular techniques based on PCR for an easy and practical implementation of insect sampling techniques in food industry (Chapter 1 & 2) and, second, two studies to move forward the knowledge for the implementation of the biological control of the stored product weevil and moth pests (Chapter 3 & 4).

1. Pest monitoring using PCR approaches

The information provided by both designed PCR approaches in this thesis greatly facilitates the surveying and identification of internal feeders and may offer important information for further pest management decisions (Chapter 1 & 2). However, some challenges to the use of these approaches can still be encountered. For example, the insect diagnosis in stored product may require 13 hours to complete all the process. This period may be acceptable to check the status of a long stored grain. On the other hand, it may be too long when deciding the acceptance of an arriving harvested grain load to be processed.

Nevetheless, most of the food industries already include PCR in their diagnositic rutines to detect food of altered origin or the presence of GMO (Malorny et al., 2004; Meyer et al., 1996, Noguchi et al. 2015). Additionally, in recent years, with the expanding use of molecular approaches, especially for medical purposes, the time neded for these techniques, as well as their price has significantly decreased and is still decreasing, facilitating the possibility of usage of these PCR approaches also for insect diagnosis.

In the present expanding world trade situation, it is important to unify insect diagnosis protocols and pest acceptance thresholds, especially when insects are hidden, present in low and non-uniform

distribution (Trematerra et al., 2013). For this reason, figure 21 proposes a protocol for insect diagnosis with the molecular PCR approaches developed in this thesis. This figure also presents a protocol for a relative newly emerged PCR approach that combines the strenghts of the multiplex and the real-time approaches: the multiplex real-time PCR.

The three proposed PCR approaches begin with a DNA extraction of a ground grain sample after a visual inspection of the grain. However, since each grain load may need an especific sampling size in order to be representative, further research regarding sampling size and sampling methodologies is still needed (Jian et al., 2014a, b). Once the sampling protocol is established, at least 3 subsamples of the determinated representative sample would be thoroughly homogenized and followed by visual inspection of the grain to detect insect adults or their products. If the presence of insects is detected a management decision will take place: reject the grain, treat the grain, downgrade the grain destination, continue the grain processing, etc.

In the case of no insect detection, the use of molecular tools is recommended. The designed multiplex PCR protocol followed by electrophoresis gel is proposed to reveal if the grain is infested by immature stages of some of the internal feeders present in the Mediterranean region (chapter 2). A positive result with this approach,

although being a qualitative analysis, may indicate a grain infestation of more than 0.1 pupae/Kg (except for *R*. *dominica* 10 pupae/kg) due to the sensitivity threshold of the approach.

On the other side, for the particular quantification of *R*. *dominica* infestation in rice, the use of the developed real-time PCR protocol may be recommended (chapter 1). As a result, a threshold cycle value (C_t) inverse to the quantity of DNA present, will be procured. Once the C_t value is compared to a provided standard curve, the adult equivalent per kilo of rice will be obtained. Then, the use of inverse predictions to the obtained number of adult equivalent per kilo of rice will allow the correlation to a determinate insect age category (egg to 2nd instar larvae, 3rd instar larvae to pupae or adult). At this point, a new question may arise: is the grain infestation superior to the pest acceptance threshold? Unfortunately, as already mentioned, the acceptance trheshold for insect presence is not unified yet neither among countries nor for industries. Then, the need for a common acceptance threshold for insect's presence is again highlighted.

This thesis has presented the technique of using *R*. *dominica* and rice as a model system for insect diagnosis in stored products with real-time PCR. However, the development of further standard curves for

other damaging pest species in different grains or processed food may be advisable.

Overall, although both proposed techniques (multiplex PCR and realtime PCR) are ready for use, further research for the optimization of faster and more economic diagnostic kits to solve the cost and real time diagnosis inconveniences is needed. It may be interesting to focus the research on the development of a real time multiplex PCR technique for insect diagnosis. This approach, proposed in figure 21, will simultaneously identify and quantify the target species in a simple PCR reaction preventing having to choose between identifying or quantifying insect infestations. Fortunately, the multiplex real-time PCR technique is already being used for medical purposes (Wittwer, 2001; Navarro, 2015, Khumalo, 2017; Mayer, 2017). Then, the optimization and adaptation of this powerful technique for insect diagnosis may be feasible. As a result of this multiplex reltime PCR technique, C_t values corresponding to each species will be obtained. These C_t values will be automatically correlationated to especific standard curves and the inverse predictions to obtain the insect age categories will be applied. The obtained information will allow to answer if the insect infestion is superior to the pest acceptance threshold for each species.

Overall, the results obtained from either of the proposed PCR approaches will proportionate escential information for management decisions: reject, treat, downgrade the grain destination or continue the grain processing toward the consumer.

On the other side, the vital state of the insect is not important for the majority of consumers: the simple presence of an insect (alive or dead) may lead to the rejection of the food. On the contrary, for further grain treatments, the vital state of the insect matters. In these situations, reserving a grain sample for at least 15 days at 30ºC and then estimating the insect adult emergence may solve this problem. Because for management decisions the vital stage of the insect infestation (dead or alive) matters, it is important to develop and include an easy methodology for the detection of insect vital stage following any of the proposed PCR approaches.

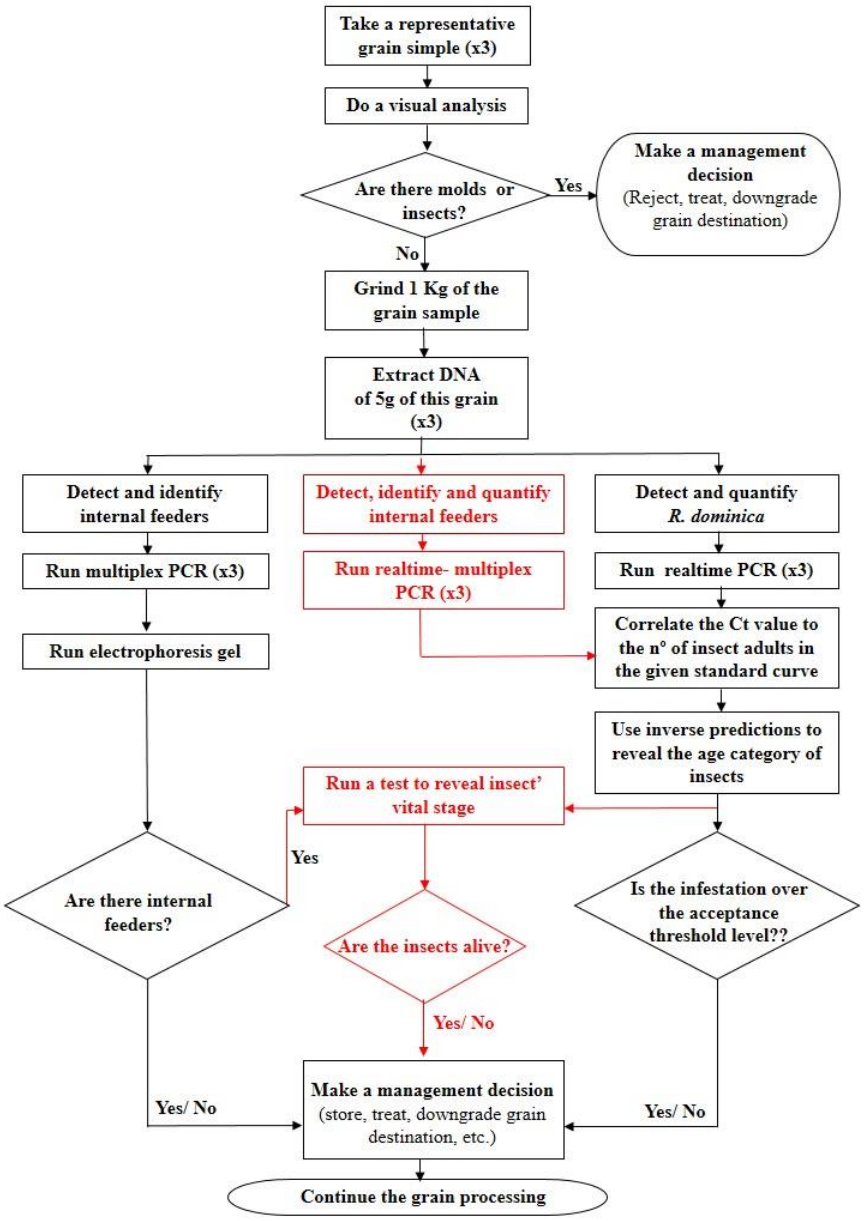


Figure 21. Proposition of sampling protocol for diagnosis of internal feeders with the designed PCR approaches in black. New steps using molecular approaches that need to be developed are in red.

2. Biological control

The effectiveness of the release of natural enemies depends on understanding the role of several factors implied in the stored product system (Arbogast et al., 1998; Hagstrum and Subramanyam, 2009, Maier et al., 1996). Therefore, detailed information on the biology, behavior and life history of the pest and the natural enemy under different conditions are required. (Hagstrum and Subramanyam, 2009; Schöller et al., 1997). The results reported in chapter 3 from the study of the effectiveness of *A calandrae* released in three different densities in 2Kg rice containers to control two of the most dangerous pests in the Mediterranean region, are of great importance. The acquired knowledge from the results of this study may encourage the use of biological control in storage facilities. This may allow producers to decrease periodical pesticide applications, which will satisfy the economy but especially consumers' demands and environmental quality. Further studies targeting other cereal grains or insect species may complete the scenario for the use of biocontrol strategies that are specific for each particular infestation case.

The introduction of molecular tools, such as proposed in the first two chapters of this thesis, in descriptive grain system studies such as the study in chapter 3, may improve, even more, the information gained from

a specific stored product system. This gain in information may facilitate a more precise and successful use of biological control. For example, the use of the designed real-time approach (chapter 1) in the rice containers infested with *R: dominica* (chapter 3) may enlarge the information about the real state of the rice infestation by also quantifying the immature stages of *R*. *dominica*.

Moreover, for an accurate biocontrol strategy, it is important to understand the host induced mortality causes. In the case of internal feeders, in which host-parasitoid interactions mainly occur inside the grain kernels, the use of molecular tools can be of interest. The employment of a newly specific designed *A*. *calandrae* primer set together with the already designed *R*. *dominica* or *S*. *zeamais* primer set in a real-time PCR approach over a period of time, may allow the discrimination of the host induced mortality causes (host feeding, unsuccessful parasitoid development, etc.) by observing an increase or maintenance of DNA quantity of the host or the parasitoid with the progress of time.

At the moment, parasitoids and predators for biological control are mainly applied by augmentation of native mass-reared insects. However, this methodology may not be the most suitable either for stored product environments or for large scale applications (King et al., 1985).

Additionally, since some of the main constraints for the use of biological control are the difficulty of implementation and the elevated price of the biological control strategies, there is a need to develop economic devices ready to use in order to mass rear and release parasitoids that are easy to apply by the operators of a food industry.

The optimized banker box system with the alternative host *G. mellonella* presented in chapter 4, allows having a progressive dispersion of *H. hebetor* adults for as long as 25 days to attack *E. kuehniella* without risk of pest infestation. It is recommended that this device be replaced by a new one after 14 days to have optimal conditions for the release of adults that can disperse freely in the warehouse or mill searching for pest insects without being stressed by transportation. This method will prevent a quick new build-up of a pest population since remaining parasitoids as well as new born ones will continue to reproduce as long as their hosts are available. In the next few years, it is foreseeable that the use of biological control using these sorts of devices will become a significant component of pest management in stored product industries.

3. Future trends

The past 20 years of postharvest biocontrol research has seen tremendous advances which additionally were more in line with industry concerns. Nonetheless, numerous challenges and opportunities still exist (Droby et al., 2009). As organic food becomes economically more attractive to producers and consumers, it will become critical to spread to producers and all concerned people in the sequence of food production, from growers to consumers, the knowledge on how to implement in accordance with legislation the technically and economically feasible IPM strategies (Campbell et al., 2003; Parkin, 1956; Schöller et al.. 1997; Van Lenteren et al., 2017). Thus, communication of economic, environmental and social successes and benefits of IPM strategies, targeting other researchers, politics, grower/ land manager and other stakeholder interests is still needed (Barrat et al., 2017).

Pest management will frequently require the application of other methods such as good sanitary measures and sophisticated monitoring techniques. New control methodologies will have elements of biological control, mass trapping, pesticide use or structural heat treatment among other strategies (Riudavets, 2017; Schöller et al., 1997; Van Lenteren et al., 2017). For example, in case of an insect infestation, the product

would be first sampled using the most suitable techniques that could integrate the use of the presented PCR approaches. Then, with the adquired knowledge of the infestation (pest species, magnitude of infestation, etc.) the most adequate approach to control the pest should be selected (biological control, physical control, etc.) and adapted to the infestation conditions. Finally, if it is convenient, a combination of several control approaches should be used to increase the pest control.

Additionally, stored product researchers must constantly make an effort to integrate newly emerged tools, resulting in the fast development of the biotechnology, and adapt them as pest control methodologies (Droby et al., 2009).

Much remains to be done, but the current situation and increasing social demand for more sustainable approaches facilitate the introduction of integrated pest management strategies in the food industry and may finally decrease the use of pesticides.

References

Arbogast, R.T., Weaver, D.K., Kendra, P.E., Brenner, R.J., 1998. Implications of spatial distribution of insect populations in storage ecosystems. Environmental Entomology 27**,** 202-216.

Barratt, B.I.P., Moran, V.C., Bigler, F., Van Lenteren, J.C., 2017. The status of biological control and recommendations for improving uptake for the future. BioControl**,** 1-13.

Belda, C., Ribes-Dasi, M., Riudavets, J., 2011. Improving pest management in pet food mills using accurate monitoring and spatial analysis. Journal of Stored Products Research 47**,** 385-392.

Campbell, J.F., Prabhakaran, S., Schneider, B., Arbogast, R.T., 2003. Critical issues in the development and interpretation of pest monitoring programs for food processing facilities. Advances in Stored Product Protection**,** 121-127.

Droby, S., Wisniewski, M., Macarisin, D., Wilson, C., 2009. Twenty years of postharvest biocontrol research: is it time for a new paradigm? Postharvest biology and technology 52**,** 137-145.

Hagstrum and Subramanyam, 2009 A Review of Stored-Product Entomology Information Sources. American Entomologist 55, 3.

Jian, F., Jayas, D.S., White, N.D.G., 2014a. How many kilograms of grain per sample unit is big enough? Part I - Comparison of insect detection and density estimation between manual probe sampling and Insector (R) system. Journal of Stored Products Research 56, 60-66.

Jian, F., Jayas, D.S., White, N.D.G., 2014b. How many kilograms of grain per sample unit is big enough? Part II - Simulation of sampling from grain mass with different insect densities and distribution patterns. Journal of Stored Products Research 56, 67-80.

King, E.G., Hopper, K.R., Powell, J.E., 1985. Analysis of systems for biological control of crop arthropod pests in the US by augmentation of predators and parasites.

Khumalo, J., Nicol, M., Hardie, D., Muloiwa, R., Mteshana, P., Bamford, C., 2017. Diagnostic accuracy of two multiplex real-time polymerase chain reaction assays for the diagnosis of meningitis in children in a resource-limited setting. PloS one, 12(3), e0173948.

Mayer, L. M., Kahlert, C., Rassouli, F., Vernazza, P., Albrich, W. C., 2017. Impact of viral multiplex real-time PCR on management of respiratory tract infection: a retrospective cohort study.Pneumonia, 9(1), 4.

Maier, D. E., Adams, W. H.; Throne, J. E., Mason, L. J., 1996.Temperature Management of the Maize Weevil, Sitophilus zeamais Motsch. (Coleoptera: Curculionidae), in Three

Locations in the United States. Journal of. Stored Product Research 32: 3, 255-273

Malorny, B., Paccassoni, E., Fach, P., Bunge, C., Martin, A., & Helmuth, R., 2004. Diagnostic real-time PCR for detection of Salmonella in food. Applied and environmental microbiology, 70(12), 7046-7052.

Meyer, R., Chardonnens, F., Hübner, P., & Lüthy, J., 1996. Polymerase chain reaction (PCR) in the quality and safety assurance of food: detection of soya in processed meat products. Zeitschrift für Lebensmitteluntersuchung und-forschung A, 203(4), 339-344.

Navarro, E., Serrano-Heras, G., Castaño, M. J., & Solera, J., 2015. Real-time PCR detection chemistry.Clinica chimica acta, 439, 231-250.

Noguchi, A., Akiyama, H., Nakamura, K., Sakata, K., Minegishi, Y., Mano, J., & Kondo, K., 2015. A novel trait-specific real-time PCR method enables quantification of genetically modified (GM) maize content in ground grain samples containing stacked GM maize.European Food Research and Technology, 240(2), 413-422.

Parkin, E.A., 1956. Stored product entomology (the assessment and reduction of losses caused by insects to stored foodstuffs). Annual Review of Entomology 1**,** 223-240.

Riudavets, J., 2017. Biological Control of stored products pests. IOBC bulletin.

Trematerra, P., 2013. Aspects related to decision support tools and Integrated Pest Management in food chains. Food Control 34**,** 733-742.

Schöller, M., Prozell, S., Al-Kirshi, A.G., Reichmuth, C., 1997. Towards biological control as a major component of integrated pest management in stored product protection. Journal of Stored Products Research 33**,** 81-97.

Van Lenteren, J.C., Bolckmans, K., Köhl, J., Ravensberg, W.J., Urbaneja, A., 2017. Biological control using invertebrates and microorganisms: plenty of new opportunities. BioControl**,** 1-21.

Wittwer, C. T., Herrmann, M. G., Gundry, C. N., Elenitoba-Johnson, K. S., 2001. Real-time multiplex PCR assays.Methods, 25(4), 430-442.

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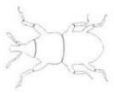
Conclusions



CONCLUSIONS

CHAPTER 1

- The designed qPCR approach is able to specifically and accurately diagnose all developmental stages of *R*. *dominica* in rice with high sensitivity (i.e. from 0.02 adults, 0.1 3rd instar to pupae or 13 egg to 2nd instar detectable per kg of rice) and accuracy (DNA was detected in more than 73% of the samples).

- The quantity of *R*. *dominica* DNA present is strongly associated with a given infestation size and with a determinated age category, increasing along with the size of the population and the age category (egg to 2nd instar larvae, 3rd instar larve to pupae and, adult.).

- We predict that this protocol adapted to a specific infestation can be used in all steps of the food production chain for the detection and quantification of *R*. *dominica* in grain with an easy interpretation of the results.

CHAPTER 2

- The developed multiplex PCR approach can universally detect and simultaneously identify the five most concerning internal feeders in the Mediterranean region (*Rhyzopertha*. *dominica*, *Sitophilus granarius*, *S*. *oryzae*, *S*. *zeamais* and *Sitotroga cerealella*) with high sensibility (0.1 pupa/ kilo of rice, except for *R*. *dominica* 10 pupae/kilo).

- The cross-reactivity test performed with the 46 other species potentially present in the stored ecosystem environment ensured the specific identification of the five target species.

- With the detection of hidden immature stages of *S*. *zeamais* in different kinds of grain (barley, oat, spelt, rice, wheat) and pasta, even when the grain is treated with CO_2 it is expected that this method will detect all target species present at any developmental stage.

- The post-mortem detection period of the technique showed to be superior to one year.

- The applicability of the developed multiplex PCR in food industries has been confirmed when testing real commercial grain samples from a pasta mill industry suggesting that the use of this tool in food control analyses for insect detection and identification would improve the quality of food and satisfy most consumer concerns.

CHAPTER 3

- The release of *A. calandrae* highly reduced *R. dominica* and *S. zeamais* host emergence and its associated rice damage, especially at 28ºC and with higher number of parasitoids released (40♀♀).

- *A. calandrae* was more effective controlling *S. zeamais* than *R. dominica*.

- The release of 10♀♀ parasitoids at 28ºC and the release of 20♀♀ at 23ºC may be enough to suppress the rice damage caused by both pests.

- The parasitoid fitness parameters such as number of parasitoid offspring, parasitoid sex ratio and body and tibia length under the

tested conditions indicated that the parasitoid is under stress when 40♀♀ are released.

- Overall, we recommend the release of 10♀♀ *A*. *calandrae* to control the pest at 28º C and the release of 20♀♀ at 23ºC for a situations of rice infestation with *R*. *dominica* or *S*. *zeamais* with similar host-parasitoid ratio.

- The adaptation of *A*. *calandrae* release densities to real *S*. *zeamais* and *R*. *dominica* rice infestations, may help to decrease the use of pesticides in food industries.

CHAPTER 4

- The use of *Galleria mellonella* in a mixture of two larval sizes as alternative host for the rearing of the parasitoid *Habrobracon hebetor* highly improves the bankerbox system based on the parasitoid rearing on the host *Ephestia kuehniella*.

- The optimized bankerbox system eliminates the risk of contamination and improves the parasitoid production.

- The system can release adult parasitoids over a period of approximately 25 days, allowing for their prolonged dispersion in the target location to effectively attack the *E. kuehniella* offered in the pest box.

- We predict that the use of this sort of ready to use devices for insect pest control will become a common practice in food industries.