EMC equation development for additional crops is needed which may include pulses, other grains and processed food products that span harvesting, drying, storage, conditioning, and processing operations.

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Stored Grain Protection: cases studies in Portugal

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Abstract

Considering the edibility of insects' species associated with storage ecosystem, chemical control methods can be easily replaced by environmental and economically sustainable alternatives.

Use of biogenerated atmospheres is an inexpensive method that tolerates insect presence. In Portugal, during one year, hermetic bags were used to store paddy under 65-75-85% relative humidity (RH) and 14-17-24ºC temperatures. Brown rice infested with *Sitophilus zeamais* adults was placed inside the hermetic bags.

Biogenerated atmosphere was naturally produced inside the hermetic bag, at 85% RH, with low O_2 and high CO_2 contents, showing that *S. zeamais* can survive but has no progeny at 14º-17ºC, or attained 100% mortality before producing progeny at 24ºC. The most abundant fungi isolated were *Alternaria alternata* and *Epicoccum nigrum*. The results showed the importance of the RH on changes in atmospheric gas content of paddy, due to biological agents' activity.

Analysing the edibility of insects species associated with stored grain, preliminary studies were carried out to evaluate the nutritional value of immatures stages of *Tribolium castaneum*. Larvae of *T. castaneum* had a content of 21.4% protein, 9.1% lipids, 8.8% fiber, and a relevant content of eight essential amino acids and also manganese and copper. The edibility of insects must be consider given their high nutritional value, low emissions of Green House Gases (GHGs), low requirements for land, and by reducing and mitigating the need for chemical control.

Keywords: insect edibility, paddy, hermetic storage, biogenerated atmosphere, *Tribolium castaneum*, *Sitophilus zeamais*

1. Introduction

Rice in Portugal and in Europe in general is a seasonal crop. Among pest species of stored rice, *Tribolium castaneum* (Herbst, 1797) is common but *Sitophilus zeamais* Motschulsky, 1855 is considered the key pest. The most common practice to control insects of stored grain is the use of fumigants to prevent and suppress insect development. The development of resistance of these two insect species to fumigants has decades of history (Champ and Dyte, 1977), and consumer concern over the use of pesticides in food oblige the research for alternative methods of insect control in order to get food product free of pesticide residues. Also, insect protein can be a good alternative to livestock production because it is more efficient feed/food conversion, lower greenhouse gas and ammonia production, less land area needed, and has potential to be grown on organic byproducts (Huis et al., 2013). Concerning producing benzoquinone-containing defensive secretions by *T. castaneum*, IARC (International Agency for Research on Cancer) officially states that no epidemiological data are available on the carcinogenicity of 1,4-benzoquinone, which consequently is not classifiable as to its carcinogenicity to humans (IARC and WHO, 1999).

On the other hand, fungal mycotoxin producers are the major cause of loss during long-term storage periods without efficient control of temperature and, above all, moisture content of stored grain (Christensen and Kaufmann, 1969; Wicklow, 1995; Fleurat-Lessard, 2017). One of the most important phenomena caused by fungi is the "hot spot" previously associated with insect infestations but currently identified as a consequence of fungal development in situations of poor storage conditions, such as high moisture content. The microclimate generated, i.e., temperature and moisture, attracts the insect populations (Fleurat-Lessard, 2017).

Based on these assumptions, preliminary trials were developed to analyze the edibility of immatures stages of *T. castaneum*. Also, trials were carried out using hermetic bags to store paddy rice, as a green and inexpensive alternative method to control insects and fungal development, under 65-75-85% RH and 14º-17º-24ºC. Moreover, rheological tests were performed on a MARS III controlled-stress rheometer to analyse the viscoelastic functions of the respective rice pastes.

The main objective is considering hermetic storage as a sustainable technology to reduce insect presence and fungal development, mitigating pesticide effect in food and feed.

2. Material and methods

2.1. Modified atmosphere

2.1.1. Sample preparation

Experiments were conducted in a warehouse located in Alcácer do Sal, Portugal. Three trials were carried out: T_1 the first trial, four months, temperature average 14ºC (December to April); T_2 the second trial, seven months, temperature average 17ºC (December to July); T_3 the third trial, four months, temperature average 24ºC (July to November).

For experiments, GrainPro® SuperGrainbag® Farm™ were used to store two rice varieties: Ronaldo, a japonica variety, and Sírio, an indica variety. Paddy rice, was stored in jute bags, as the control.

In all experiments, the two varieties were stored as paddy and submitted to three different relative humidities: 67, 75 and 85% RH, at three different average temperature (14, 17 and 24ºC). The RH and temperature were monitored by Hobo ® Data loggers, with probes placed inside the bags.

Both jute bags and SuperGrainbag® have a capacity of 50 kg, and for each treatment and variety three replications were carried out, for a total of 48 samples per trial.

At the end of all experiments, CheckpointII Portable O_2 and CO_2 Gas Analyzer was used to assess gas contents, at the bottom and top of each bag, totalizing six measures per treatment. The gas content is expressed in % by volume in air. After, the bags were opened and paddy samples were collected, samples were taken to be dehusked and milled to analyze water activity (a_w), with three replications per treatment, and rheology tests were performed. HygroPalm HP23 Rotronic was used to estimate a_w.

2.1.2. Bioassays

To evaluate insect response to each treatment, *Sitophilus zeamais,* the maize weevil, was chosen because it is the main pest of rice in Portugal (Carvalho et al. 2012). *Sitophilus zeamais* was originally collected from Portuguese rice mills and reared in climatic chambers, at 25±2ºC and 70% RH, at laboratory of Instituto Superior de Agronomia, University of Lisbon.

For experiments, 20 g of brown rice were infested with ~20 one-week-old *S. zeamais* adults and placed inside paper bags. One paper bag was set up inside of each paddy bag, totalling three replications per treatment.

2.1.3. Mycoflora analysis

From samples collected at the end of T_1 and T_2 trials, three samples were collected in sterilized containers and taken into the laboratory. In the laboratory, the rice samples were subdivided into samples with 100 grains. The surfaces of these grains were disinfected with 1% sodium hypochlorite for five minutes, as described by Pitt and Hocking (2009) and Magro et al. (2008). Ten disinfected grains were placed on Petri dishes with 20 mL of Potato Dextrose Agar (PDA) medium with chloramphenicol (1%). There were ten replicates for each sample.

2.1.4. Rheology measurements

Rheology tests were carried out for T1 trial and performed on a MARS III controlled-stress rheometer (Haake) coupled with a temperature controlling Peltier unit, using 35-mm-diameter serrated parallel plates and 0.5-mm gap. Aqueous flour suspensions (10% w/w) were held 5 min at 20ºC, between the plates, before testing. Stress sweeps were performed to ensure that all measurements were within the viscoelastic region. Then, the rheological study using SAOS (Small-Amplitude Oscillatory Shear) was performed according to previously optimized conditions (Torres et al., 2014).

2.2. Tribolium castaneum analysis

Moisture content was determined by placing approximately 2 g of each sample in a drying oven at 60ºC for at least 48 hours until constant weight. Protein was determined using the Dumas method as described by Saint-Denis & Goupy (2004), using a LECO FP-528 (LECO, St. Joseph, USA) calibrated with EDTA. The conversion factor of 6.25 was used to calculate total protein values. Total fat was determined by extraction with petroleum ether of around 0.5 g of sample using a Soxtec HT apparatus, and total fibre was determined according to the Weende method by extraction of non-fibre components from 0.5 g sample with sulphuric acid (0.2 M) and potassium hydroxide (0.2 M), followed by acetone washing in a FibreTec apparatus. Amino acids determination was performed by HPLC (Agilent 1100 HPLC, Agilent, Palo Alto, USA), using a Phenomenex Gemini ODS C18 110 Å column (4.6 × 150 mm, 5 µm, Phenomenex Inc., Torrence, USA) and a fluorescence detector according to the method described in Henderson et al. (2000). Mineral elements were determined by flame atomic absorption spectrophotometry (Unicam Solaar M, Thermo Electron GmbH, Dreieich, Germany) following acid digestion of 0.5 g of dried sample in 7.5 mL nitric acid and 2.5 mL hydrochloric acid using an SCP Science heat block (1.5 hours at 110 ºC).

2.3. Data analysis

All the computations and graphs were performed with software R (R Core Team, 2017). Function lm was used to fit and test for significance of the linear models.

3. Results and Discussion

3.1. Hermetic storage and *Sitophilus zeamais*

The average temperatures were similar for the same experiment and type of rice, but the individual days showed different values that are summarized in Table 1: T_1 had a temperature average of 14ºC, and was never higher than 20ºC; T_2 had an average temperature of 17ºC with 43 days above 20ºC and 13 days above 25ºC; and T_3 had a temperature average of 24ºC with 103 days above 20ºC of which 60 days were over 25ºC and 34 days above 27ºC.

Table 1- Number of days of each experiment, mean temperature, and number of "hot days".

	Trial	**[T_1]**	**[T_2]**	**[T_3]**
*** days**		**139**	**215**	**121**

Temperature average (ºC)	14±1	17±4	24±3
* days with Temp>20ºC	0	43	103
* days with Temp>25ºC	0	13	60
* days with Temp>27ºC	0	0	34

**days*- the number of days with a determined mean temperature.

Tables 2 and 3 report the results of the population growth of *S. zeamais* in each type of rice, Indica and Japonica, under different conditions of temperature, RH, atmosphereic composition, and water activity. When we consider the progeny, the first 20 weevils adults used, per replication, were eliminated from results, to better understand if there were offspring produced during trials. Under hermetic conditions at low O_2 and high CO_2 contents in trials in which the RH was 85%, at all temperatures there were no progeny although the initial insects were still alive. In trials at RH of 75% under hermetic conditions at medium oxygen and CO_2 contents, progeny were found, and the number of F1 individuals was dependent on temperature. In trials at RH of 67% under hermetic conditions, there was no change of the atmospheric content, and at 14 and 17ºC there were fewer adults alive than the trials at 75% RH. The population showed a significant increase when the temperature average was 24ºC.

Table 2 – Indica rice. Average number of *Sitophilus zeamais* adults: alive, dead, and progeny (alive + dead-20) for each pair of temperature and relative humidity (RH) conditions. Values correspond to averages across the replications, with small bags containing rice initially contaminated with 20 insects. The amounts of oxygen (O_2, %), carbon dioxide (CO_2, %) and water activity (a_w, %) at the end of each experiment are also shown.

Trial	Temp (ºC)	RH (%)	CO_2	O_2	a_w	*S. zeamais* alive	*S. zeamais* dead	Progeny
[T_1]	14	67	1.2	20.4	0.5	4.3	44.5	28.8
	14	75	3.9	18.3	0.5	13.7	37.3	31.0
	14	85	20.6	4.4	0.5	10.3	7.7	0.0*
[T_2]	17	67	1.5	19.4	0.4	8.5	33.8	22.3
	17	75	9.2	10.5	0.4	24.5	46.0	50.5
	17	85	23	0.8	0.5	0.7	20.3	1.0
[T_3]	24	67	1.4	19.9	0.6	84.0	93.3	157.3
	24	75	6.5	14.4	**0.7**	86.3	64.0	130.3
	24	85	21.8	2.6	**0.7**	0.0	20.0	0.0

*at end less than 20 insects were found

Table 3 – Japonica rice. Average number of *Sitophilus zeamais* adults: alive, dead, and progeny (alive + dead-20) for each pair of temperature and relative humidity (RH) conditions. Values correspond to averages across the replications, with small bags containing rice initially contaminated with 20 insects. The amounts of oxygen (O_2, %), carbon dioxide (CO_2, %) and water activity (a_w, %) at the end of each experiment are also shown.

Trial	Temp (ºC)	RH (%)	CO_2	O_2	a_w	*S. zeamais* alive	*S. zeamais* death	Progeny
[T_1]	14	67	1.3	20.3	0.6	1.3	69.7	51.0
	14	75	3.5	18.7	0.5	12.5	41.0	33.5
	14	85	17.3	7.4	0.6	27.7	9.3	17.0
[T_2]		67	1.6	19.3	0.5	3.3	32.3	15.7
		75	4.0	16.9	0.5	36.5	20.0	36.5
	17	85	26.7	0.2	0.4	0.0	25.3	5.3
[T_3]	24	67	1.4	19.8	0.6	108.0	71.0	159.0
	24	75	3.8	17.8	0.6	16.0	43.0	39.0
	24	85	22.0	3.8	0.7	0.0	20.0	0.0

3.2. Hermetic storage and mycoflora analysis

Thirteen species of fungi were found on paddy hermetically stored during T_1 trial with average temperatures of 14ºC, and 20 species were found after seven months of storage with average temperatures of 17ºC [T_2]. Fungi identified in more than 20 grains were *Alternaria alternata*, *Aspergillus flavus, A. fumigatus, A. niger, Epicoccum nigrum, Nigrospora.* sp, and *Rhizopus* sp. After four months of storage at 14ºC (T_1), the majority of the fungi identified were associated with field species.

After seven months of storage and higher average temperature (17 ºC) (T_2), *Aspergillus spp*. became more abundant and dominant relative to field fungi species. From all samples collected, the a_w was lower than 0.6 and was considered secure for the non-development of mycotoxins, as the husk is most certainly a barrier to protect grain from water changes (Fig.1).

3.3. Hermetic storage and rheological measurements

Rheological tests were carried out at end of trial T_1. Figure 2 shows the gelling point (when G′ overcomes G′′) of 10% (w/w) pastes of japonica and indica rice flour heated from 20 to 90ºC, from paddy flours kept under the three different RHs. For the japonica rice paste, there is a slight decrease in the gelling point temperature with the increase of RH: hermetic storage at 67% RH and control started gelling at about 87ºC; hermetic storage under 75% and 85% RH, started gelling at 85 and 82ºC, respectively. For the indica rice pastes, the results were reversed: the control and hermetic storage at 85% RH started gelling at 85ºC, and both hermetic conditions of 67% and 75% RH paddy pastes started gelling at 82ºC.

Figure 3 shows the mechanical spectra of the paddy pastes by variety and RH conditions. The paste obtained from rice stored at lower RH values, i.e., 67%, is slightly more structured than rice stored at higher humidity and control, i.e., the values of the viscoelastic parameters G′ (storage or elastic modulus) and G′′ (loss or viscous modulus) are slightly higher for the pastes from rice stored under lower RH conditions, showing a better structured paste (Nunes et al., 2006).

3.4. T. castaneum chemical analysis

Singh and Sinha (1977) studied the changes in protein levels in the developmental stages of *Sitophilus oryzae* (L.) and *S. granarius* (L.) reared on whole wheat at 30°C and 70% RH. In S. *oryzae* life cycle, the average values of this nutrient (percentage of dry body weight) ranged between 50.0 (L4) and 78.3% (adult). In *S. granarius* life cycle, the comparable protein values ranged between 47.0% (prepupa) and 75.7% (adult). The protein contents in S. *oryzae* increased up to the prepupal stage, declined slightly in pupae, and increased again in adults; whereas in *S. granarius* it constantly increased through the life cycle stages up to adult emergence.

The larvae of *T. castaneum* also is particularly rich in several essential elements confirming that the enrichment of different flours with this insect will improve its global nutritional value.

The values for the nutritional analysis presented in this work (Table 4) are typical of other insects from the same order, with a relatively high protein content, although the total fat is lower than the usual values, taking into account that these values are highly dependent on species and on the feed type (Ghosh et al., 2017).

Regarding the amino acid analysis, the larvae of *T. castaneum* have a higher amino acid content compared to the regular flour confirming that the presence of this insect will enrich it in several amino acids. Leucine was the most abundant essential amino acid followed by valine and threonine. Among these three essential amino acids, threonine is strictly indispensable since it is not transaminated and its deamination is irreversible (Belluco et al., 2013).

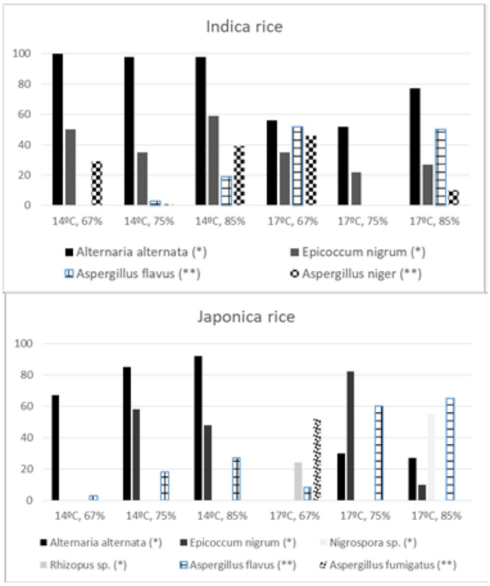

Figure 1 - Fungi incidence in 100 grains tested in indica and japonica rice stored for four months at 14ºC temperature average and stored for seven months at 17ºC temperature average. (*) field fungi (**); storage fungi.

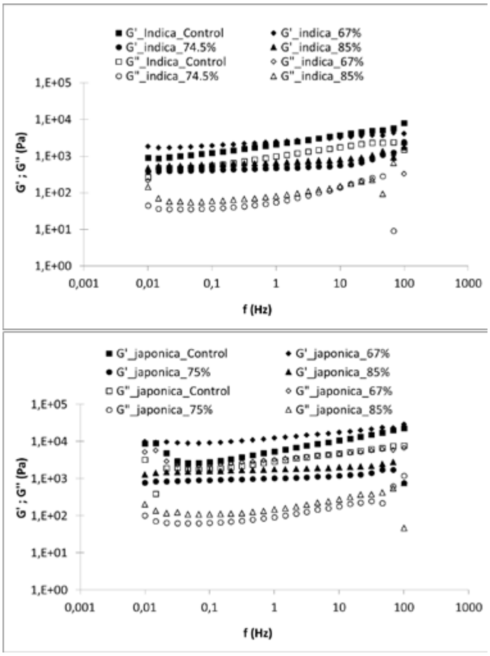

Figure 2- Indica and japonica rice: Mechanical spectra at 20ºC (G'- elastic modulus; G''- viscous modulus).

Table 4 – Chemical and nutritional composition of *Tribolium castaneum* larvae.

Water content (%)	42.23 ± 2.11	**Total fat (%)**	9.07 ± 4.09
Protein (%)	21.37 ± 0.45	**Fibre (%)**	8.76 ± 1.07
Amino acids (%)		**Mineral elements (mg/kg)**	
Histidine	0.64 ± 0.06	Fe	71.3 ± 8.1
Threonine	0.97 ± 0.06	Cu	6.1 ± 1.2
Valine	1.00 ± 0.07	Zn	48.2 ± 1.7
Lysine	0.84 ± 0.10	Mn	6.7 ± 0.5
Methionine	< LQ	Mg	41.5 ± 1.8
Leucine	1.23 ± 0.07	Na	199.5 ± 25.2
Isoleucine	0.71 ± 0.06	K	219.1 ± 8.6
Tryptophan	< LQ		
Phenylalanine	0.90 ± 0.07		

LQ: limit of quantification, 9 pmol/µL

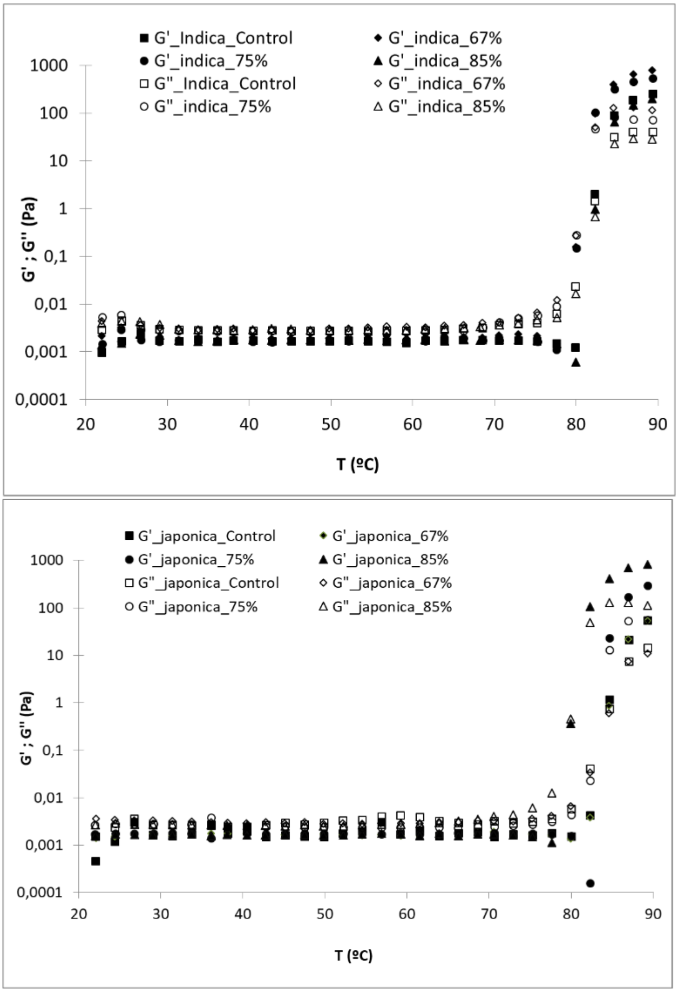

Figure 3 - Indica and japonica: **Starch gelatinization**. Heating from 20ºC to 90ºC at 2ºC/min (G′ elastic modulus; G′′ viscous modulus).

López-Vergé et al. (2013) evaluated the protein content of *Tenebrio molitor, Ephestia kuehniella*, and *Tribolium confusum* on a dry matter basis, and the results obtained in crude protein ranged from 42.47 to 58.77% and in ether extract ranged from 24.99 to 34.13%, supporting the idea that they can be incorporated into the feed diet. The same authors carried out a feeding trial in order to compare the effect of adding larvae of insect species *Sitophilus zeamais* to the diet on the performance parameters. Animals fed the insect-infested diet had higher final body weight (*P*=0.015) and higher average daily feed intake (ADFI, P=0.015) compared to animals fed the untreated (control) diet (López-Vergé et al., 2013).

Reuters announced in November 23, 2017, that a Finnish baker launched bread made from crushed crickets explaining that it «*offers consumers a good protein source and also gives them an easy way to familiarize themselves with insect-based food*».

4. Conclusion

Concerning the studies of hermetically stored paddy under different environmental conditions, the results showed that RH is the key factor for the modified atmosphere, attained only on trials under

75 and 85% RH at any average temperature, mainly due to respiration of paddy and fungi. As the time of storage increased, more fungal species developed, mainly associated with stored products. However, considering the determined a_w value was always below 0.6, the environment was considered safe from mycotoxin development. The modified atmosphere produced inside the hermetic bag with low O_2 and high CO_2 contents at 85% RH and at average temperatures of 14 and 17ºC demonstrated that *S. zeamais* can survive but produce no progeny. At the same conditions but higher average temperature of 24 ºC, ~100% mortality of *S. zeamais* occurred but progeny still were produced.

The increase in respiration rate at higher RH values, reduced the viscoelastic functions and changed the starch gelatinization point of indica and japonica rice.

The enrichment of different flours with larvae of *T. castaneum* can improve its global nutritional value.

It is our intention to break the misconception that insects are always harmful and to contribute positively to the concept of edible insects in the sector of stored products. It can be used for the incorporation of their protein as food/feed ingredients, and also to allow some tolerance to their presence during the storage period in order to suppress the use of chemical control methods, contributing to the sustainability of the environment and human health and animal welfare.

Following this concept, hermetic storage can be one of the sustainable and green methodologies to be used on grain storage. Storing paddy hermetically at low RH, did not change atmospheric content but maintained the viscoelastic functions of the rice pastes, low fungal incidence, and reduced insect population growth.

Acknowledgments

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Survey of dermestids of the genus *Trogoderma* in grain storages in Spain

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Several *Trogoderma* species of the family Dermestidae are important pests of stored products. Among them, *Trogoderma granarium* Everts, is one of the most harmful pests of cereal grains for all countries that are major exporters of agricultural commodities and for their trading partners (Athanassiou et al., 2016). Therefore, in most countries a very strict quarantine legislation exists to prevent the introduction of this pest (Myers and Hagstrum, 2012).

Trogoderma granarium is considered an endemic species in the southern Mediterranean region, and it has been intercepted or eradicated in many European countries. Nevertheless, global warming and the increase in international trade of raw materials are favoring its expansion. The establishment of *T. granarium* can likely occur in countries with more than 4 months per year with an average temperature higher than 20ºC (EPPO, 2011). However, temperatures in storage facilities can be higher than in open field, so there is also a risk of establishment in colder climatic areas.

According to the EPPO, *T. granarium* is present in Spain with a restricted distribution. But, while it has been detected in the country, there is no evidence of its establishment. It was found in 1952, but, after that record, there have been no new records of its presence (Banks 1977, Rebolledo and Arroyo 1993). Therefore, it is important to know whether *T. granarium* is present or not in Spain to take the necessary measures for its eradication or management. In the present study, a survey of the species of the genus *Trogoderma* has been conducted to determine the species present in grain storage facilities in Spain and their phytosanitary importance.

In 2016 and 2017, we sampled with traps baited with the pheromone of *Trogoderma* spp. in fifteen warehouses and grain silos along the Spanish Mediterranean coast. Monthly samples were collected in each sampling location using five PC floor traps placed in the storage facilities and three probe traps inserted in the grain piles. Taxonomic keys were used for the identification of the specimens found, as well as *T. granarium*-specific molecular markers by conventional PCR analysis.

A total of 3,276 *Trogoderma* specimens were captured in almost all locations sampled. However, no *T. granarium* were found. The majority of them were *T. inclusum* Leconte, and some were *T. variabile*