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Lethal and sublethal effects of stored grains insects after the exposure to diatomaceus earth treated grains

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Thesis submitted to the Entomology Graduate Program of the Universidade Federal de Viçosa in partial fulfillment of the requirements for the degree of *Doctor Scientiae*.

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Assent:

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This thesis is entirely dedicated to my husband Thiago F. Ferreira and my daughter Helena S. Ferreira. I also dedicate this thesis to my parents, Angela A. P. dos Santos and Sebastião R. S. Filho, my sister Kamilla M. S. Vilela and to the memory of my grandfather Sebastião R. dos Santos.

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“Didn’t I order you? Be strong and brave! Do not panic, nor be discouraged, for the Lord, your God, will be with you where you walk".
(Joshua 1:9)

ABSTRACT

FERREIRA, Karla Daiana dos Santos, D.Sc., Universidade Federal de Viçosa, December, 2019. **Lethal and sublethal effects of stored grains insects after the exposure to diatomaceus earth treated grains**. Adviser: Eugenio Eduardo de Oliveira. Co-adviser: Haddi Khalid.

The management of stored grain pests is still highly dependent on the use of synthetic pesticides. However, concerns about possible contamination and the emergence of insect resistance are encouraging the search for safer control alternatives. Among these alternatives, diatomaceous earth (DE) has been drawing attention lately for causing various effects on pest insects, thus reducing the economic losses caused by these organisms in storage units. Different species of pest insects are negatively affected when exposed to DE, and a better understanding of the action of DE on different insects (e.g., susceptibility differences and behavioral differences) is necessary in order to establish and apply appropriate management strategies. Given the above, the present study takes an approach with investigations aimed at better understanding the differences in the lethal and sublethal effects of DE in two important stored pests, *Sitophilus zeamais* (M.) and *Sitophilus oryzae* (L.) (Coleoptera: Curculionidae). In the first chapter we examined different effects of insects when exposed to DE-treated maize grains. Our results showed that survival, population growth rate and other factors (e.g., walking behavior, preference, grain intake, oviposition, etc.) are affected by exposure to DE. In addition, we verified differences in susceptibility of the studied species in which DE is more effective in controlling *S. zeamais*. In the second chapter we intensified the study of *S. zeamais* and *S. oryzae* exposure to DE now using treated rice grains and also evaluated the effect of sublethal exposure in male and female as well as the persistence of DE action. In addition to causing mortality and walking impairments, we observed that sublethal exposure to DE can cause harm to offspring and that sublethally exposed females appear to be more affected. Again, the *S. zeamais* species was more susceptible to DE. Thus, in the third chapter we sought to examine possible differences in the morphology, location, distribution and abundance of sensilla associated with the antennae and legs of *S. zeamais* and *S. oryzae* males and females. Some differences in sensilla length were observed for sex and species. Moreover, important interspecific differences were found for certain types of sensillae that were more abundant in *S. oryzae* and often were also more present in males. Overall, the results showed that DE is an efficient alternative for controlling *Sitophilus* spp., not only causing satisfactory weevil mortality,

but also affecting other behaviors (e.g., walking, feeding, reproductive behavior, etc.). damage to the activity and development of these pests. Despite the effects on both species and although more experimentation is required, the most efficient results of DE in *S. zeamais* may be due to several factors such as the morphological differences found in the sensilla of the different species of *Sitophilus* spp. studied here, differences in responses and behavior of these species.

Keywords: inert dust; grain storage; integrated pest management

RESUMO

FERREIRA, Karla Daiana dos Santos, D.Sc., Universidade Federal de Viçosa, dezembro de 2019. **Efeitos letais e subletais da exposição à terra de diatomáceas em insetos de grãos armazenados**. Orientador: Eugenio Eduardo de Oliveira. Coorientador: Haddi Khalid.

O manejo de pragas de grãos armazenados ainda é altamente dependente do uso de pesticidas sintéticos. Contudo, preocupações com possíveis contaminações e o surgimento de resistência de insetos vêm incentivando a busca por alternativas de controle mais seguras. Entre essas alternativas, a terra de diatomáceas (DE) vem chamando atenção ultimamente por causar diversos efeitos nos insetos pragas, reduzindo assim as perdas econômicas causadas por esses organismos nas unidades de armazenamento. Diferentes espécies de insetos pragas são afetadas negativamente quando expostas à DE e a melhor compreensão sobre a ação da DE em diferentes insetos (e.g., diferenças de suscetibilidade e diferenças comportamentais) se faz necessário a fim de se estabelecer e aplicar adequadas estratégias de manejo. Diante do exposto, o presente estudo faz uma abordagem com investigações conduzidas objetivando melhor compreender as diferenças nos efeitos letais e subletais da DE em três importantes pragas dos grãos armazenados, *Sitophilus zeamais* (M.) e *Sitophilus oryzae* (L.) (Coleoptera: Curculionidae). No primeiro capítulo nós examinamos diferentes efeitos ocasionados aos insetos quando expostos a grãos de milho tratado com DE. Nossos resultados mostraram que a sobrevivência, taxa de crescimento populacional e outros fatores (e.g., comportamento de caminhamento, preferência, consumo de grãos, oviposição, etc.) são afetados pela exposição à DE. Além disso, verificamos diferenças na suscetibilidade das espécies estudadas no qual a DE se mostra mais efetiva no controle de *S. zeamais*. No segundo capítulo intensificamos o estudo da exposição de *S. zeamais* e *S. oryzae* à DE, agora, utilizando grãos de arroz tratados e, avaliamos também, o efeito da exposição subletal em macho e fêmea, bem como, a persistência da ação da DE. Além de causar mortalidade e prejuízos no caminhamento, observamos que a exposição subletal à DE pode trazer prejuízos à descendência e que fêmeas expostas subletalmente parecem ser mais afetadas. Novamente, a espécie *S. zeamais* se mostrou mais suscetível à DE. Assim, no terceiro capítulo buscamos examinar possíveis diferenças na morfologia, localização, distribuição e abundância das sensilas associadas às antenas e pernas de machos e fêmeas de *S. zeamais* e *S. oryzae*. Algumas diferenças no comprimento de sensilas foram observadas para sexo e espécie. E ainda, importantes diferenças interespecíficas foram

encontradas para determinados tipos de sensilas que estavam mais abundantes em *S. oryzae* e, muitas das vezes, estavam também mais presentes em machos. De forma geral, os resultados mostraram que a DE é uma alternativa eficiente no controle de *Sitophilus* spp., não apenas causando mortalidade satisfatória dos gorgulhos, mas afetando também outros comportamentos (e.g., comportamento de caminhada, de alimentação, reprodutivos, etc.) causando danos à atividade e ao desenvolvimento dessas pragas. Apesar dos efeitos em ambas as espécies e, embora necessite de mais experimentação, os resultados mais eficientes da DE em *S. zeamais* podem ser devidos a diversos fatores como as diferenças morfológicas encontradas nas sensilas das diferentes espécies de *Sitophilus* spp. aqui estudadas, pois fornecem evidências das diferenças nas respostas e comportamentos dessas espécies.

Palavras-chave: pó inerte; armazenamento de grãos; manejo integrado de pragas

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SUMMARY

INTRODUCTION

Agricultural pests are mainly controlled with the use of synthetic insecticides, but their indiscriminate use has altered the control efficiency with the emergence of resistant insect pest populations, also allowing for environmental contamination (Isman et al., 2011; Guedes et al., 2016). In this sense, products of natural origin occur as an alternative for insect pest control because they have efficiency and use advantages because they do not offer toxic residues in the environment and apparently do not compromise insect control over time (Isman et al., 2011; Lorini, 2008; Aktar et al., 2009; Sousa et al., 2008).

Postharvest grain storage is one of the biggest challenges in which any lapse can lead to irreparable losses as it is the final product to be marketed. Most of these losses are caused by insect pests and there are two important groups of insects that attack stored grains and their subproducts, namely the Coleoptera (beetles, weevils or weevils) *Sitophilus zeamais, Sitophilus oryzae, Tribolium castaneum, Lasioderma serricorne and the Lepidoptera* (Moths) *Sitotroga cerealella, Ephestia kuehniella, Ephestia elutella* and *Plodia interpunctella* (EMBRAPA, 2011). However, beetles receive greater attention for causing greater damage and justifying most preventive control (Faroni and Silva, 2008; Guedes et al., 2016). Thus, the use of chemical insecticides has been justified and practiced in warehouses, but their use has presented restrictions as outbreaks of resistance and / or contamination generated by these products (Guedes et al., 2016; Aktar et al., 2009). With the prohibition of DDT for agricultural purposes, and due to the intense use of insecticides such as phosphine and pyrethroids, resistant populations were strongly selected causing control failures (Guedes et al., 1994, 1995; Ribeiro et al., 2003; Araújo et al., 2011; Daglish et al., 2014; Fragoso et al., 2003).

New research in the harvesting and storage segment is very important for the conservation of grain obtained by farmers, since it is essential that the quality of the grains is preserved, keeping them healthy, clean and free of pesticide residues, used to combat the diseases. pests that always attack stored grains. In this sense, natural insecticides have been suggested as a potential control alternative, since the development and the search for new alternatives for weevil control and other stored product pests have been encouraged (Phillips and Throne, 2010; Shah and Khan, 2014; Kavallieratoset al., 2015). In this sense, the use of alternative methods such as

inertproducts (e.g., diatomaceous earth) has been used and studied for proper pest management (Phillips and Throne, 2010; Subramanyam and Roesli, 2000; Shah and Khan, 2014; Lorini,2008; Fields and Muir, 1996; Watkinsand Norton,1947; Ebeling,1971).

Diatomaceous earth (DE), extracted from sedimentary rocks of diatom algae, is among the post-inert with the greatest control potential (Shah and Khan, 2014). The DE is a natural product extracted from sedimentary rocks of diatomaceous algae carapaces, represents the most popular inert dusts that have drawn focus and attention to being chemically unreactive and safe to vertebrates (Shah and Khan, 2014; Raju and Rom,1998; Korunic, 1998). Silica has the ability to kill insects by adsorption and abrasiveness, causing them to lose body water until they die (Subramanyam and Roesli, 2000). However, mortality occurs over a variable period, depending on the pest species (Banks and Fields,1995) and other factors (e.g., temperature, humidity, origin of DE formulation, etc.), in addition to sublethal exposure and their interactions with arthropods present important knowledge gaps (Guedes and Cutler, 2014; Guedes et al., 2015, 2016).

The corn weevil, *Sitophilus zeamais* Motsch., and rice, *Sitophilus oryzae* L. (Coleoptera: Curculionidae), are internal primary pests capable of damaging healthy and intact grains in which the development of immature phases (egg, larva and pupa) occurs inside of the grains (Lorini et al., 2015; Lorini, 2008). Sitophilus spp. cause high damage because larvae and adults can to destroy completely cereal grains and they have high reproductive capacity, cross-infestation, are polyphagous and have the ability to penetrate to great depths in grain mass (Gallo et al., 2002; Lorini, 2008). In Brazil, *S. zeamais* and *S. oryzae* are considered the main pests of cereal grains and stored products causing quantitative and qualitative losses that potentiate economic damages (Caneppele et al., 2003). Quantitative losses include the reduction of the dry mass due to the insect consumption and the increase of the production cost due to the necessity of the control practice (Caneppele et al., 2003), while losses and changes in quality may occur due to the depreciation nutritional values by grain consumption, heating (fermentation) of the product during storage, dissemination of microorganisms favoring pathogen infestation, mycotoxin production, reduction of germination power and vigor in seeds and contamination of grain mass by the presence of fungi and secondary pests (Caneppele et al., 2003).

The bean weevil, *Acanthoscelides obtectus* Say (Coleoptera: Chrysomelidae), is also an important pest that grow on common beans (Alvarez et al., 2005; Lorini et al., 2015), economically important crop in Brazil (FAO, 2017). Adult bean weevils lay their eggs externally in field pods or stored grains, and newly emerged larvae penetrate, feed and grow inside the bean grains (Caswell, 1981). The consequence of the damage caused by these insect pests is also quantitative losses due to the grains consumption and the adoption of control practices, and qualitative losses due to depreciation of nutritional properties and the presence of residues left in the grain mass (Alkan et al., 2019). Sitophilus spp. and *A. obtectus* also stand out as important pests for being able to infest grain in the field or storage units and for rapidly increasing the level of infestation due to their small size and high reproductive capacity (Lorini et al., 2015).

Thus, the present dissertation focused on the use of a DE formulation (Insect®) against these three weevil species, *S. zeamais* and *S. oryzae* and *Acanthoscelides obtectus* to better understand the responses of these species to this tool. Firstly, survival tests, locomotion, free choice, reproductive capacity (e.g., population rate of increase), grain consumption and effects on the offspring of these species were performed using corn grains treated with different doses of DE against *S. zemais* and *S. oryzae.*

In the background, we use DE-treated rice grains to intensify the study of the different responses generated by species by assessing not only mortality and walking behavior, but also the persistence of DE and the effects on reproductive capacity, grain consumption and progeny (e.g., respiratory rate, insect weight, sex ratio) of these two species from combinations of couples treated sublethally or not with DE. Since it was possible to show differences in the response to DE treatment between the studied species, we used scanning electron microscopy (SEM) to examine possible differences in antenna and leg morphology, as well as their associated sensilla, for the two weevils, *S. zeamais* and *S. oryzae*, which could partly explain behavioral differences regarding management strategies and thus avoid possible control failures in storage units. Finally, mortality/survival rate, reproductive capacity, consumption was evaluated for *Acanthoscelides obtectus* using red bean grains treated with different doses of DE. For *Acanthoscelides obtectus* the same formulation did not show effective control, highlighting the variation of the DE action between different insects

and the importance of conducting studies of the insecticidal efficacy for different products.

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1. CHAPTER 1

Differences in susceptibility and behavior of two major stored product pests, *Sitophilus zeamais* and *Sitophilus oryzae* (Coleoptera: Curculionidae), after exposure to diatomaceous earth treated maize grains

ABSTRACT: Weevil management in storage facilities is highly dependent on the use of chemical insecticides. However, due to the resistance occurrence and the risks associated with these control tools, the use of these products has been restricted, making room for alternative products (e.g., post-inert). Among these alternatives, diatomaceous earth (DE) have been lately drawing attention for causing diverse effects on pest insects, thus reducing economic losses caused by these organisms in storage units. The aim of this study was to evaluate the DE effects as a potential tool in the control of *S. zeamais* and *S. oryzae*. Testes of Survival, locomotion, free choice, reproductive capacity, grain consumption and effects on these species off spring were performed using different doses of DE (0, 1, 3 and 6g DE/kg maize grains). Higher DE concentrations compromised survival time and caused higher mortality rates (70-100%), with remarkable control of *S. zeamais* that was more susceptible to DE ($P < 0.001$). There was a significant reduction in grain loss ($P < 0.001$), reaching 60% and 30% in the highest concentrations tested (3 and 6 g / kg). DE treatments reduced the population growth of both species, and lower progeny suppression rates were obtained for *S. zeamais* ($P = 0.003$). However, DE treatment did not affect body mass and sex ratio of the progeny ($P > 0.05$), but CO_2 production was lower in the *S. zeamais* treated progeny ($P = 0.042$), and effect not observed for *S. oryzae* ($P > 0.05$). *S. zeamais* also had its walking behavior affected by walking less after exposure to DE, an effect that was not clearly evident for *S. oryzae* ($P > 0.05$). Moreover, only *S. zeamais* showed aversion to DE treated grains at concentrations of 3 and 6g / kg. Both species had oviposition affected by exposure to DE, in which larval development and adult emergence were only observed in controls. The results showed that DE is an efficient alternative in the control of *Sitophilus* spp., not only causing satisfactory mortality of the weevils, but also affecting the reproductive and walking behaviors, causing damage to the activity and development of these pests. However, DE has been shown to be more effective in controlling *S. zeamais* and further studies are still needed to

understand the reasons behind this differential activity of DE in relation to various stored product pests.

Key words: Weevil maize. Weevil rice. Storage losses. Inert dusts. Alternative control.

1.1. INTRODUCTION

The maize weevil *Sitophilus zemais* Motsch. and the rice weevil *Sitophilus oryzae* L. (Coleoptera: Curculionidae) are widespread insects that occur in warm areas around the world and attack stored grains intact (e.g., corn, wheat, rice, sorghum), as well can cause harm in many processed products, such as cereals, pasta, and dehydrated cassava (Dal Bello et al., 2000; Danho et al., 2002). Although these two species are very similar in morphological characters (distinguished only by the study of the genitalia or by molecular characters), and can occur simultaneously in storage units, different behaviors and responses to various factors can be expected (Hidayat et al., 1996; Peng et al., 2003).

It is known that these species cause expressive qualitative and quantitative losses to stored commodities (Dal Bello et al., 2000), which requires the adoption of control strategies. Historically, the control of the stored-grain insect pests, as maize and rice weevils, relied mainly on the use of chemical/synthetic insecticides of broad spectrum and fumigants. However, with the prohibition of DDT for agricultural purposes, and due to the overreliance on both phosphine and pyrethroids insecticides, resistant populations were strongly selected (Daglish et al., 2014; Fragoso et al., 2003; Guedes et al., 1995; Ribeiro et al., 2003). Thus, due to increasing insect resistance populations, the restricted number of registered active ingredients and the detrimental effects posed to the environment and human health, safer alternative control methods have been developed (e.g., low and high temperature, sanitation, biological control, and natural products).

The uses of inert dusts that are dry chemically unreactive powders have been suggested as natural product alternative to stored grain pest control (Ebeling, 1971; Fields and Muir, 1996; Phillips and Throne, 2010; Shah and Khan, 2014; Subramanyam and Roesli, 2000; Watkins and Norton, 1947; Wille et al., 2019). The diatomaceous earth (DE), which is a natural product extracted from sedimentary rocks of diatomaceous algae carapaces, represents the most popular inert dusts that have drawn focus and attention to be chemically unreactive and safe to vertebrates (Raju and Rom, 1998; Korunic, 1998; Shah and Khan, 2014).

Different species of insect pests are adversely affected when exposed to DE with a recognized effect against important stored products pests, such *Rhyzopertha dominica*, *Oryzaephilus surinamensis*, *Tribolium* spp., *Sitophilus* spp. (Arthur, 2002;

Chanbang et al., 2007; Kavallieratos et al., 2007; Malia et al., 2016b; Nwaubani et al., 2014; Shah and Khan, 2014), thus the DE has been recommended for important stored product weevils. However, its use remains controversial as its activity does vary with the insect (e.g., arthropod species, population, growth stage of pest species), DE deposit, environmental conditions (e.g., application method, temperature and humidity) and grains conditions (e.g., grain moisture and origin) (Aldryhim, 1993; Chanbang et al., 2007; Fields and Korunic, 2000; Malia et al., 2016b; Rigaux et al., 2001). Thus, the interactions of DE powders with arthropods are still uncovered and exhibit important knowledge gaps (Guedes et al., 2016, 2015; Guedes and Cutler, 2014).

Therefore, the aim of this study was to evaluate a DE formulation as an alternative control against two important insect species of the stored cereal grains *S. zeamais* and *S. oryzae* through investigating its effects on the survival rate, walking behavior, repellence to treated maize grains, reproductive capacity (oviposition, larval development and adults emergence), grain consumption and effects on these species offspring, as well as evidence possible differences in the response to DE treatment among the studied species, which can explain differences and control failures in storage units.

1.2. MATERIAL AND METHODS

1.2.1. Overall experimental conditions: Insects and DE formulation

Adult weevils of *S. zeamais* and *S. oryzae*, not sexed, were collected randomly and used as needed in all the experiments carried out. Both populations were maintained in glass containers in rearing facilities under controlled conditions (25±2°C, 70±10% relative humidity, 14 h: 10 h lighting regime [D: L]) and reared on insecticide-free maize grains (approx. 13% moisture content) at the Departamento de Entomologia da Universidade Federal de Viçosa, Viçosa, Minas Gerais, Brazil.

Diatomaceous earth Insecto R (Natural Insecto Products, Orange, CA, USA) was used for the bioassays with register and recommendation to control stored product pests in Brazil at the dose of 1g/kg (Química (2015)). Insecto R is a formulation of marine origin containing 86.7% SiO_2 and particle size distribution between 0.82 and 52.33 mm, but centering between 9 and 30 mm (Subramanyam, 1993). The DE

treatments were applied to whole maize grains using a stainless still cylinder coupled to a heavy-duty rotator (ROTO-TORQUE model 7637, Cole-Parmer, Vernon Hills, IL, USA).

1.2.2. Mortality rate and Time-mortality (survival) biossays

In order to verify the efficacy of DE against *Sitophilus* spp., mortality tests were performed for each species using the dosages of 1, 3 and 6 g of DE / kg of maize grains, and untreated grains to the control. Five replicates consisting of one plastic flask (300 ml) each containing 50 g of grains, received 20 unsexed adult insects and the mortality was assessed after 14 days of exposure. Survival tests were performed in the same conditions to the mortality tests; however, the mortality was daily assessed within 35 days after the beginning of the exhibition.

1.2.3. Instantaneous growth ratio (ri), grain consumption (grain loss) and progeny effects

For investigate the DE effect on the population rate of increase (ri) and on the grain consumption, a bioassay was performed using plastic flasks of 500 ml capacity where 50 insects were left to colonize 200 g of maize. Four replicates were used for each treatment (0, 1, 3 and 6 g of DE/ kg of maize grains) and for each species. After 60 days, the total number of insects and the final grain mass was recorded. The instantaneous growth rate (ri) was calculated for each specie using the formula $ri=[In(Nf/Ni)]/DT$, where Nf and Ni are the final and initial numbers of live (adults) insects, respectively, and DT is the duration of the experimental in days (Walthall and Stark, 1997).

In addition, the progeny (F1) obtained from this bioassay was used for analysis of sexual ratio (e.i., male: female ratio), body mass, and respiratory activity (i.e., CO_2 production). To determine the body mass, the insects of each sex were individually weighed on an ultra-precision electronic scale (model XS3DU, Mettler Toledo, Columbus, OH, USA). Respirometry bioassays were carried out using a CO_2 Analyzer TR2 (Sable Systems International, Las Vegas, NV, USA) following previously described methods (Oliveira et al., 2007; Guedes et al., 2006; Haddi et al., 2015). The average respiratory rate (CO_2 production) of the offspring was measured from 15

replicates to each specie and sex. The insects were placed in chambers (25 ml) connected to a completely closed system, to which the chambers were connected for 90 min before being injected CO_2 free air for 2 minutes (600 ml/minutes). The air current directed the CO_2 produced by insect respiration to an infrared reader that was connected to the system, which allowed for the immediate quantitation of CO_2 production.

1.2.4. Walking behavior

To evaluate the effects on walking behavior, bioassays were mounted under the same survival test conditions and kept in the rearing conditions for 14 days. After this period, the surviving insects were individually transferred from the treatments to Petri dishes (9cm x 2cm) lined with filter paper (replaced after each recording) and their walking activity was video recorded for 10 minutes by a video camera coupled to a computer equipped with video tracking system (VideoTrack System, Viewpoint LifeSciences, Montreal, Canada). A threshold of 0.1 cm/s was used to distinguish slow from fast walking. The camera was positioned 30 cm from the Petri dish and video analysis was performed under incandescent light and temperature of 25±2°C. Twenty insects were used for each treatment and the parameter evaluated was stops number, distance covered (cm), walking time (s) and velocity time (cm/s).

1.2.5. Repellence

Free-choice tests were executed using arenas with four choice opportunities as described in (Jumbo et al., 2014). Briefly, each arena was composed by one central plastic pot connected to four plastic pots with PVC pipes in a cross shaped design. Treated and untreated maize were added to the outside pots (two opposite pots containing untreated maize while the two other containing treated maize). Fifth insects were released into the central pot. Five replicates (arenas) were made for the treatments with 1 and 3 g/ kg of maize grains, for each species. The number of insects in each pot of the arenas was recorded after 24-hour period.

1.2.6. Oviposition, larval development and emergency

Couples of newly emerged insects (up to 7 days old), previously separated by sex, were in this bioassay. Test tubes (10 ml) received five corn kernels treated with DE (1, 3 and 6 g of DE/ kg of maize grains) or untreated control and a couple of *Sitophilus* spp. Twenty repetitions per species were set up and oviposition was monitored every three days, when the oviposited grains were replaced by five new grains (with DE or non-DE, depending on the treatment). Grain exchanges occurred for 30 days or until the death of the couple or death of the female. All samples, after the exchanges, were duly identified, analyzed in a stereoscopic loupe, to count the number of oviposition points, and kept in ideal conditions for larval development. Daily, insect mortality was monitored and egg density assessments were also performed every three days (initial phase-soon after the three-day period for oviposition) and larval density for up to 35 days. Evaluations of larvae (and also eggs) were performed by digital X-ray images of the five-grain group using a specimen radiography system equipped with a digital camera 14-bit MX-20 (Faxitron X-Ray Corp., Wheeling, IL, USA). Ten radiographs per sample were collected over 35 days and after that period the samples were kept in adequate conditions for adult emergence, which was counted at the end of the experiment.

1.2.7. Statistical analyses

The survival data was analyzed used Log-Rank test to obtain survival curves and the treatments were compared using and Kaplan-Meier Survival Analysis (SSI, 2008). The others data were subjected to analysis of variance (ANOVA). All means comparisons, whenever necessary, were performed by applying Tukey test at 5% probability using SigmaPlot Software (SSI, 2008).

1.3. RESULTS

Mortality of Sitophilus spp. after the 14-day exposure period significantly increased compared to the control in a dose dependent ($F= 392.108$; $P <0.001$). The species showed different mortality rates when exposed to DE ($F= 17.070$; $P <0.001$), where *S. zeamais* was more susceptible to treatments with higher mortality way

reaching over 80% and 100% with 3 and 6 grams of DE, wich that did not differ from each other (P = 0.317). For *S. oryzae* mortality also increased as a function of the dosages which differed from each other (P <0.05), however lower mortality rates were achieved (Figure 1A).

Our results demonstrate that the survival of *S. zeamais* and *S. oryzae* decreased significantly in relation to control (P <0.001) when they had to colonize DE-treated grain masses. Despite the effect on both species, S*. zeamais* showed higher susceptibility to DE with significant differences for all doses tested (P <0.001) and showed a rapid reduction in survival with population suppression in the period around 20 days for treatments with doses of 3 and 6g of TD / kg of grains. There was also a difference between species for the tested doses (P <0.001). *S. oryzae* had an average survival time was 25, 24 e 23 days to 1, 3 and 6 grams of TD / kg of grains (P> 0.05), respectively, while for *S. zeamais* had this time reduced to 19, 9 e 10 days (P> 0.001), respectively. Significant difference for control was not observed for *S. oryzae* and *S. zeamais* (P = 0.972), in which they presented average survival time of 28 e 31 days, respectively (Figure 1B).

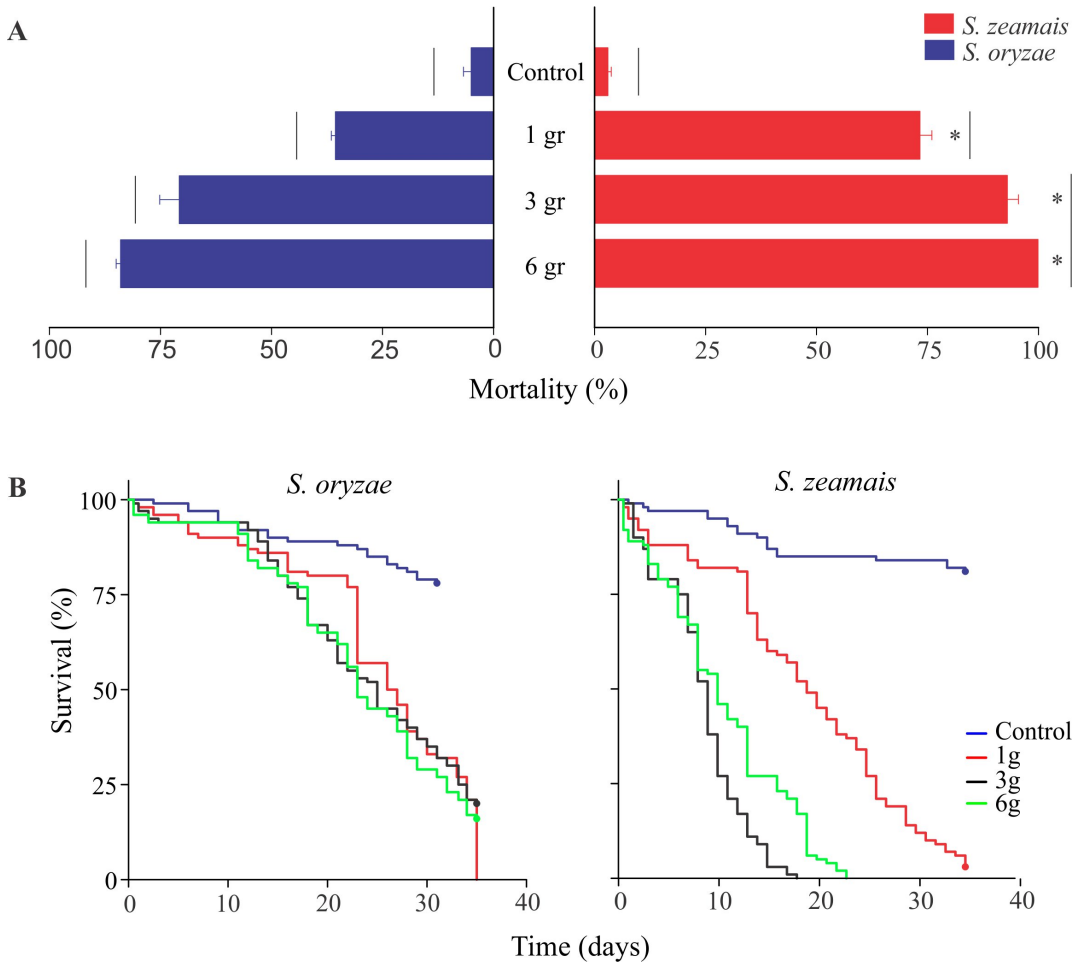

Figure 1.1: (**A**) Mortality and of adults insects of *S. oryzae* and *S. zeamais* after 14 days of exposure to DE at 0, 1, 3 and 6 g/kg of maize grains. (**B**) Survival of *S. zeamais* and *S. oryzae* on exposure to DE dosages at 0, 1, 3 and 6 g/kg of maize grains. Different vertical bars indicate significant differences at 5% level by Tukey test. *Indicate difference between species at 5% level by Tukey test.

Grain consumption decreased significantly after 60 days of exposure for the different DE-dosages for both species (F = 485.677; *P <0.001*) with a reduction of up to 60 and 30% in grain loss at dosages of 3 and 6 g DE / kg of maize grains when compared to the control. Significant differences (F = 7.340; *P = 0.001*) between species for treatments were also observed (Figure 2A).

After the exposure period of 60 days, the results showed that the application of DE results in a significant (F = 73.007; *P <0.001*) decrease in the population growth rate (*ri*) compared to the control for both species, however increment in the population dose did not result in differences in growth rate (*P> 0.05*). *S. zeamais* was more affected for DE doses registering a larger fall in the population growth rate than in *S.*

oryzae (F = 8,246; *P = 0.003*), as it shows in Figure 2B. Exposure to 6 g DE / kg of maize grains did not give progeny.

Sex ratio for both species was not affected by DE exposure (F = 0.376; *P = 0.692*) and there was no difference between species (F = 0.398; *P> 0.677*) that maintained the 1:1 ratio (male: female). Treatment with DE did not affect the body mass of the two populations (F = 0.142; *P = 0.868*) and although *S. oryzae* had lower body mass than *S. zeamais* (F = 316.055; *P <0.001*), no difference between sex was observed (F = 0.816; *P = 0.367*) (Figure 1C). No differences were found for sex in CO2 production (F = 1.575; *P = 0.216*), but there was a significant difference between species (F = 11.547; *P = 0.001*). *S. zeamais* presented higher CO2 production capacity under control conditions (*P <0.001*), however, CO2 production by *S. zeamais* progenies exposed to DE was lower than control (F = 3.413; *P = 0.042*), effect not observed for *S. oryzae* (*P> 0.05*) (Figure 2).

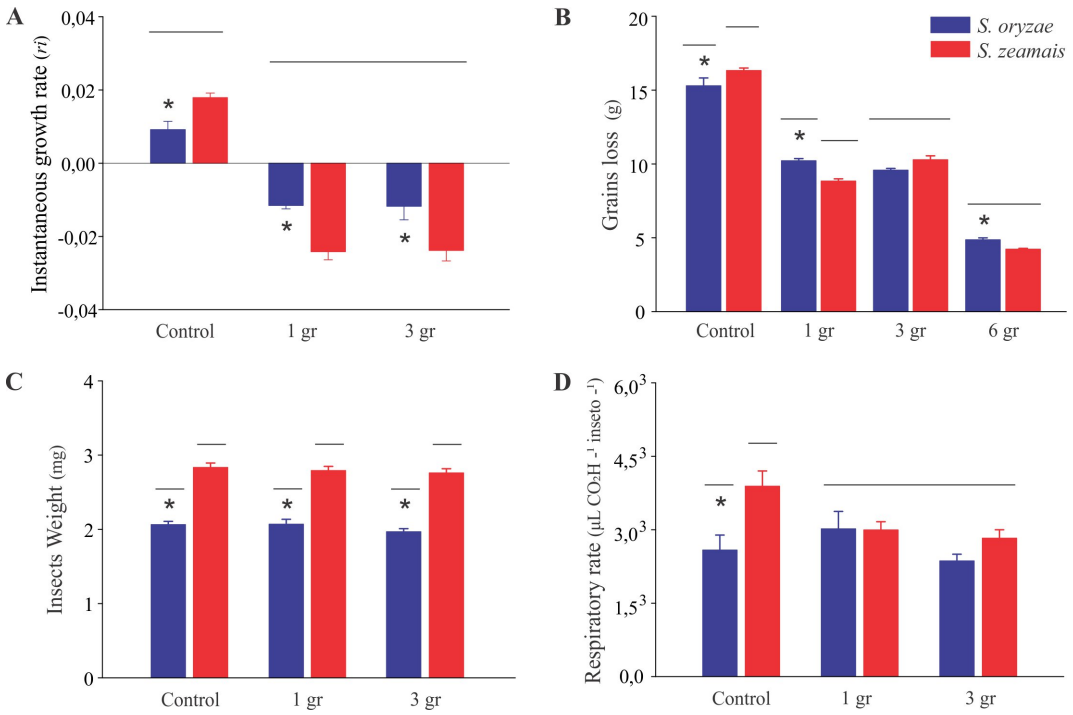

Figure 1.2: Instantaneous population growth rate (r_i), grains loss and physiological changes in progeny after 60 days of exposure to DE at the dosages of 0, 1, 3 and 6g / kg of maize grains. (**A**) Instantaneous growth rate, (**B**) Grain loss, (**C**) Insect weight and (**D**) Respiratory rate. Different horizontal bars indicate significant difference at 5% level by Tukey test. * Indicate difference between species at 5% level by Tukey test.

Regardless of dose, *S. zeamais* had the number of stops (F = 6.499; P = 0.03), distance walked (F = 8.761; P <0.001), walking time (F = 4.084; P <0.022) and walking velocity (F = 13.311; P <0.001) affected when exposed to DE and, in addition, there were no surviving insects at a dose of 6 g of DE/ kg of maize grains. These effects were not clearly evident for *S. oryzae* (P> 0.05) (Figure 3), only a significant effect was observed on walking velocity at a dosage of 1 g of DE/ kg of maize grains, which did not happen for the higher doses. Some significant differences between species could be observed as a function of the treatment for the distance walked (F = 3.973; P = 0.021), walking time (F = 3.464; P 0.035) and walking velocity (F = 3.627; P= 0.030).

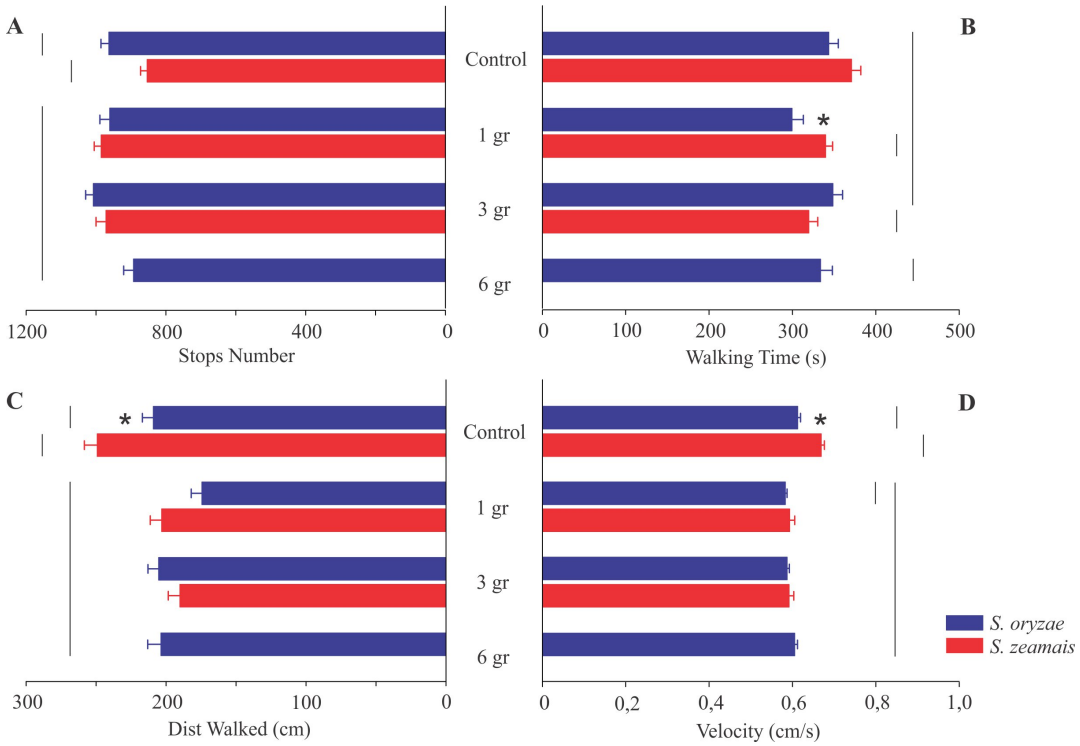

Figure 1.3: Walking behavior to *S. oryzae* and *S. zeamais* after 14 days of exposure to DE at the dosages of 0, 1, 3 and 6g / kg of maize. (**A**) Number of stops, (**B**) Walking time, (**C**) Distance traveled and (**D**) Walking speed. Different vertical bars indicate significant difference at 5% level by Tukey test. * Indicate difference between species at 5% level by Tukey test.

The repellent effect of DE in the free-choice test was noticeable (F = 3,999; *P = 0.032*) only for *S. zeamais* in the highest dosages (3 and 6 g/kg), as it shows in Figure 4. Differences in species choice behavior were observed for doses of 3 (*P = 0,001*) and 6 (*P = 0,008*) g TD/kg of maize grains, *S. zemais* showed higher aversion to treated corn than *S. oryzae*.

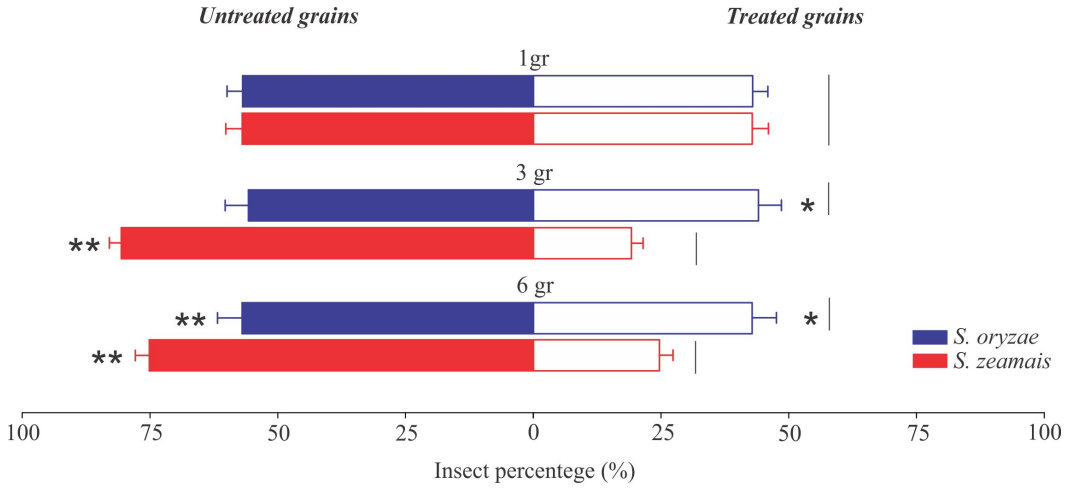

Figure 1.4: Repellence of DE-treated maize to *S. zeamais* and *S. oryzae* at 1, 3 and 6 g/kg of maize graisn. Different vertical bars indicate significant differences at 5% level by Tukey test. *Indicate difference between species to untreated and treated at 5% level by Tukey test. ** Indicate difference between treated and untreated in species at 5% level by Tukey test.

The oviposition of Sitophilus spp. was also affected by exposure to DE. Under control conditions, *S. zeamais* presented higher capacity of oviposition-able holes (F = 7.751; P = 0.007), however, the number of holes decreased significantly for both species when the grains were treated with 1 g of DE/ kg of maize grains (F = 35.793; P <0.001) (Figure 5A). Effective oviposition (egg-containing holes) was significantly affected (F = 12,300; P <0.001) only for *S. zeamais* and differences between species were not observed (F = 1,280; P = 0.261) (Figure 5B). For treatments with 3 and 6 g all insects died after 3 days of exposure and no oviposition was observed.

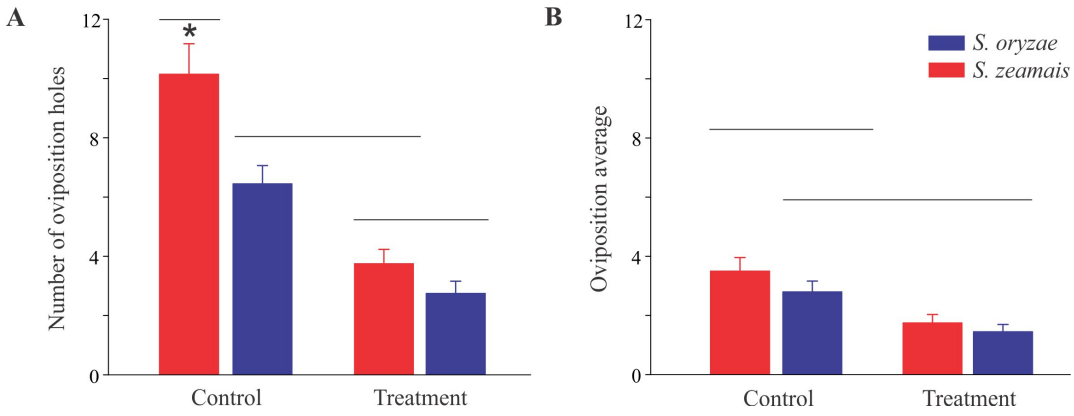

Figure 1.5: Oviposition capacity of *S. zeamais* and *S. oryzae* by exposure to DE at dosages of 0 and 1 g/kg of maize grains. (**A**) Average number of oviposition holes, with or without eggs, (**B**) Average number of egg holes. Different horizontal bars indicate significant difference to species and treatment at 5% level by Tukey test. * Indicate difference between species at 5% level by Tukey test.

Although oviposition occurred in grains treated with DE, larval development (Figure 6C and 6D) and adult emergence were only observed in controls with the number of adults corresponding to the number of larvae. *S. zeamais* showed greater larval development capacity and, consequently, more emerged adults with a total of 20 emerged adult insects, while for *S. oryzae* only 5 adult insects were obtained. These data had no statistical difference between species and interaction (species x treatment) (F = 3.740; *P> 0.05*), however there was a statistically significant difference regarding treatment (F = 12.118; *P = 0.001*).

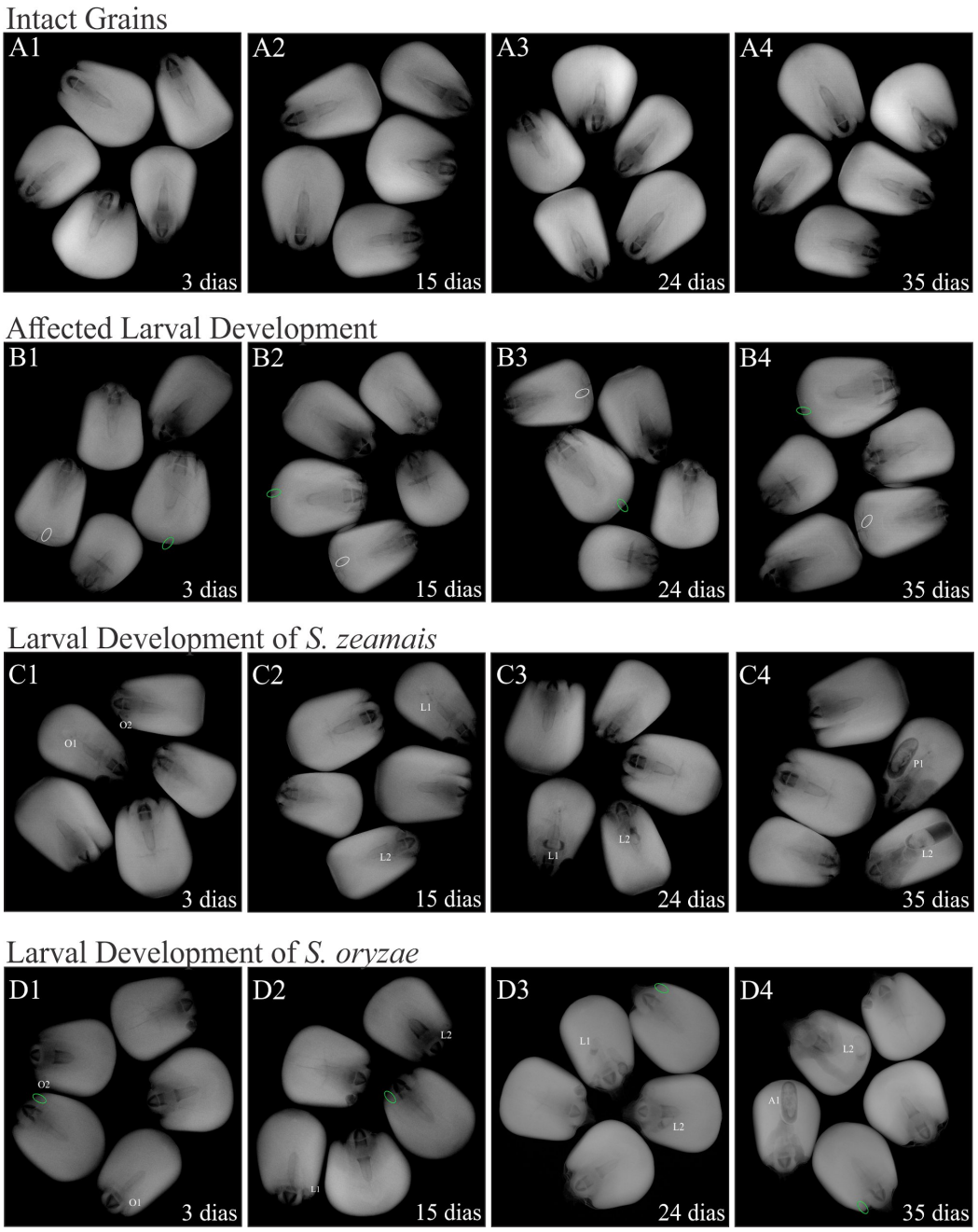

Figure 1.6: Illustrative sequence of X-ray pictures for 3, 15, 24 and 35 days after oviposition of *S. zeamais* in grains treated with DE (1 g / kg) or not showing (**A**) intact grains without oviposition, (**B**) DE-treated grains with affected *S. zeamais* larval development, (**C**) DE-untreated grains with normal larval development for *S. zeamais* and (**D**) *S. oryzae*. The undeveloped larvae are circulated by white lines. The void holes are circulated by green lines. E refers to Egg; L refers to Larva; P refers to Pupa.

1.4. DISCUSSION

Insect mortality and survival were affected by the DE formulation tested, but there was a difference in effect for the two weevil species. The high efficiency to control on the *S. zeamais* is in concordance with previous studies (Doumbia et al., 2014; Malia et al., 2016b; Nwaubani et al., 2014; Wille et al., 2019). The mortality caused by DE is attributed principally to physical properties of the diatom particles (Arthur and Throne, 2003). For example, the dust resembles pieces of glass that cause abrasive damage to the cuticle, including sensillae and pores, in addition, silica content and DE internal micropores are capable not only of damaging the insect integument, but also act to absorb the cuticle wax layer which contributes to the reduction of body water content by compromising water balance and increasing the water loss rate of these insects (Doumbia et al., 2014; Ebeling et al., 1961; Malia et al., 2016b).

The highest mortality rates (70% -100%) were obtained when insects were exposed to higher concentrations. Treatment at a concentration of 1 g/kg (recommended dose) had low efficiency (less than 40% and 70% for *S. oryzae* and *S. zeamais*, respectively) after 14 days of exposure. Control failures with the use of low DE concentrations have been reported in other studies (Wille et al., 2019), however is possible to adopt strategies that combined with low DE concentrations increase their insecticidal effect as a combination with additives (Korunic, 1998; Quarles, 1992), high temperature (Chanbang et al., 2007), biological control agents (Ashraf et al., 2017; Athanassiou et al., 2016; Athanassiou and Steenberg, 2007), plant extracts (Ulrichs et al., 2000; Yang et al., 2010; Jumbo et al., 2014; Ziaee et al., 2014), and even low concentrations of synthetic insecticides (Athanassiou et al., 2016; Athanassiou and Korunic, 2007; Machekano et al., 2017). Differences in the weevils control obtained for the mortality and survival tests (14 days after the beginning of the DE exposure) can be attributed to the evaluation methodology, in which during the daily evaluation there may be losses of the product compromising its effect.

The instantaneous population growth rate (r*i*) allowed us to evaluate the effect of DE on the capacity of *Sitophilus* spp. to increase their population over a given exposure period (Walthall and Stark, 1997). Although the population reduction was satisfactory for both species, DE was more effective on *S. zeamais*. There were no significant decreases with increasing doses from 1 g to 3 g of DE / kg maize grains,

however, as for 6 g no progeny were obtained it is possible to assume that the population of this insect decreased to extinction for the smallest doses. These data agree with those obtained by (Wille et al., 2019) who found increases in control of *S. zeamais* progeny as a function of dose, reducing more than 90% at a dosage of 2 g / kg. Another factor to be considered in reducing ri is that, besides mortality, the short survival period of adult insects and the treated grains would bring sufficient impediments to oviposition, preventing progeny. Fields (1998) emphasizes that the action of DE is slower than that of synthetic insecticides, which may allow oviposition and progeny production, even in small numbers. Feeding failures can be explained not only by insect population reduction and its progeny, but also due to insect difficulty in walking, adhering to and staying on DE-treated grains (Athanassiou et al., 2008).

DE can also cause insect aversion, as reported by (Vardeman et al., 2007) and observed in the present study. Despite the satisfactory population reduction, DE did not affect the body mass and sex ratio of *Sitophilus* progeny. Nevertheless, significant changes in the respiratory rate of *S. zeamais* offspring were observed and this was probably due to continued exposure of the progeny to DE. These results reinforce that the physical action of DE hardly generates physiological response that can be transferred to the offspring (Shah and Khan, 2014).

In addition to a lethal effect on insects, non-lethal effects such as walking impairment and also aversion to DE-treated grains were observed. However, these effects were evidenced only for *S. zeamais* which reinforces its greater susceptibility to DE. Since DE causes damage to the walking behavior of the weevils, therefore, damage to the development and establishment of these insects is expected. Thus, changes in feeding behavior (e.g., searching for food) and reproduction (e.g., searching for partners and places for oviposition) may also occur through the action of DE. Generally, inert powders are insect repellent due to the physical presence of the powder (White et al., 1966) and the repellency is dependent on the applied dosage (Bartlett, 1951; Flanders, 1941; Korunic, 1998). Thus, the repelling effect of DE may explain the interference in the behavior of choice of *S. zeamais* insects, as also reported by (Vardeman et al., 2007).

Reduction in *Sitophilus* spp. oviposition capacity may have been caused by several impairments caused by DE, including early mortality, difficulty walking, aversion to treated grains and loss of perception capacity (e.g., damage to the sensilla and pores) as shown in the present study and others studies (Malia et al., 2016b,a;

Vardeman et al., 2007; Wille et al., 2019). The early mortality found in this bioassay, especially for 3 and 6 grams, can be attributed to the age of the insects used. Newly emerged insects (up to 7 days old) may have less sclerotized cuticles, thus less resistant and more susceptible to damage from DE.

Despite the effects on both species and although it needs more experimentation, the most efficient results of the DE on *S. zeamais* demonstrated in the present investigation may be due to possible morphological differences (e.g., cuticle thickness and constitution, as well as pore and sensilla density and distribution) between the two species. Behavioral differences should also be considered (Hidayat et al., 1996), and the *S. oryzae* habit is another important factor to be highlighted, whereas this species, having rice as its main host could be provided with better tools to mitigate the effects of DE. This is due to the fact that rice grains have a silica-rich coating (peel), which would be more similar with DE (Patil et al., 2014). It is noteworthy that with a physical mode of action the development of physiological resistance is considered unlikely (Golob, 1997; Shah and Khan, 2014), however some studies indicate the possibility that some species present or develop some tolerance (Fields, 2003; Rigaux et al., 2001; Vayias and Stephou, 2009; Vayias et al., 2008).

Shah and Khan (2014) points out that this resistance to DE can be considered as a behavioral and not a physiological resistance, since it is generally based on the ability to avoid contact with DE particles. For example, less mobile insects of the *Tribolium castaneum* specie are more tolerant because they reduce contact with DE particles and have higher survival rates (Rigaux et al., 2001). Other studies show that individual and population variations may contribute to behavioral responses and thus contribute to the susceptibility of *S. zeamais* to insecticides (Malia et al., 2016a; Morales et al., 2013). Malia et al. (2016a) found that low-active weevils for walking less minimized contact with DE and had a high survival rate and, alternatively, highly active weevils due to the greater surface escape capacity DE-treated also had considerable survival rates. A study conducted by Adarkwah et al. (2012) showed possible resistance and physiological differences for *Sitophilus oryzae* when exposed to DE. The insect populations used here had no previous contact with the DEs, so we can disregard that the *S. oryzae* population has acquired resistance to the DE, but is possible to consider that behavioral (e.g., rate of movement and reaction to the DE presence) as well as structural characteristics (e.g., smaller size and specific cuticle characteristics, as thickness and / or rigidity of the integument) may have contributed

to its better performance and tolerance to DE (Fields and Korunic, 2000; Shah and Khan, 2014).

In general, the highest concentrations of DE tested (3 and 6g) provided the greatest effects, however, the DE formulations registered in Brazil have recommendations that vary between 0.5 and 1.0 g / kg (Fields and Korunic, 2000; Shah and Khan, 2014). It is known that high DE concentrations should be avoided as they can damage grains and impair storage (Koruni´c, 2016; Korunic, 1998; Korunic et al., 1996). Even so, we suggest that the lethal and non-lethal effects of higher concentrations should be better evaluated in future studies, since in storage areas the DE homogenization may fail with grain mass parts containing higher DE content than others (escape areas).

DE is an important alternative in the control of *Sitophilus* spp. causing not only satisfactory mortality, but also behavioral alteration, which can cause damage to the activity and development of these pests. DE, like all insecticide products, has advantages and disadvantages for insect control in stored grain. The direct action of DE on insects is slower than synthetic insecticides, which eliminate insects faster, reducing the chances of reproduction (Fields, 1998). However, synthetic insecticides can cause serious damage such as resistance development, food and feed contamination, and environmental problems (Aktar et al., 2009). And in contrast, DE leaves no toxic residues in grains and products and is not toxic to humans, animals and the environment. Thus, this information is important in integrated pest management programs, with the possibility of combining the DE application technique with other safe techniques (e.g., grain cooling or heating, biological control, natural products etc.) (Athanassiou et al., 2008; Athanassiou and Steenberg, 2007; Chanbang et al., 2007; Adarkwah et al., 2018) for the control of weevils and other product pests stored in storage units.

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2. CHAPTER 2

Persistence and sex-related differences in toxicity and behavioral responses of two stored grains weevils, *Sitophilus zeamais* and *Sitophilus oryzae* (Coleoptera: Curculionidae), after exposure to diatomaceous earth treated rice grains

ABSTRACT: The weevils *Sitophilus zeamais* and S*itophilus oryzae* (Coleoptera: Curculionidae) are important grain insect pests stored in Brazil and worldwide. Chemical insecticides (e.g., pyrethroids, organophosphates and fumigants) are commonly used to protect stored grains against attack by these pests, however due to limitations by contamination and insect resistance development alternative control methods (e.g., temperature, radiation, essential oils, inert powders, etc.) are required. Alternative forms of control include the use of inert dusts, such as diatomaceous earth (DE). Thus, the objective of this work was to evaluate an DE formulation as an alternative control for two important species of stored grain insects (*S. zeamais* and *S. oryzae*), investigating its lethal and sublethal effects by assessing mortality, walking behavior, DE persistence, reproductive capacity, grain consumption and progeny effects of these species from combinations of couples treated or not with DE using different concentrations (0, 1, 2, 3 and 6 g / DE / kg of rice grains). Higher mortality was obtained for *S. zeamais* than *S. oryzae* ($P < 0.05$) reaching 100% control for the highest concentrations tested (3 and 6 g). Effects on walking behavior for *S. zemais* were also observed ($P < 0.05$), but not for *S. oryzae* ($P > 0.05$). The DE showed greater persistence in its action with the increase exposure period of *S. zeamais*, maintaining a control above 80% for the highest concentrations tested for the storage period of up to 5 months. By evaluating the offspring and grain consumption from the formation of couples by the combination of males and females, treated or not, it was possible to verify not only the greatest DE effect on *S. zeamais* population growth, but also that treated females are more harmed than treated males. Couples with treated females had lower numbers of offspring and, consequently, lower grain consumption, and in contrast, couples with treated males had progeny increases and high grain consumption rate. Effects on progeny for sex ratio were not observed ($P > 0.05$), however, some changes in insect weight and respiratory rate were observed ($P < 0.05$),

but with uncertainties about the role of DE in changing these parameters. The results showed that the DE was efficient in the control of the stored grain weevils, being able to be used in the medium and long term storage. However, we also concluded that *S. oryzae* specie have greater tolerance to DE and that, besides *S. zeamais* species being more susceptible, females when sublethally exposed are more affected than males, and that these differences in response to DE-treatment, at the species and individual level, can explain differences in control and mitigate control failures in storage units.

Key words: Weevil maize. Weevil rice. Pest control. Grãos armazenados. Inert dusts.

2.1. INTRODUCTION

The maize weevil (*Sitophilus zeamais* Motschulsky) and rice weevil (*Sitophilus oryzae* L.) are the main pest species of cereals and stored products. Due to the ability to cause irreparable damage to the final product to be marketed, preventive chemical control by spraying liquid insecticides or dressing on already infested grains using liquid or solid fumigants (e.g., phosphine) has been used and justified (Arthur,1994). However, its use has shown restrictions as outbreaks of resistance and /or contamination arise (Aktar et al., 2009; Guedes et al., 2016).

With the increasing number of insect resistant populations to synthetic insecticides (Boyer et al., 2012; Daglish et al., 2014; Fragoso et al., 2003; Guedes et al., 1995; Ribeiro et al., 2003) and concerning current contamination of products and the environment, efficient control of stored grain pest insects can be achieved by combining different strategies that shape Integrated Pest Management (Hagstrum and Phillips, 2017). Various insect control methods are usable in stored grains, with emphasis on physical methods (e.g. temperature, radiation, inert powders) (Athanassiou and Steenberg, 2007; Chanbang et al., 2007; Fields and Muir, 1996; Subramanyam and Roesli, 2000).

The use of chemically unreactive inert dusts has resurfaced as an important and promising alternative in the control of stored grains pest insects (Kavallieratos et al., 2007; Phillips and Throne, 2010; Shah and Khan, 2014; Subramanyam and Roesli, 2000). Due to its greater control potential, the siliceous diatomaceous earth (DE) is among the most popular inert dusts that have drawn focus and attention for its safety to vertebrates and do not release contaminants into the environment (Korunic, 1998; Raju and Rom,1998; Shah and Khan, 2014).

DE is a natural product extracted from shell sediments of the diatom algae and has silica dioxide as its main component (Chanbang et al., 2007; Lorini, 2008; Subramanyam and Roesli, 2000). Silica has the ability to kill insects by adsorption and abrasiveness, causing them to lose body water until they die (Subramanyam and Roesli, 2000). However, mortality occurs over a variable period, depending on the pest species and other factors (e.g., temperature, humidity, origin of DE formulation, etc.) (Aldryhim,1993; Banks and Fields, 1995; Chanbang et al., 2007; Fields and Korunic, 2000; Malia et al., 2016b; Rigaux et al., 2001) and in addition to sublethal exposure

and their interactions with arthropods present important knowledge gaps (Guedes et al., 2015, 2016; Guedes and Cutler, 2014).

Therefore, the aim of this study was to evaluate a DE formulation as an alternative control against two important insect species of the stored cereal grains (*S. zeamais* and *S. oryzae*) through investigating its lethal and sublethal effects. Was avalieted the mortality, walking behavior, DE persistence and, in addition we evaluate the reproductive capacity, grain consumption and effects on the progeny of these species from combinations of couples treated or not with DE.

2.2. MATERIAL AND METHODS

2.2.1. Overall experimental conditions: Insects and DE formulation

Adult weevils of *S. zeamai*s and *S. oryzae,* not sexed, were collected randomly and used as needed in all the experiments carried out. Both populations were maintained in glass containers in rearing facilities under controlled conditions (25 ±2◦C, 70 ±10% relative humidity, 14 h: 10 h lighting regime [D: L]) and reared on insecticide-free maize grains (approx. 13% moisture content) at the Departamento de Entomologia da Universidade Federal de Viçosa, Viçosa, Minas Gerais, Brazil.

Diatomaceous earth Insecto® (Natural Insecto Products, Orange, CA, USA) was used for the bioassays with register and recommendation to control stored product pests in Brazil at the dose of 1g/kg (Química, 2015). Insecto® is a formulation de marine origin containing 86.7% SiO2 and particle size distribution between 0.82 and 52.33 µm, but centering between 9 and 30 µm (Subramanyam, 1993). The DE treatments were applied to whole rice grains using a stainless still cylinder coupled to a heavy-duty rotator (ROTO-TORQUE model 7637, Cole-Parmer, Vernon Hills, IL, USA).

2.2.2. Mortality rate of the *S. zeamais* and *S. oryzae*

In order to verify the efficacy of DE against Sitophilus spp., mortality tests were performed for each species using the dosages of 1, 3 and 6 g of DE / kg of rice grains, and untreated grains to the control. Four replicates consisting of one plastic flask (300

ml) each containing 50 g of grains, received 25 unsexed adult insects and the mortality was assessed after 14 days of exposure.

2.2.3. Walking behavior of the *S. zeamais* and *S. oryzae*

To evaluate the effects on walking behavior, bioassays were mounted under the same survival test conditions and kept in the rearing conditions for 14 days. After this period, the surviving insects were individually transferred from the treatments to Petri dishes (9cm x 2cm) lined with filter paper (replaced after each recording) and their walking activity was video recorded for 10 minutes by a video camera coupled to a computer equipped with video tracking system (VideoTrack System, Viewpoint LifeSciences, Montreal, Canada). A threshold of 0.1 cm/s was used to distinguish slow from fast walking. The camera was positioned 30 cm from the Petri dish and video analysis was performed under incandescent light and temperature of 25±2◦C. Twenty insects were used for each treatment and the parameter evaluated was stops number, distance covered (cm), walking time (s) and velocity time (cm/s).

2.2.4. Reproductive success, grain consumption (grain loss) and progeny effects

Newly emerged insects (up to 10 days old), previously separated by sex, were exposed to DE-treated grains (0, 1, 3 and 6 g DE / kg grains rice) for 3 and 7 days and the surviving insects were used in the bioassay. Ten treatments were assembled with the following couple combinations: Control, 1g treated male and female (1g), 1g treated male (1g TM), 1g treated female (1g TF), 3g treated male and female (3g), 3g treated male (3g TM), 3g treated female (1g TF), 6g treated male and female (6g), 6g treated male (6g TM) and 6g treated female (6g TF). Glass flasks (20 ml) received 10 g of grains rice and a couple of Sitophilus spp. Twenty-two repetitions per species and treatment were set up and the total number of insects, the final grain mass and de reproductive success (ability to generate progeny by instantaneous growth rate (ri) were recorded after 50 days of storage. The (ri) was calculated for each trateament using the formula $ri = [\ln(N_f / N_i)]/\Delta T$, where Nf and Ni are the final and initial numbers of live (adults) insects, respectively, and ΔT is the duration of the experimental in days (Walthall and Stark,1997).

The progeny (F1) obtained from this bioassay was used for analysis of sexual ratio (e.i., male: female ratio), body mass, and respiratory activity (i.e., CO2 production). To determine the body mass, the insects of each sex were individually weighed on an ultra-precision electronic scale (model XS3DU, Mettler Toledo, Columbus, OH, USA). Respirometry bioassays were carried out using a CO2 Analyzer TR2 (Sable Systems International, Las Vegas, NV, USA) following previously described methods (Guedes et al.,2006; Haddi et al.,2015; Oliveira et al.,2007). The average respiratory rate (CO2 production) of the offspring was measured from 15 replicates to each specie and sex. The insects were placed in chambers (25 ml) connected to a completely closed system, to which the chambers were connected for 90 min before being injected CO2 free air for 2 minutes (600 ml/minutes). The air current directed the CO2 produced by insect respiration to an infrared reader that was connected to the system, which allowed for the immediate quantitation of CO2 production.

2.2.5. Persistence of DE

Rice grains were treated with DE at 1, 3 and 6 g / kg of grain and then stored for later evaluation of the persistence of DE after 1, 2, 3, 4, 5 and 6 months. Clean grains were stored under the same condition as treated grains and used for control. After the respective storage periods, the treated or untreated grains were used to assemble the bioassays. Five replicates consisting of one plastic flask (100 ml) each containing 20 g of grains, received 20 unsexed adult insects and the mortality was assessing after 3, 7 and 14 days of exposure.

2.2.6. Statistical analyses

The survival data was analyzed used Log-Rank test to obtain survival curves and the treatments were compared using and Kaplan-Meier Survival Analysis (SSI,2008). The others data were subjected to analysis of variance (ANOVA). All means comparisons, whenever necessary, were performed by applying Tukey test at 5% probability using SigmaPlot Software (SSI,2008).

2.3. RESULTS

Sitophilus spp. were affected by the Insect® DE formulation. After 14 days of exposure, significant increases in mortality of both species were observed as a function of increases in DE concentration (F = 12.562; P <0.001). *S. zeamais* was more affected by DE with mortality higher than 95% for the highest concentrations (3 and 6g), which did not differ from each other (P = 0.840), whereas for *S. oryzae* a mortality of 75% was reached for the highest concentration tested.

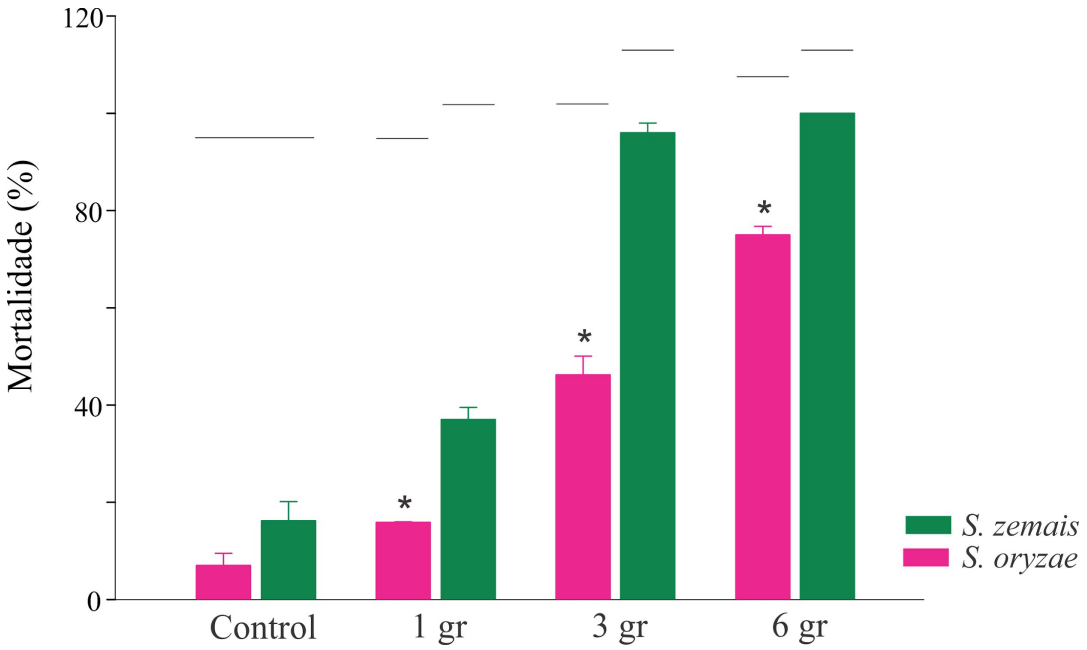

Figure 2.1: Mortality (%) of adults insects of *S. oryzae* and *S. zeamais* after 14 days of exposure to DE at 0, 1, 3 and 6 g/kg of rice grains. Different vertical bars indicate significant differences at 5% level by Tukey test. *Indicate difference between species at 5% level by Tukey test.

After exposure to DE, *S. zeamais* had its walking behavior affected and besides for the concentration of 6g / kg there were no surviving insects to use in the tests. Significant increases in the stops number were observed at 1g / kg (F = 4.906; P = 0.011) and, regardless of dose, significant reductions were observed in the distance traveled (F = 6.591; P = 0.003) and walking speed (F = 10,006; P <0.001), however the walking time did not have significant differences (F = 2.855; P = 0.066). Significant effects (P> 0.05) on *S. oryzae* walking were not observed and the higher concentration

tested did not compromise the insects obtaining for the analyzes. Significant differences between species as a function of treatment occurred in the number of stops (F = 3.767; P = 0.026) for the control (P <0.001) and 3 g (P = 0.023), and in the walking speed (F = 5.896; P = 0.004) for the control (P <0.001).

For the evaluation of the persistence of the DE-effect, significant interactions (P <0.05) were observed for species vs storage period, species vs concentration, and storage period vs concentration (Table 1). However, the interaction between species vs storage period vs concentration was not observed after 3 days of exposure (F = 1.073; P = 0.381) (Table 1).

Table 2.1: Analysis of variance of mortality of *S. zeamais* and *S. oryzae* for different concentrations and storage periods after 3, 7 and 14 days of exposure.

Sources of variation	Exposure Period					
	3 days		7 days		14 days	
	F	P value	F	P value	F	P value
Specie VS Stored	4.869	<0.001*	6.818	<0.001*	3.249	0.004*
Specie VS Conc.	17.059	<0.001*	67.335	<0.001*	64.900	<0.001*
Stored VS Conc.	11.757	<0.001*	26.344	<0.001*	58.859	<0.001*
Especie VS Stored VS Conc.	1.073	0.381	7.792	<0.001*	9.078	<0.001*

* Significant at 5% of probability

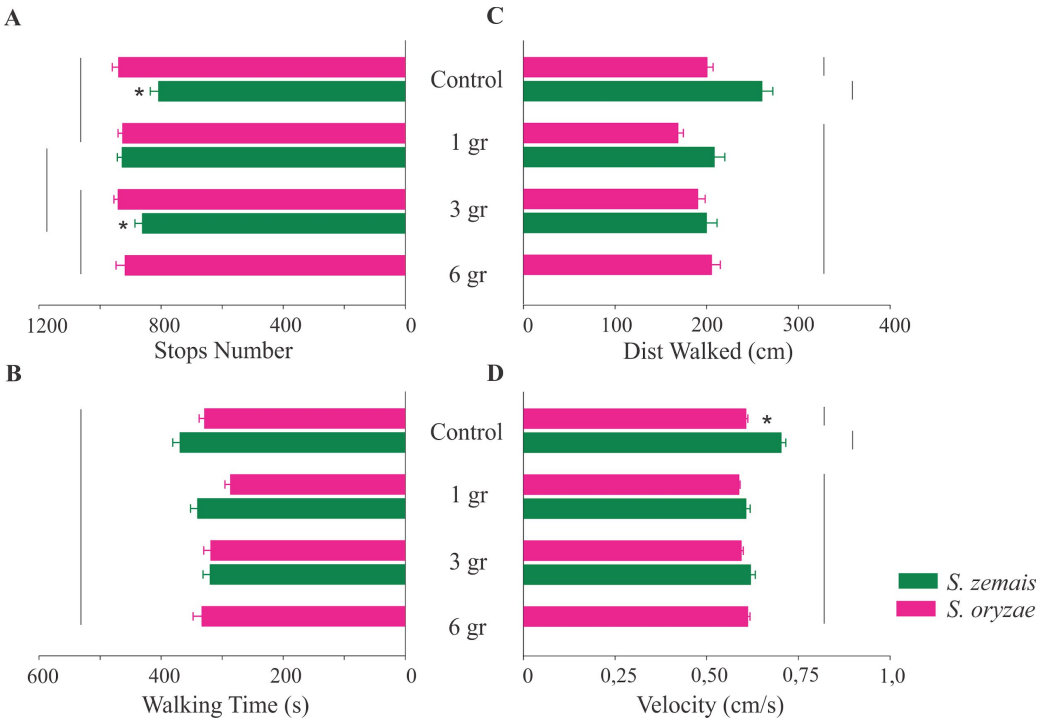

Figure 2.2: Walking behavior to *S. oryzae* and *S. zeamais* after 14 days of exposure to DE at the dosages of 0, 1, 3 and 6g / kg of rice grains. (**A**) Number of stops, (**B**) Walking time, (**C**) Distance walked and (**D**) Velocity. Different vertical bars indicate significant difference at 5% level by Tukey test. * Indicate difference between species at 5% level by Tukey test.

For the differences concentrations and both species the storage period affected the control efficiency of Sitophilus spp. (Figure 3). Although for 3 days of exposure to DE the mortality rate was low (less than 20 and 30% for S. oryzae and *S. zeamais*, respectively), both species had their mortality compromised as a function of the period storage showing significant mortality reductions (P <0.05) for grains treated with 1 and 3g stored for 2 months or more. However, *S. zemais* exposed to the highest concentration (6g / kg) had a significant reduction in mortality only for the storage period of 4 months or more (Figure 3). For storage periods of 4 months or more, 0% DE-persistence was found for both species and all concentrations tested, which did not differ from the control (P> 0.05). The increase in the exposure period to 7 and 14 days generated considerable 728 increases in the mortality rate of both species (Figure 3) and significant 64 Diatomaceous earth effects at species and sex level for Sitophlius spp. losses in the mortality of *S. zemais* and *S. oryzae* at a concentration of 1g / kg occurred for storage by 2 months or more, while for 3g / kg occurred for 4 months or more. However, at the highest concentration (6 g) significant reduction in mortality occurred for storage for 4 months or more (7 exposure days) and 6 months (14 exposure days) for both species (Figure 3).

Fifty days after sublethal exposure of insects to DE and combinations of couples there was a high insect mortality at higher concentrations, which resulted in a decrease in the number of repeats that obtained progenies and that could be used in the respective analyzes. The population growth rate (ri) was significantly compromised for both species for 3 days (F = 2.529; P = 0.008) and 7 days of exposure (F = 5.983; P <0.001). *S. zeamais* was most affected by the increase in the DE concentrations with significant reduction when females and / or males were treated at higher concentrations for 3 days of exposure (3g FT (P <0.001) and 6g FT (P = 0.020)] and 7 days of exposure rates [3g FT (P <0.001) and 6gMT (P <0.001)] in which there were larger reductions in population growth rate (ri). For 7 days of exposure treated males at 6g / kg (6g MT) also had significant reduction (P <0.001) in ri which did not differ from treated females at higher concentrations (3g FT and 6g FT), as shown in Figure 2A. *S. oryzae* did not show a response pattern as funtion to increases in concentration, presenting ri significantly different from control only to 3g (P = 0.035) for 3 days of exposure and 1g FT (P = 0.001). Differences between species as a function of treatment occurred for 3g (P = 0.042), 3g FT (P = 0.002), 6g MT (P = 0.009) for 3 days of exposure and 1g FT (P = 0.032), 3g (P = 0.013), 3g MT (P = 0.005), 3g FT (P

<0.001), 6g MT (P <0.001) and 6g FT (P <0.001) for 7 days of exposure (Figura 4A).

Grain consumption (loss of grains) had a significant effect for treatments for both exposure times [(3 days: F = 3.494; P <0.001) (7 days: 65 Diatomaceous earth effects at species and sex level for Sitophlius spp. F = 4.807; P <0.001)] and non-significant effect for *S oryzae* (P> 0.05). For 3 days of exposure, higher grain consumption rates for *S. zeamais* were reached for 3g MT (2.260.24 g), which differed (P <0.001) from the control (1.370.14 g) and 6g MT (1.840.17 g), which did not differ (P = 0.450) from the control, whereas for 7 days of exposure the Control, 1g and 3g MT showed higher grain consumption rates (1.680.07 g, 1.380.07 g, 1.610.09 g, respectively), which did not differ from each other (P> 0.05). Grain consumption by *S. oryzae* had no significant effect between treatments (P> 0.05) for both exposure times. There was also significant difference (P <0.05) between species in different treatments for 3 [except to 3g FT (P = 0.091) and 6g (P = 0,076)] and 7 days of exposure [except to 6g FT (P = 0,472)] (Figure 4B).

Sex ratio did not present significant interaction for species vs concentration (F = 0.853; P = 0.568), species vs exposure period (F = 1.418; P = 0.234), exposure vs concentration (F = 1.784; P = 0.068) and species vs exposure period vs concentration (F = 0.568; P = 0.824), thus maintaining the 1: 1 aspect ratio (male: female) (Figura 5A).

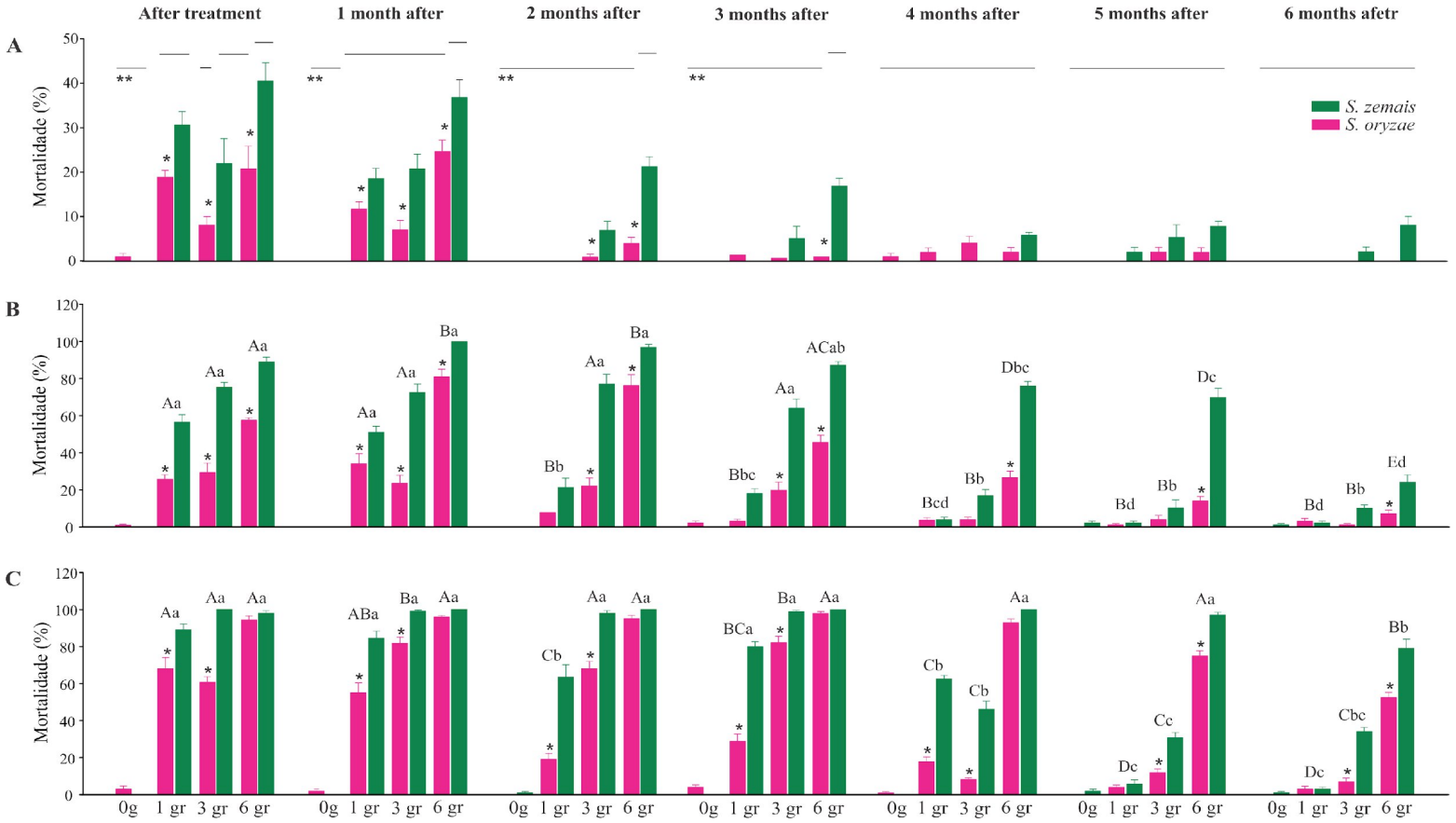

Figure 2.3: Mortality (%) of adults insects of *S. oryzae* and *S. zeamais* after (**A**) 3, (**B**) 7 and (**C**) 14 days of exposure to DE at 0, 1, 3 and 6 g/kg of rice grains used immediately after treatment (after treatment) or previously stored for 1, 2, 3, 4, 5 and 6 months after treatment. Different vertical bars indicate significant differences for species and concentration as a function of storage time at 5% level by Tukey test. * Indicate difference between species as an of concentration at 5% level by Tukey test. ** Indicate difference between species significant difference between species as a function of storage time at 5% level by Tukey test. Different capital letters indicate significant difference for *S. oryzae* in concentration as a function of storage time at 5% level by Tukey test. Different lower case letters indicate significant difference for *S. zeamais* in concentration as a function of storage time at 5% level by Tukey test.

Regarding CO2 production no significant effects were observed for sex according to species and treatment, for both exposure periods 3 (F = 1.609; P = 0.117) and 7 days (F = 0.731; P = 0.693) and no significant difference was observed between specie vs treatment vs exposure time (F = 1.013; P =0.433). However, a positive interaction between species and treatment was observed for 3 days of exposure (F = 2.472; P = 0.012), which was not observed for 7 days of exposure (F = 1.577; P = 0.127). Thus, *S. zeamais*, in the exposure period of 3 days, showed significant changes in CO2 production from the 3g FT treatment (2.93 ±0.14 μL CO2H−1 insect−1) with significant increases (P = 0.037). respiratory rate which differed from the control (2.43 ±0.06 μL CO2H−1 insect−1), while for *S. oryzae* there was a significant difference (P = 0.034) only between Parental (F0) (1.8 ±0.05 μL CO2H−1 insect−1) and the control (1.23 ±0.04 μL CO2H−1 insect−1) with no treatment effect. Significant differences between species occurred 789 for all treatments for the 3 day exposure period (Figure 5B).

Body mass of both species did not differ significantly between sexes for 3 days (F = 0.453; P = 0.501) and 7 days of exposure (F = 1.298; P = 0.255). However, significant differences for species as a function of treatment were observed for the 3 (F = 5.757; P <0,001) and 7 (F = 4.242; P <0,001) exposure periods. Parental insects (F0) of both species presented higher body mass *[S. zeamais* (3.09±0.04 g); S. oryzae (1.84±0.03 g)] which differed statistically from the other treatments for 3 and 7 days of exposure (P <0.001). For the 3-day period of exposure *S. zeamais* had in the treatments Control (2.13±0.03 g) and 3g (2.05±0.03 g) the lowest values of body mass, which did not differ from each other (P = 0.848), and S. oryzae had in the Control treatment (1.05±0.03 g) the lowest body mass which differed from the treatments 1g FT (1.24±0.05 g; P = 0.015), 3g (1.27±0.02 g; P = 0.002), 3g FT (1.27±0.03 g; P = 0.002) and 6g FT (1.26±0.02 g; P = 0.003). For 7 days of exposure, *S. zeamais* had in the treatment 1g FT (2.12±0.04 g) the lowest body mass that differed from the treatments 3g FT (2.26±0.03 g; P = 0.006), 6g (2.32±0.03 g; P = 0.007) and 6g FT (2.31±0.03 g; P = 0.017), and *S. oryzae* again had the treatment Control (1.08±0, 02 g) with lower body mass differing from the treatments 808 1g FT (1.02±0.02 g; P = 0.013), 3g (1.13±0.01 g; P = 0.002), 3g FT (21.24±0.02 g; P = 0.002) and 6g FT (1.26±0.03 g; P = 0.002) Significant differences (P<0.05) between species occurred for all treatments for the 3 and 7 day exposure period (Figure 6) .

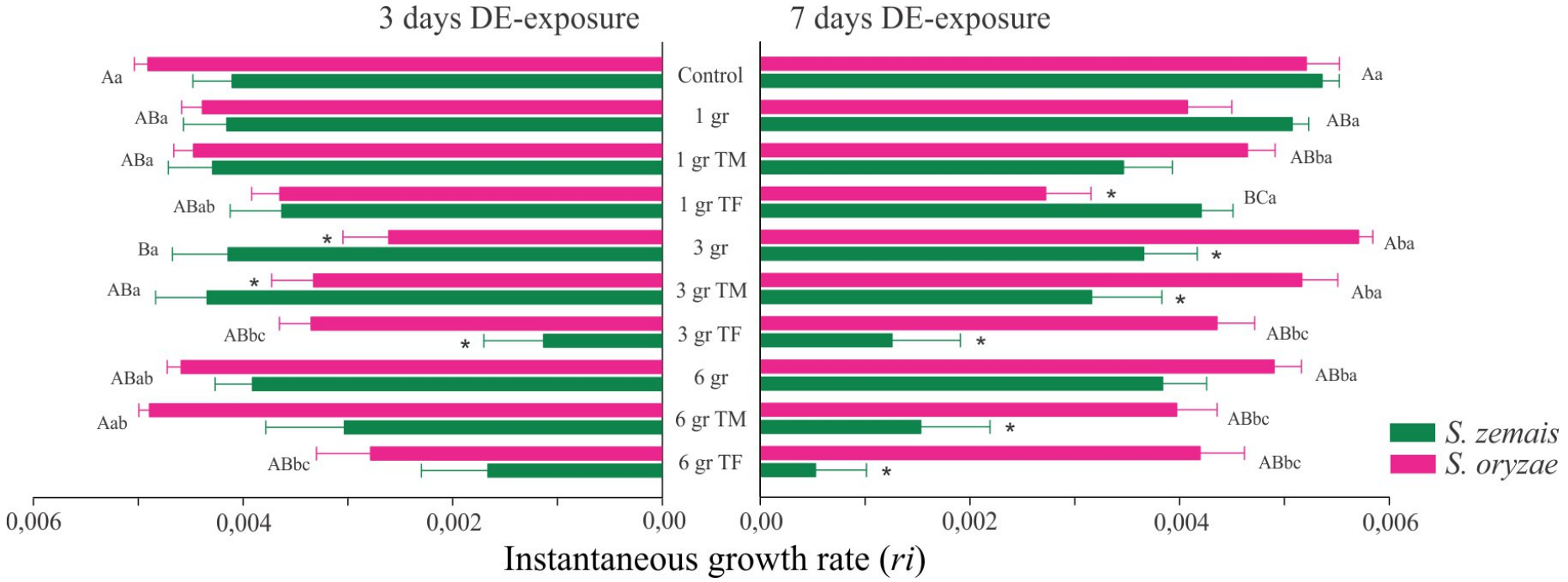

Figure 2.4: Instantaneous population growth rate (r_i) and for *S. oryzae* and *S. zeamais* couples after 50 days of exposure to DE at the dosages of 0, 1, 3 and 6g / kg of rice grains. TM refers to treated male and TF refers to treated female.
* Indicate difference between species at the concentration at 5% level by Tukey test.
Different capital letters indicate significant difference for *S. oryzae* as a concentration at 5% level by Tukey test. Different lower case letters indicate significant difference for *S. zeamais* as a concentration at 5% level by Tukey test.

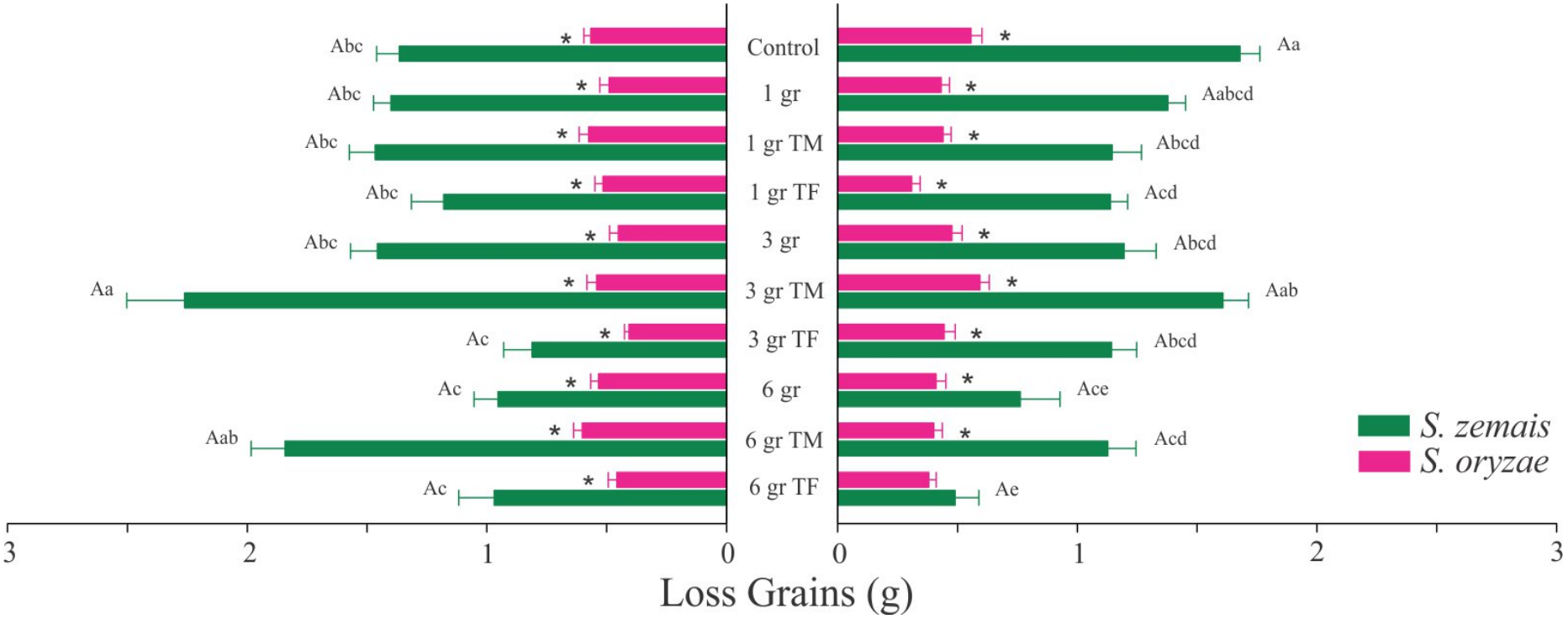

Figure 2.5: Grain loss (g) for *S. oryzae* and *S. zeamais* couples after 50 days of exposure to DE at the dosages of 0, 1, 3 and 6g / kg of rice grains. TM refers to treated male and TF refers to treated female.
* Indicate difference between species at the concentration at 5% level by Tukey test.
Different capital letters indicate significant difference for *S. oryzae* as a concentration at 5% level by Tukey test. Different lower case letters indicate significant difference for *S. zeamais* as a concentration at 5% level by Tukey test.

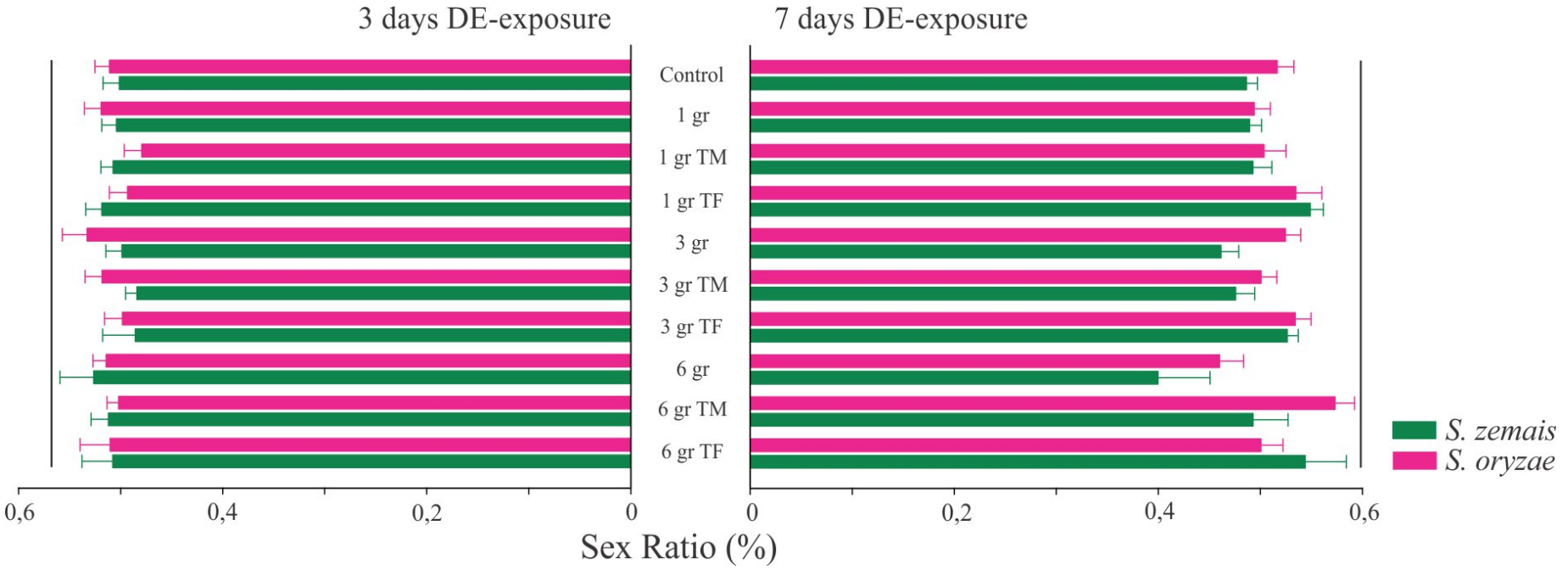

Figure 2.6: A) Sex ratio (%) for *S. oryzae* and *S. zeamais* after 50 days of exposure to DE at the dosages of 0, 1, 3 and 6g / kg of rice grains. TM refers to treated male and TF refers to treated female. Equal bars indicate significant difference at 5% level by Tukey test.

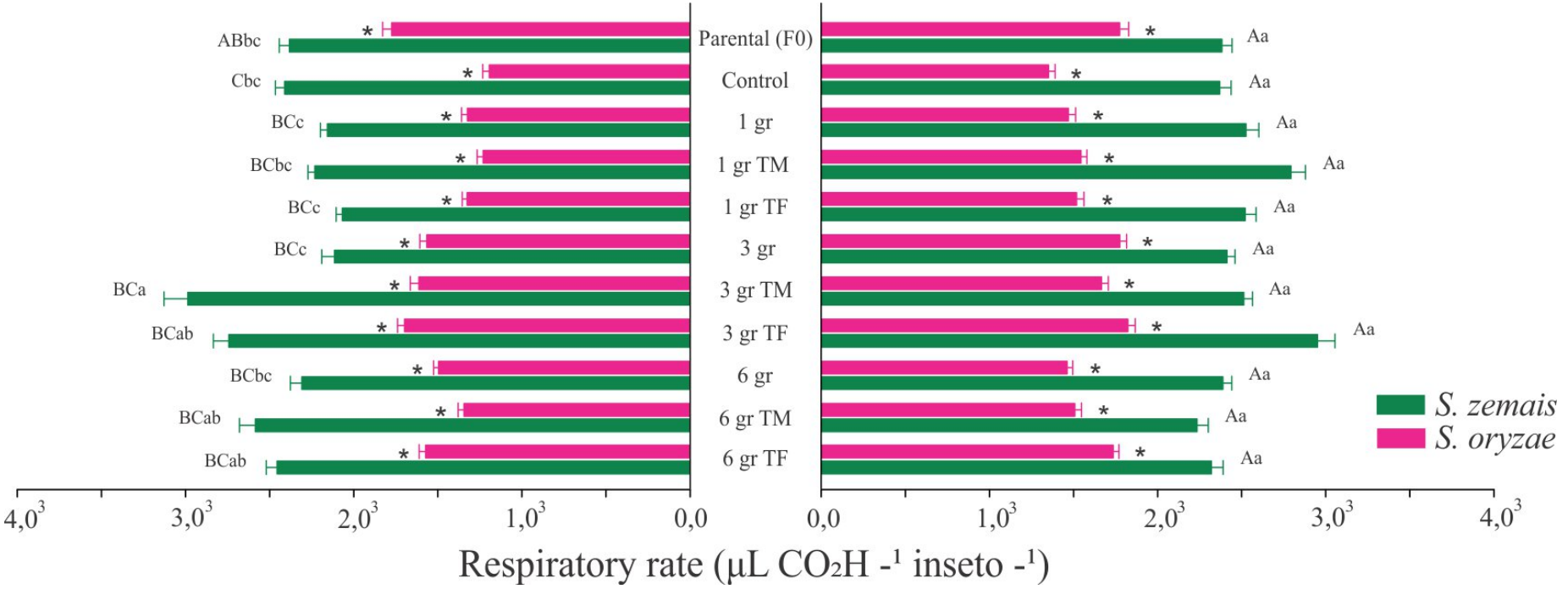

Figure 2.7: Respiratory rate (μL CO_2H^{-1} $insect^{-1}$) for *S. oryzae* and *S. zeamais* after 50 days of exposure to DE at the dosages of 0, 1, 3 and 6g / kg of rice grains. Parental (F0) refers to insects coming from breeding maintenance; TM refers to treated male and TF refers to treated female.

* Indicate difference between species at the concentration at 5% level by Tukey test.

Different capital letters indicate significant difference for *S. oryzae* as a concentration at 5% level by Tukey test. Different lower case letters indicate significant difference for *S. zeamais* as a concentration at 5% level by Tukey test.

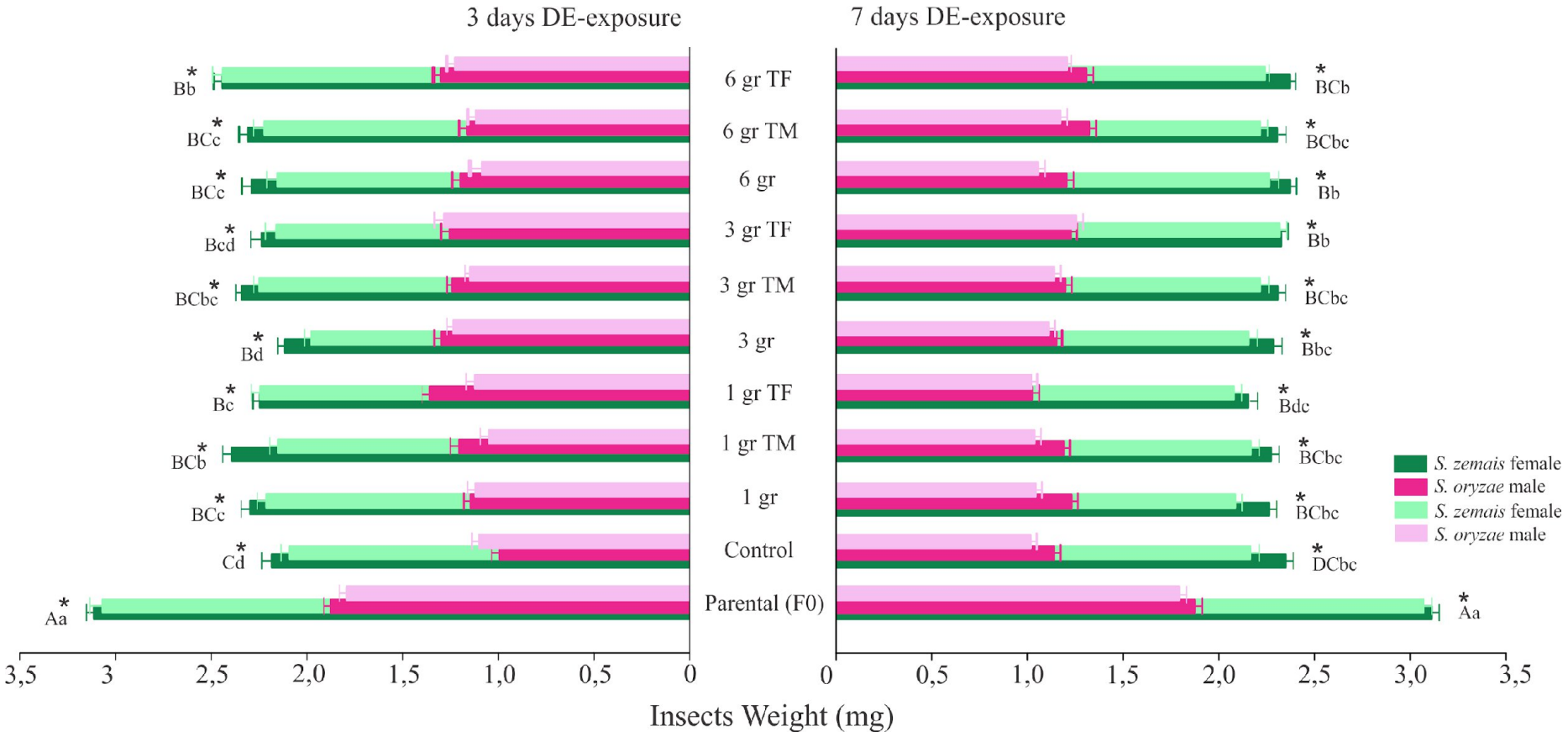

Figure 2.8: Insect weight (mg) for *S. oryzae* and *S. zeamais* couples after 50 days of exposure to DE at the dosages of 0, 1, 3 and 6g/ kg of rice grains. Parental (F0) refers to insects coming from breeding maintenance; TM refers to treated male and TF refers to treated female.

* Indicate difference between species at the concentration at 5% level by Tukey test.

Different capital letters indicate significant difference for *S. oryzae* as a concentration at 5% level by Tukey test. Different lower case letters indicate significant difference for *S. zeamais* as a concentration at 5% level by Tukey test.

2.4. DISCUSSION

Although of the tested populations, *S. zeamais* and *S. oryzae*, were affected by DE at the 3 tested concentrations, satisfactory control (high mortality rate, greater than 95%) occurred only for *S. zeamais* at the highest tested concentrations (3 and 6 g / kg), while the highest mortality rate of *S. oryzae* reached only 75% for the 6g / kg treatment. The efficiency of DE in controlling Sitophilus spp. has been reported in other studies (Doumbia et al.,2014; Malia et al.,2016b; Nwaubani et al.,2014; Wille et al.,2019). Diatomaceous earth has silica dioxide as its main component and mortality is associated with the physical and abrasive properties of DE particles that compromise and increase insect water loss rate (Arthur and Throne, 2003; Banks and Fields,1995; Chanbang et al., 2007; Doumbia et al., 2014; Ebeling et al.,1961; Malia et al., 2016b; Subramanyam and Roesli, 2000).

Sublethal effects on *S. zeamais* walking behavior reinforced the greater susceptibility of this species to DE. DE causes damage to the insect integument (Ebeling,1971; Ebeling et al.,1961; Korunic,1998) and these could cause impairments in mobility and, consequently, impairments in food (eg, search for food, permanence on grain, etc.) and reproduction (e.g., searching for partners, oviposition sites, etc.) which could have a 1 long-term effect on your establishment in grain mass.

In general, the increased storage period of DE-treated rice grains reduced the persistence of DE efficiency for the species and concentrations tested. For the short exposur period evaluation (3 exposure days) significant reductions in control rate occurred for grains treated at 1 month storage or longer, while in later evaluations reductions in control efficiency occurred for grains treated at 4 months of storage or more. Wille et al. (2019) also observed loss of efficiency in the control of *S. zeamais* by grains treated at 1 g / kg and stored for periods of 1, 2 and 3 months, in which there was a considerable reduction in the mortality rate (up to 80%). Storage conditions may affect the insecticidal effect of Des, as DE particles tend to absorb moisture from the environment which reduces their effectiveness in insect control (Fields and

Korunic,2000; Subramanyam and Roesli,2000; Wille et al., 2019). However, other studies by Wille et al. (2019) have shown that prior grain treatment with DE has efficacy as a method to protect grain from future infestations.

The instantaneous population growth rate (ri) allowed us to evaluate the effect

of DE on the ability of couples of Sitophilus spp. generate progeny over a given exposure period (Walthall and Stark,1997). Although some significant population reductions were observed for *S. oryzae*, no pattern was detected as a function of the treated sex or increased DE concentration. In contrast, for *S. zeamais* significant decreases were observed for increasing doses especially for the combination of couples that had the female previously exposed to DE for 3 or 7 days of exposure.

Efficient control of *S. zeamais* progeny by the action of DE also described in other studies (Fields, 1998; Wille et al., 2019) may be due not only to the immediate mortality of the treated insects, but also to the damage added to the survival short period. As reported in this study and other studies (Malia et al., 2016 a, b; Vardeman et al., 2007; Wille et al., 2019) changes in the walking behavior (e.g., movement and maintenance impairments insect damage to the grain), damage to the integument, especially in pores and sensillae (e.g., impaired ability to perceive) and aversion to treated grains would bring sufficient impediments to copulation, oviposition, thus preventing offspring.

Based on the progeny (ri) generated by the different combinations of couples, feeding failures followed different patterns for each species studied. Thus, no difference was observed for *S. oryzae* highlighting its greater tolerance to the action of DE and that sublethal exposures of the male or female do not affect their offspring. However, for *S. zeamais* feeding failures were greater with increasing DE concentration and sublethal exposure of females. *S. zeamais* females were more affected by DE than males, not only with lower progeny, but also because when we had couples with males who were treated (and untreated females) a high population growth rate and high loss grain were observed, being even superior to the control treatment (untreated couple).

Despite the effect on population growth, DE did not significantly affect the progeny sex ratio of *S. zeamais* and *S. oryzae*. However, some changes in body mass and respiratory rate were observed for both species. For example, progenies from combinations of couples where male or female, or both, were exposed to the high concentration of DE showed significant in creases in respiratory rate. These physiological changes in the progeny of *S. zeamais* and *S. oryzae* were initially not expected, because once the mode of action of DE in physical the development of physiological response / resistance is considered unlikely (Golob,1997; Shah and Khan, 2014). The most presumably here is that the substrate had affected the insect

development, since these variables (insect weight and respiratory rate) were relatively higher for insects from stocking (Parental F0). For example, the insect populations used in these bioassays were kept in maize grains which may have allowed a higher Parent (F0) growth and due to the smaller size of the rice grains, and possible nutritional differences, the progenies developed in the grains rice, had their limited growth.

Although these two species are very close and morphologically similar (Hidayat et al., 1996; Peng et al., 2003), and future trials are required, the greater susceptibility of *S. zeamais* to DE showed in this study may be due to the characteristics inherent tO the species (e.g., size, behavior),structural differences at the integument level (e.g., thickness, cuticle pores, sensilla, etc.) or behavioral differences (e.g., mobility, lifestyle/main host) (Hidayat et al.,1996; Adarkwah et al., 2012). For example, the *S. oryzae* specie has rice grains as its main host, and because these grains have a silica rich coating (Patil et al., 2014), *S. oryzae* could be provided with better mechanisms or structures that minimize the DE action. It has also been observed that some insects may have greater tolerance to DE due to their walking behavior and thus the ability to avoid direct contact with DE particles (Malia et al., 2016a; Rigaux et al., 2001; Shah and Khan, 2014). Less mobile insects have greater tolerance to DE, since less movement reduces the contact of insects with DE providing higher survival rates (Rigaux et al., 2001). Malia et al. (2016a) still emphasize that less active maize weevils had reduced DE contact and a high survival rate and, alternatively, highly active weevils also had considerable survival rates due to their greater ability to escape from the DE treated surface.

In this context, the species studied here showed differences in walking behavior (data analyzed by video tracking system) in which *S. zeamais* showed more mobility (distance traveled and walking speed) may be strongly related to its greater susceptibility to DE, because they would be more exposed for being more mobile generating greater friction / contact between the integument and the DE particles. Structural characteristics may also have favored the greater tolerance of *S. oryzae* to DE. For example, their lower body mass may result in a smaller contact surface for 920 the action of DE and other cuticular differences between these species (e.g., thickness / stiffness and cuticle constitution, as well as pore and sensilla density and distribution, etc.) could also be contributing for such susceptibility differences (Fields and Korunic, 2000; Shah and Khan, 2014).

The concentrations that provide the greatest effects (e.g., mortality, progeny prevention, etc.) shown here in this study and in other studies (Wille et al.,2019) are higher than the recommended concentrations, which range from 0.5 to 1.0 g/kg (Brasil, 2019). This is because, among other factors, higher DE concentrations can bring obstacles and disadvantages in commercial use as its undesirable effect on grain flow, bulk density or weight (Korunic´, 2016; Korunic´ et al., 2017). However, DEsmay have a justified application in farms and for structural (multi-surface) treatments in the grain and food industry (Korunic´, 2016; Wille et al., 2019). Another possibility for the use of DE at low concentrations is its combination with other insect pest control methods such as grain mass heating or cooling, biological control, use of natural products, and even the use of low doses of synthetic insecticides etc. (Adarkwah et al., 2017, 2018; Athanassiou and Steenberg, 2007; Athanassiou et al., 2008; Chanbang et al., 2007; Kavallieratos et al., 2010; Korunic´ and Rozman, 2010). Given the above, diatomaceous earth proved to be an important tool in the management of Sitophilus spp., especially *S. zeamais* causing not only lethality but also changes in the individual that may cause damage in the development and establishment of these pests in grain mass.

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3. CHAPTER 3

Morphological characteristics and distribution of sensilla on antennae and legs in male and female of the *Sitophilus oryzae* and *Sitophilus zeamais* (Coleoptera: Curculionidae)

ABSTRACT: The corn weevil, *Sitophilus zeamais* (M.), and rice weevil *Sitophilus oryzae* (L.) (Coleoptera: Curculionidae), are important pests of stored cereals and often show differences in response to environment and to treatment for the control in storage units. The insects have cuticular structures and epidermal cells, the sensillae, that function as sense organs playing an important role in the stimuli perception and behaviors (e.g., communication, food selection, mate search, oviposition). The type, morphometry, abundance and distribution of sensillae on the antennae and legs of *S. zeamais* and *S. oryzae* in both sexes, were analyzed by scanning electron microscopy techniques. Antenna and legs morphology were similar in both sexes and specie and six sensilla types were characterized, including chaetica (antennae: subtypes SC1, SC2 and SC3; legs: subtypes SC1, SC4, SC5 and SC6), basiconica (antennae: subtypes SB1, SB2 and SB3; legs: SB1), trichodea (antennae: subtypes ST1 and ST2; legs: subtypes ST3, ST4, ST5 and ST6), coelonic (SCo), cavity sensillar and squamiformia (only present legs). The possible functions of these sensillatypes are discussed based on their morphology and distribution, as well as based in previously research. The chaetica, tricoid and basiconic sensillae are considered as chemo-and/ormechanoreceptor, squamiformia sensillae have only mechanoreception function and coelonic and cavity sensillar would function as thermo- and/or hygrorecptory. Some differences in the mean length were identified for sex (SC5, SC6, ST2 and SCa) and specie (SC1 antennal, SC5 and SB1 for legs) ($P < 0.05$). Interspecific differences were found especially for abundance of SC1 (for legs), SC3, ST1 and SB1 (for antennae) that were more present in *S. oryzae* with possible differences between sex ($P < 0.05$). These differences on the types of sensory organs are undoubtedly important for behavioral diversity and for insect survival, and can further clarify differences in interspecific responses and provide a basis for the development of management strategies. Therefore, future studies (e.g., transmission electron microscopy (TEM), electrophysiological, proteomics) are needed to confirm these proposed functions.

Key words: Scanning electron microscopy. Weevils. Sexual dimorphism. Antennal sensilla. Tarsal sensillae.

3.1. INTRODUCTION

The maize and rice weevil, *Sitophilus zemais* Motsch and Sitophilus oryzae Linnaeus (Coleoptera: Curculionidae), are a serious pest of stored grains have been dispersing worldwide. Classified as cosmopolitan and primary pests, these beetles infestation results in a substantial reduction in the quantity and quality of the grains and products (Danho et al.,2002; Athanassiou et al.,2017). These species present the same life history, but they have differences in some behavior and in their grains exploration strategies (Longstaff et al.,1981; Athanassiou et al.,2017). The insects can perceive environmental stimuli through their olfactory and chemosensory organs, the sensory structures (e.i., sensilla), that play an important role in various behaviors in the different stages of their life cycle (De Bruyne and Baker, 2008; Keil and Steinbrecht, 1984; Schneider, 1964).

The sensillae can be classified based on their morphometric characteristics (e.g., chaetica, tricoidea, basiconica, coelonic, etc.) and based on their functions, as perception of chemical molecules (olfactory /mechanoreception), perception of body and wings position, substrate, surface (tactile/mechanoperception) and perception of the temperatura and humidity (thermo- and hygroreception) (Zacharuk,1985). Like this, the sensillae are structures important responsible to sensitivity of the insects to chemicals and tactiles signals in and are principally present in the antennae, mouthpieces, and legs (De Bruyne and Baker, 2008).

Insect antennae are important in the detection of several stimuli involving various types of sensilla with different functions to find suitable habitats, food sources and mating opportunities (Schneider, 1964). Antennal sensilla of different species, including beetles, have been characterized in numerous studies using electron microscopic techniques (Ali et al., 2016; Ravaiano et al., 2014; Isaac et al., 2015) and the type, distribution and abundance might vary according to species, sex, castes, but also to the adaptation necessity, insect behavior and geographical distribution (Chen et al., 2003; Esteban et al., 2005; Euzébio et al., 2013; Faucheux et al., 2006; Nakanishi et al., 2009; Nascimento et al., 2013; Ravaiano et al., 2014). Alabi et al. (2014) emphasize the evolution of insets favored many structural changes to pest of storage, as its legs with sensillas, because its distribution in confined spaces need a precise and fast locomotion with the recognition of the environment.

However, very few studies have focused on the insets legs, including the search for difference between *S. zeamais* and *S. oryzae* in antennae and legs parts. Thus, this study used the scanning electron microscopy (SEM) to examine antennal and legs morphology with their associated sensilla in two weevils, *S. zeamais* and *S. oryzae*, and to characterized and determined the localization, distribution and abundance of the sensilla on the both sexes.

3.2. MATERIAL AND METHODS

3.2.1. Insects

Adults of *S. zeamais* and *S. oryzae*, one day old, were collected from 1020 maize grains maintained at the Departamento de Entomologia da 1021 Universidade Federal de Viçosa, Viçosa, Minas Gerais, Brazil. They were 1022 reared in 1000 mL plastic jars containing about 500 g maize grains in the laboratory, and maintained at 25 [± 2 ◦ 1023 C], 75% relative humidity with 1024 14:10 light: dark photoperiod.

3.2.2. Scanning electron microscopy (SEM)

Freshly emerged adult beetles had their right-side antennae and legs removed. Antennae were carefully excised from de antennal sockets while the legs were cut out on the tibia base. Immediately after the cuts, antennae and legs were placed in alcohol 70% for one day. After that, antennae and legs were dehydrated in a graded alcohol series of 70 to 99% and in hexamethyldisilazane (HMDS), each case for 5 min submerged, dried in a critical point dryer, mounted on aluminum stubs with double-sided adhesive tapes. The stubs were maintained in hermetic sealing with silica gel and, immediately before the observation, were sputter coated with gold (metallized) in a using a sputter-coating device. The samples had their ventral surface examined using a LEO VP1430 SEM at the Núcleo de Microscopia e Microanálises at UFV (NMM/UFV).

3.2.3. Statistical analyses

The sensilla from the ventral surface of the antennae and legs of male and female, for both two species, were identified, counted and measured. The morphological terminologies of Schneider (1964), Zacharuk (1985) Differences in antenna and leg sensilla for *S. zeamais* and *S. oryzae* and Ali et al. (2016) were applied in the classification of sensilla types, based on the external shape and size. For the counting and measurement of the sensilla length we use the ImageJ R software (Rasband, 2018). Measurements (µm) obtained from photomicrographs of the 15 individual sensilla of the same type and of at least three samples were used to calculate the means. Data obtained on the distribution of the different types of sensilla on male and female of *S. zeamais* and *S. oryzae* were analyzed using t-test ($P < 0.05$) with SigmaPlot R (Systat-Software, 2008).

3.3. RESULTS

3.3.1. General description of antenna and leg of *S. zeamais* and *S. oryzae*

The antennae and legs of *S. zeamais* and *S. oryzae* are similar in shape, including for male and female. These species have geniculate antenna that consist of the scape, pedicel and flagellum with six flagellomeres. All flagellomeres, except for the last one characterized in an oval club, have a more conical base (Figure 1). The antennal surface is covered with scales, which are more evident on the pedicel and in the flagellomeres previous to the club. All legs (foreleg, midleg and hindleg) were also similar for male and female to both species, and consist of cursorial legs with coxa, trochanter, femur, tibia and tarsus (Figure 2). The legs are also covered with scales and different sensilla to the segments, which are more evident on the tibia and tarsus. Difference in the lengths of antennae and legs, and its segments, are demonstrated in Table 1 e 2, respectively.

Table 3.1: Mean length (*µ*m) of antennal segments of *S. zeamais* and *S. oryzae*.

Species	Scape	Pedicel	Flagelomere						Total
			1^{th}	2^{nd}	3^{rd}	4^{th}	5^{th}	6^{th}	
S. zeamais	936.09*	360.62*	103.66*	79.82*	54.96*	51.89*	58.44*	60.56*	171.57*
	(±23.68)	(±11.15)	(±4.14)	(±2.76)	(±2.09)	(±2.05)	(±1.62)	(±1.74)	(±2.89)
S. oryzae	772.97	297.67	77.81	60.85	42.43	44.02	45.62	51.18	152.73
	(*µ*6.18)	(±2.22)	(±0.69)	(±0.88)	(±0.98)	(±1.01)	(±1.07)	(±1.66)	(±0.79)

Values are means standard deviation (SE) number of each segment
* Means in the same column significantly different between species at 5% level by Tukey test

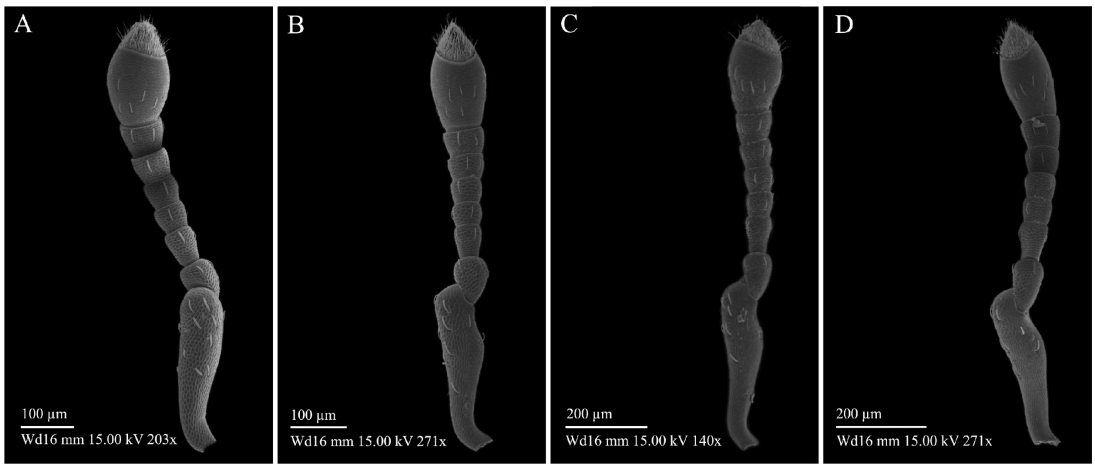

Figure 3.1: Scanning electron microscopy (SEM) showing the longitudinal morphology view of male and female antennae of *S. oryzae* and *S. zeamais*. (**A**) Antenna of *S. oryzae* female; (**B**) Antenna of *S. oryzae* male; (**C**) Antenna of *S. zeamais* female; (**D**) Antenna of *S. oryzae* male. Sc refers to scape; Pe refers to pedicel; Fl refers to flagellomere (1^{th}-6^{th}). Scale bars: (A-B) 100 *µ*m; (C-D) 200 *µ*m.

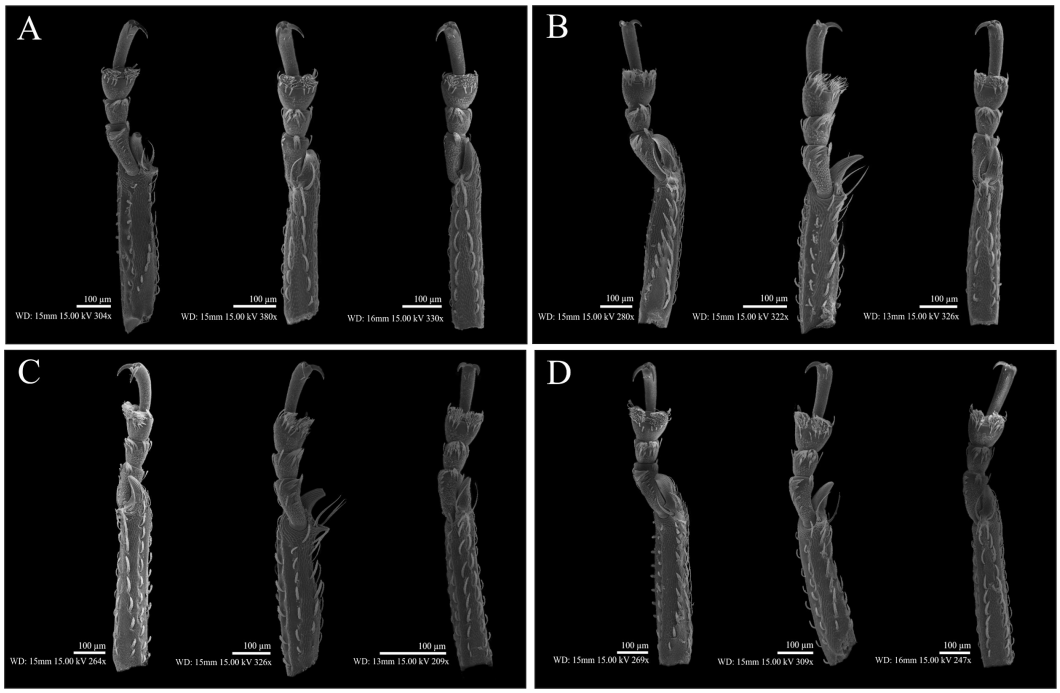

Figure 3.2: Scaning electron microscopy (SEM) showing the morphology longitudinal view of different legs types (Forleg, midleg and hindleg) of *S. oryzae* female (**A**) and male (**B**) and *S. zemais* female (**C**) and male (**D**). T refers to tibia; S refers to spur; T refers to tarsomere; C refers to claws. Scale bars: (A-D) 100 *µ*m.

Table 3.2: Mean length (*μ*m) of legs segments of *S. zeamais* and *S. oryzae*.

Forleg	Tibia	Spur	Tarsu			Claws	Total
			1th Segment	2nd Segment	3rd Segment		
S. ZEAMAIS	542.24* (±10.45)	122.21 (±6.49)	154.72 (±5.96)	76.22* (±1.21)	101,77 (±1.53)	158,18 (±3.61)	1026.06** (±16.17)
S. oryzae	445.21 (±7.56)	65.26 (±17.76)	133.58 (±2.23)	64.63 (±1.26)	89.66 (±1.48)	151.917 (±3.72)	849.50 (±16.69)

Midleg	Tibia	Spur	Tarsu			Claws	Total
			1th Segment	2nd Segment	3rd Segment		
S. ZEAMAIS	446.36** (±12.70)	98.33 (±2.50)	129.61 (±3.36)	71.29 (±1.93)	99.12 (±1.95)	164.45 (±2.94)	905.93** (±14.04)
S. oryzae	334.96 (±8.38)	84.94 (±1.78)	106.17 (±5.09)	66.74 (±1.78)	105.21 (±10.52)	144.28 (±3.14)	757.38 (±8.12)

Hindleg	Tibia	Spur	Tarsu			Claws	Total
			1th Segment	2nd Segment	3rd Segment		
S. ZEAMAIS	527.45** (±16.27)	112.37 (±5.19)	141.36 (±6.48)	77.55 (±2.40)	103.42 (±1.41)	172.63 (±5.83)	1.019,76** (±20.38)
S. oryzae	393.57 (±6.33)	87.65 (±2.33)	125.26 (±3.15)	68.27 (±0.99)	91.35 (±1.16)	155.32 (±2.92)	828.14 (±5.09)

Values are means ±standard deviation (SE) number of each segment for the different legs (n=3), forleg, midleg and hindleg.

* Means significantly different between species at 5% level by Tukey test.

** Means significantly different between species at 0,1% level by Tukey test

3.3.2. Characterization and morphometric description of sensilla types

Ten sensilla subtypes were differentiated on the antennae of male and female, for both two species. Three types of sensilla chaetica (SC1, SC2, SC3), two types of sensilla trichoid (ST1, ST2), three types of sensilla basiconic (SB1, SB2, SB3), one type of sensilla coelonic (SCo) and one type of sensilla cavity (SCa) (Figure 3). The greatest number and variety of 1065 sensilla where observed on the oval club apex, while in most other 1066 segments occurs only one or two types of sensilla (Figure 3 B and F). The distribution of the different antennal sensillae on the antennal segments is showed in the Table 3. Morphological features of antennal sensilla of *S. zeamais* and *S. oryzae* on each segment were evaluated by SEM images and this is summarized in Table 4. For the legs of *S. zeamais* and *S. oryzae* were identified twelve sensilla types in male and female, for both two species. Four types of sensilla chaetica (SC1, SC4, SC5, SC6), four types of sensilla trichoid (ST3, ST4, ST5, ST6), one type of sensilla basiconic (SB1), one type of sensilla squamiformia (SS), one type of sensilla coelonic (SCo) and one type of sensilla cavity (SCa) (Figure 4). In the ventral surface of the pulvilli were also observed lots of 1077 tenent setae (Figure 4). The distribution of the different sensillae's legs on 1078 the legs segments is showed in the Table 5. Morphological features of legs sensilla of *S. zeamais* and *S. oryzae* on each segment were evaluated by SEM images and this is summarized in Table 6.

Sensilla chaetica (SC)

Sensilla chaetica 1 (SC1): The SC1 occurs in all segments of the antennas and legs (forleg, midleg and hindleg) for both sexes of *S. zeamais* and *S. oryzae*. The SC1 was indentified by its grooved surface, like toothed-tree leaves, on the curved distal region wich had a blunt tip. Theirs basal parts were connected to the cuticle surface in a small pin. The length to antennal SC1 presented difference ($P<0.05$) between specie, with higher length to *S. zeamais* specie (26.80±0.67 µm), and between female to species studied (28,88±0,89 µm). Difference to species for the SC1 present in legs occurred only to forleg (*S. zeamais*: 42.32±1.25 µm; *S. oryzae*: 35.80±1.39 µm), being that its average length was greater than for the SC1 found in antennas.

Sensillas chaeticas 2 and 3 (SC2 and SC3): These are sparsely distributed in the

last segment of the flagellum for both species and sexes, but SC3 type was hardly observed in *S. zeamais* females. These sensilla are morphologically similar, having rounded and erect bristles before being ramified two (SC2) or three (SC3) branches of uneven length. There was no difference in mean length for SC2 and SC3 between species and sex (P> 0.05), in wich the SC2 and SC3 length for *S. zeamais* was 14.26 µm (±0.28) and 13.17 µm (±0.61) and for S. oryzae it was 13.17 µm (±0.24) 1100 and 12.24 µm (±0.35).

Sensillas chaetica 4 and 5 (SC4 and SC5): The SC4 occurs in the first and second segment of the tarsus, while SC5 occurs in the second and third segments, on all legs to both sexes of *S. zeamais* and *S. oryzae*. These sensilla are morphologically similar, with differences in length and thickness. They had a rounded base and a flattened apex from which several branches emerged. The sencilla SC5 had longer branches that appeared more distally. No difference in SC4 length was observed, as shown in Table 5. However, SC5 length differed (P <0,05) between specie to midleg (*S. zeamais*: 30.93±0.87 µm; S. oryzae: 25.75±0.52 µm) and between sex to *S. zeamais* forleg and between species to the male hindleg (Table 5).

Sensilla chaetica 6 (SC6): Present only to the apex of the claws of all legs (forleg, midleg and hindleg) to both sexes and species. the SC6 type was distinguished by its flattened shape that can be expanded distally or proximally. The SC6 almost have no projection angle and being tilted toward the cuticle surface appear to lie on the cuticular surface. The SC6 length differed between sex to *S. zeamais* hindleg (Table 5).

Sensilla Trichoid (ST)

Sensillas Trichoids 1 and 2 (ST1 and ST2): Present at the tip of the 6th flagellomere, sensillas ST1 and ST2 were simple, branchless bristles and occurred in large quantities. The ST1 are sharp-tipped, nearly straight or slighty curved. The ST2 are shoter than ST1 a blunt-tipped and, sometimes, more curved. No difference in ST1 length was observed, as opposed to ST2 length that differed (P <0.05) between sex to *S. zeamais* (Table 4).

Sensilla Trichoid 3 (ST3): Located in pairs in the tibia of all legs (forleg, midleg and

hindleg) to both sexes and species, the ST3 type sensilla ischaracterized by being long bristles that appear to be fused distally. Emerging from a deep well, they occur as four cast bristles with curvature into the tibia. Difference ($P < 0.05$) on the ST3 length occurred between species to hindleg *(S. zeamais*: 91.37±2.17 µm; *S. oryzae:* 112.99±1.70 µm).

Sensilla Trichoid 4 (ST4): The ST4 also occurs in the tibia below the spurof all legs (forleg, midleg and hindleg) to both sexes and species. The ST4 is a single, robust, long, sharp-pointed bristle. No difference in ST4 length was observed, as shown in Table 5.

Sensilla Trichoid 5 (ST5): The ST5 occurs in the 1st tarsomere of the forlegs to both sexes and species. Like ST4, ST5 is a single, pointed bristle, but more robust and longer. ST5 occurred in large quantities in a limited space and were numerous and too dense to be counted. There were no differences in length between species or sex for ST5 (Table 5).

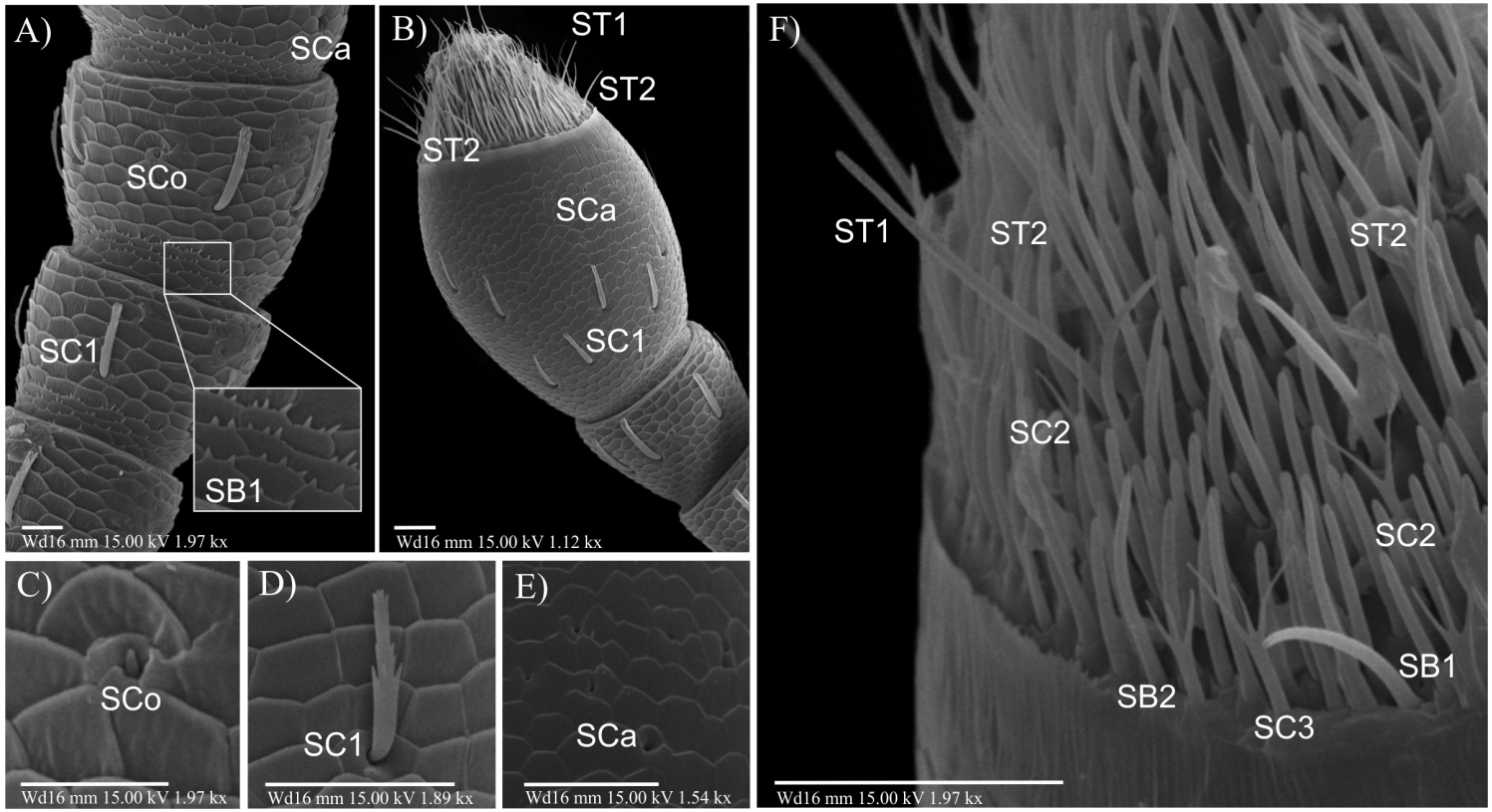

Figure 3.3: Scanning electron microscopy (SEM) of the antennal sensillae in male and female of S. oryzae and *S. zemais*. (**A**) detail of 4th and 5th flagellomere showing SC1 (sensilla cahetica type 1), SB1 (sensilla basiconic type 1), SCo (sensilla coelonic) and SCa (sensilla cavity) sensillas types; (**B**) detail of 5th and 6th flagellomere; (**C**) detail of SCo on the 5th flagellomere (**D**) detail of SC1 on the 4th flagellomere; (**E**) detail of SCa on the 6th flagellomere (**F**) detail of the last flagellomere showing SC2 (sensilla chaetica type 2), SC3 (sensilla chaetica type 3), ST1 (sensilla trichodea type 1), ST2 (sensilla trichodea type 2), SB2 (sensilla basiconic type 2), (SB3 sensilla basiconic type 3) sensillas types. Scale bars: (A and C) 10 μm; (B, D, E and F) 20 μm.

Table 3.3: Distribution of sensilla on the ventral surface of the *S. zemais*, *S. oryzae* and *S. granarius* antennae.

Specie	Sex	Antenal Segments							
		Scape	Pedicel	1st F	2nd F	3rd F	4th F	5th F	6th F
S. ZEMAIS	**Male**	SC1	SC1 SB1	SC1 SB1 SCa	SC1 SB1 SCa	SC1 SB1 SCa	SC1 SB1 SCa	SC1 SB1 SCo SCa	SC 1-2-3 SB 1-2-3 ST 1-2 SCa
	Female	SC1	SC1 SB1	SC1 SB1 SCa	SC1 SB1 SCa	SC1 SB1 SCa	SC1 SB1 SCa	SC1 SB1 SCo SCa	SC 1-2-3* SB 1-2-3 ST 1-2 SCa
S. oryzae	**Male**	SC1	SC1 SB1	SC1 SB1 SCa	SC1 SB1 SCa	SC1 SB1 SCa	SC1 SB1 SCa	SC1 SB1 SCo SCa	SC 1-2-3 SB1-2-3 ST 1-2 SCa
	Female	SC1	SC1	SC1	SC1	SC1	SC1	SC1	SC 1-2-3

			SB1	SB1 SCa	SB1 SCa	SB1 SCa	SB1 SCa	SB1 SCo SCa	SB 1-2-3 ST 1-2 SCa
S. oryzae*	**Male**	SC1	SC1 - 	SC1 - -	SC1 - -	SC1 - -	SC1 - -	SC1 - -	SC 1 SB 2-3 ST 1-2 -
	Female	SC1	SC1 - 	SC1 - -	SC1 - -	SC1 - -	SC1 - -	SC 1 - -	SC1 SB 2-3 ST 1-2 -
S. granarius*	**Male**	SC1	SC1 - 	SC1 - -	SC1 - -	SC1 - -	SC1 - -	SC1 - -	SC 1 SB 2-3 ST 1-2 -
	Female	SC1	SC1	SC1	SC1	SC1	SC1	SC 1	SC1

			-	-	-	-	-	-	SB 2-3
				-	-	-	-	-	ST 1-2
								-	-
S. granarius**	**Male**	-	-	-	-	-	-	-	SC 2-3
			-	-	-	-	-	-	ST 1
				-	-	-	-	-	SB 3
								-	-
	Female	SC1	SC1	SC1	SC1	SC1	SC1	SC1	SC1-2-3
			-	-	-	-	-	-	-
				-	-	-	-	-	-
								-	-

* related by Fouda et al. (2016): SC1 refers to SC; SB2 refers to SB1; SB3 refers to SB2; ST1 refers to ST1; and ST2 refers to ST2.
** related by Ali et al. (2016): SC1 refers to SCH II with occurrence in female without description of the wich segments occur; SC2 and SC3 refers to SCH V and SCH I with occurrence only in male; ST1 refers to SB1 with occurrence only in male; SB3 refers to SBI1.

Table 3.4: Comparative morphology of antennal sensilla types of *S. zeamais* and *S. oryzae*.

Sensilla	Specie	Morphological characteristics of sensilla				
		Length (μm)		Shape	Tip	Socket
		M	F			
SC1	*S. zeamais**	24,72 (±0,85)	28,88** (±0,89)	Curved bush	Thin tips	Raised
	S. oryzae	22,04 (±0,54)	21,79 (±0,37)			
SC2	*S. zeamais*	13,99 (±0,29)	14,55 (±0,53)	Straight with 2 tips	Sharp	Raised and tight
	S. oryzae	12,73 (±0,44)	13,60 (±0,21)			
SC3	*S. zeamais*	13,09 (±0,75)	13,58 (±0,00)	Straight with 2 tips	Sharp	Raised and tight
	S. oryzae	11,96 (±0,31)	12,85 (±0,43)			

ST1	*S. zeamais*	25,82 (±0,68)	29,76 (±0,86)	Straight	Blunt	Tight
	S. oryzae	27,20 (±0,31)	28,42 (±0,33)			
ST2	*S. zeamais*	21,371 (±0,59)	25,91** (±1,01)	Straight or slightly curved	Blunt	Tight
	S. oryzae	22,73 (±0,41)	22,67 (±0,60)			
SB1	**S. zeamais**	1,37 (±0,07)	1,17 (± 0,08)	Straight and short	Cone	Wide
	S. oryzae	1,16 (±0,06)	1,34 (±0,06)			
SB2	*S. zeamais*	13,49 (±0,24)	14,30 (±0,29)	Straight and robust	Cone	Raised and tight
	S. oryzae	14,06 (±0,28)	14,32 (±0,27)			

SB3	*S. zeamais*	16,98 (±0,42)	17,83 (±0,21)	Robust slightly curved	Cone slightly curved	Raised and tight
	S. oryzae	17,34 (±0,37)	17,51 (±0,34)			
SCo	*S. zeamais*	2,04 (±0,07)	1,69 (±0,04)	Short and robust	Small cone	Sunken and flexible
	S. oryzae	1,59 (±0,06)	1,63 (±0,09)			
SCa	*S. zeamais*	0,57 (±0,03)	0,89** (±0,05)	Pore	Pore	Sunken pit
	S. oryzae	0,55 (±0,02)	0,50 (±0,02)			

Values are means standard deviation (SE) length and width of each antennal sensilla type SC1, SC2, SC3 refer to sensilla chaetica types 1-3; ST1 and ST2 refer to sensilla trichodea types1-2; SB1, SB2 and SB3 refer to sensilla basiconica types 1-3; SCo refers to sensilla coelonic; SCa refers to sensilla cavity. A refers to 'antenna';
L refers to 'leg'. The length of SCa refers to opening length.
* Mean significantly different between species at 5% level by Tukey test.
** Mean significantly different between sex at 5% level by Tukeytest.

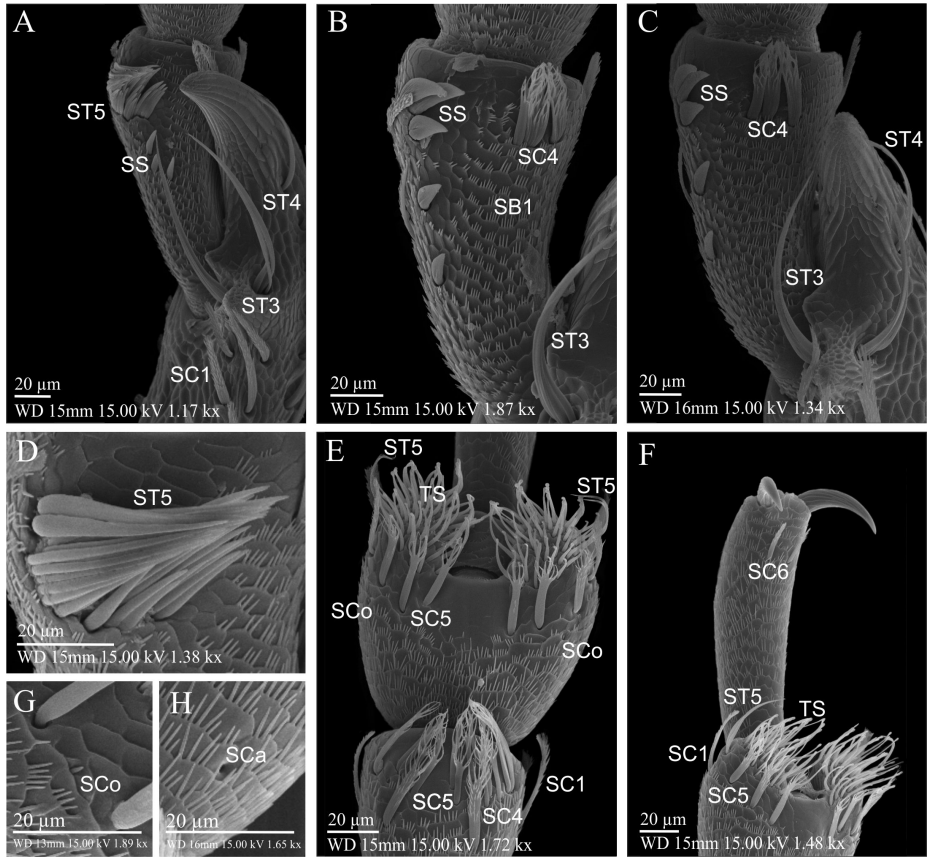

Figure 3.4: Scanning electron microscopy (SEM) of the legs sensilla in male and female of *S. oryzae* and *S*. zemais. (**A**) Forleg with end portion of the tibia, spur and 1th tarsomere showing SC1 (sensilla cahetica type 1), SC4 (sensilla cahetica type 4), ST3 (sensilla trichodea type 3), ST4 (sensilla trichodea type 4), ST5 (sensilla trichodea type 5), SB1 (sensilla basiconic type 1) and SS (sensilla squamiformia); (**B**) Midleg showing SC1, SC4, ST3, SB1 and SS sensilla types. (**C**) Hindleg showing SC1, SC4, ST3, ST4, SB1 and SS sensilla types. (**D**) detail of ST5 on the 1th tarsomere; (E) 2nd and 3^{sd} tarsomere showing SC1, SC4, SC5 (sensilla chaetica type 5), ST6 (sensila trichodea type 6), SB1, SCo (sensilla coelonic) and TS (tenent setae). (**F**) Claws showing SC6 (sensilla chaetica type 6). (**G**) detail of SCo sensilla type on the 3rd tarsomere. (**H**) detail of SCa (sensilla cavity) on the 3^{sd} tarsomere. Scale bars: (A-H) 20 μm.

Table 3.5: Distribution of sensilla on the ventral surface of the *S. zemais* and *S. oryzae* legs.

Specie	Sex	Leg Type														
		Forleg					Midleg					Hindleg				
		Tibia	1st T	2nd T	3rd T	Claws	Tibia	1st T	2nd T	3rd T	Claws	Tibia	1st T	2nd T	3rd T	Claws
S. ZEAMAIS	**Male**	SC 1	SC 1-4	SC 1-4-5	SC 1-5	SC 6	SC 1	SC 1-4	SC 1-4-5	SC 1-5	SC 6	SC 1	SC 1-4	SC 1-4-5	SC 1-5	SC 6
		ST 3-4	SB 1	SB 1	SB 1	SB 1	ST 3-4	SB 1	SB 1	SB 1	SB 1	ST 3-4	SB 1	SB 1	SB 1	SB 1
		SCa	ST 5	SCa	ST 6	SCa	SCa	ST 5	SCa	ST 6	SCa	SCa	ST 5	SCa	ST 6	SCa
			SS		SCo			SCa		SCo			SCa		SCo	
			SCa		SCa					SCa					SCa	
	Female	SC 1	SC 1-4	SC 1-4-5	SC 1-5	SC 6	SC 1	SC 1-4	SC 1-4-5	SC 1-5	SC 6	SC 1	SC 1-4	SC 1-4-5	SC 1-5	SC 6
		ST 3-4	SB 1	SB 1	SB 1	SB 1	ST 3-4	SB 1	SB 1	SB 1	SB 1	ST 3-4	SB 1	SB 1	SB 1	SB 1
		SCa	ST 5	SCa	ST 6	SCa	SCa	ST 5	SCa	ST 6	SCa	SCa	ST 5	SCa	ST 6	SCa
			SS		SCo			SCa		SCo			SCa		SCo	
			SCa		SCa					SCa					SCa	
S. oryzae	**Male**	SC 1	SC 1-4	SC 1-4-5	SC 1-5	SC 6	SC 1	SC 1-4	SC 1-4-5	SC 1-5	SC 6	SC 1	SC 1-4	SC 1-4-5	SC 1-5	SC 6
		ST 3-4	SB 1	SB 1	SB 1	SB 1	ST 3-4	SB 1	SB 1	SB 1	SB 1	ST 3-4	SB 1	SB 1	SB 1	SB 1
		SCa	ST 5	SCa	ST 6	SCa	SCa	ST 5	SCa	ST 6	SCa	SCa	ST 5	SCa	ST 6	SCa
			SS		SCo			SCa		SCo			SCa		SCo	
			SCa		SCa					SCa					SCa	
	Female	SC 1	SC 1-4	SC 1-4-5	SC 1-5	SC 6	SC 1	SC 1-4	SC 1-4-5	SC 1-5	SC 6	SC 1	SC 1-4	SC 1-4-5	SC 1-5	SC 6
		ST 3-4	SB 1	SB 1	SB 1	SB 1	ST 3-4	SB 1	SB 1	SB 1	SB 1	ST 3-4	SB 1	SB 1	SB 1	SB 1
		SCa	ST 5	SCa	ST 6	SCa	SCa	ST 5	SCa	ST 6	SCa	SCa	ST 5	SCa	ST 6	SCa
			SS		SCo			SCa		SCo			SCa		SCo	
			SCa		SCa					SCa					SCa	

Table 3.6: Comparative morphology of legs sensilla types of the *S. zemais* and *S. oryzae* legs.

Sensilla	Specie	Morphological characteristics of sensilla								
		Length (μm)						Shape	Tip	Socket
		Forleg		Midleg		Hindleg				
		M	F	M	F	M	F			
SC1	*S. zeamais*	41,47 (±1,52)	43,16** (±2,09)	41,25 (±1,38)	45,12 (±2,38)	44,56 (±2,02)	44,21 (±1,93)	Curved bush	Several	Overlapping branches
	S. oryzae	35,64 (±2,16)	35,97 (±1,78)	37,84 (±1,68)	44,80 (±2,29)	41,65 (±1,27)	43,34 (±2,10)			
SC4	*S. zeamais*	33,37 (±1,73)	30,45 (±2,15)	36,22 (±1,94)	36,48 (±2,29)	39,72 (±1,70)	36,64 (±1,16)	Little outwardly curved bush	Several branches	Sunken
	S. oryzae	35,21 (±0,95)	36,90 (±0,57)	34,92 (±0,59)	33,03 (±0,67)	32,88 (±2,33)	38,87 (±1,64)			
SC5	*S. zeamais*	27,07* (±0,81)	22,06 (±1,32)	31,85 (±0,94)	30,02*** (±1,40)	32,07**** (±0,61)	29,32 (±0,60)	Straight bush	Several branches	Sunken
	S. oryzae	26,94 (±0,61)	29,89 (±1,00)	27,01 (±0,46)	24,48 (±0,83)	26,15 (±0,77)	29,35 (±1,02)			
SC6	*S. zeamais*	15,47 (±0,49)	16,32 (±1,04)	23,12 (±1,04)	14,18 (±1,57)	14,64 (±1,03)	28,02* (±2,54)	Folded bush	Blunt	Deep pits
	S. oryzae	16,00 (±1,04)	15,39 (±1,34)	20,35 (±0,52)	13,92 (±1,45)	18,53 (±0,99)	18,86 (±2,10)			
ST3	*S. zeamais*	99,74 (±4,79)	91,71 (±4,48)	101,93 (±2,91)	102,69 (±5,85)	109,77 (±2,40)	115,41* (±2,34)	Long and curved	Very sharp	Sunken
	S. oryzae	89,37	88,25	90,98	87,52	89,70	93,05			

		(±3,69)	(±3,57)	(±1,97)	(±1,80)	(±2,54)	(±3,55)			
ST4	*S. zeamais*	58,65 (±2,69)	59,80 (±1,48)	50,95 (±5,33)	57,21 (±4,75)	59,67 (±2,84)	64,91 (±7,58)	Flattened with curved base	Very sharp	Deep pits
	S. oryzae	59,77 (±3,43)	39,57 (±0,60)	43,92 (±4,15)	48,27 (±0,97)	48,04 (±4,45)	55,34 (±1,29)			
ST5	*S. zeamais*	33,06 (±1,55)	42,16 (±2,38)	–	–	–	–	Straight and robust	Elongated cone	Sunken
	S. oryzae	42,89 (±2,98)	43,09 (±2,56)	–	–	–	–			
ST6	*S. zeamais*	44,46 (±2,34)	49,23 (±2,34)	42,86 (±2,85)	43,10 (±2,12)	40,22 (±2,48)	44,75 (±2,25)	Long and curved	Sharp	Sunken
	S. oryzae	40,16 (±2,23)	42,32 (±1,53)	38,30 (±1,37)	32,35 (±2,08)	36,58 (±3,96)	39,59 (±2,92)			
SB1	*S. zeamais*	3,70** (±0,14)	4,71 (±0,22)	4,86 (±0,25)	5,18 (±0,18)	5,02 (±0,26)	5,22 (±0,29)	Straight and short	Fingers like	Wide
	S. oryzae	5,16 (±0,25)	4,61 (±0,22)	4,42 (±0,27)	4,15 (±0,23)	4,72 (±0,28)	4,71 (±0,21)			
SS	*S. zeamais*	15,30 (±0,73)	15,43 (±0,76)	23,81 (±1,63)	23,17 (±1,98)	23,40 (±2,14)	25,99* (±1,59)	Flattened	Cone like	Wide and flexible
	S. oryzae	15,46 (±0,69)	15,98 (±0,93)	19,59 (±1,23)	15,58 (±1,09)	15,51 (±0,76)	20,00 (±2,20)			
SCo	*S. zeamais*	1,62 (±0,13)	1,87 (±0,11)	1,80 (±0,13)	1,49 (±0,06)	1,69 (±0,12)	1,77 (±0,14)	Short and robust	Small cone	Sunken and flexible
	S. oryzae	1,52 (±0,07)	1,62 (±0,21)	1,76 (±0,04)	1,83 (±0,08)	1,83 (±0,04)	1,74 (±0,09)			

SCa	*S. zeamais*	0,99 (±0,05)	1,29 (±0,08)	0,85 (±0,07)	0,96 (±0,05)	1,32 (±0,09)	1,06 (±0,14)	Pore	Pore	Sunken pit
	S. oryzae	1,05 (±0,12)	1,48 (±0,08)	0,94 (±0,07)	0,99 (±0,09)	1,09 (±0,14)	1,06 (±0,07)			
TS	*S. zeamais*	21,52 (±0,79)	17,56 (±0,69)	22,07 (±1,25)	21,54*** (±0,73)	19,35 (±0,93)	22,97 (±0,63)	Long bristles	Spatula-like	
	S. oryzae	20,43 (±0,83)	19,83 (±0,97)	19,12 (±0,83)	16,29 (±0,57)	18,69 (±0,90)	20,09 (±0,97)			

Sensilla Trichoid 6 (ST6): ST6 is characterized by the overlapping of two long bristles with abrupt curvature into the segment. They occur in pairs in the third segment of the tarsus of all legs (forleg, midleg and hindleg) to both sexes and species. ST6 also showed no difference in mean length (Table 5).

Sensilla Basiconic (SB)

Sensilla Basiconic 1 (SB1): Sensilla SB1 were found appearing from the scales present in antennas and legs (forleg, midleg and hindleg) for both sexes of *S. zeamais* and *S. oryzae*. In the antennas SB1 were present at the base of the flagellomeres, except in the 1st flagellomer. In the legs SB1 were present along all segments, with tibia excision. The SB1 was characterized by being simple bristles, without branching, like a long cone and variable length. No difference in the mean length of antennal SB1 was observed. However, occurred difference in the mean length of the SB1 between species present in male forleg (Table 5).

Sensillas Basiconics 2 and 3 (SB2 and SB3): Present at the tip of the 6th flagellomere, sensillas SB2 and SB3 were similar to ST1 and ST2, however shorter with a finger-like appearance and located on small cuticle elevations. No difference in the mean length for SB2 and SB3 was observed (Table 4).

Sensilla Squamiformia (SS)

Present in the 1st segment of the tarsus in all legs for both sexes and species, the SS was characterized as a flattened bristle without branching. For the forlegs, SS appear spaced below the heap of ST5, whereas for the midlegs and hindlegs the SS also appear closer and replacing the ST5, absent in these segments. Significant differences in mean length for species or sex did not occur for SS (Table 5).

Sensilla Coelonic (SCo)

Sensillas SCo1 were observed in the 5th 1168 flagellomer of antennas and in the 3rd segment of the tarsus for all legs (forleg, midleg and hindleg), for both sexes

of *S. zeamais* and *S. oryzae.* The SCo occurred as individual cone short in small circular pits of the cuticle. There are no significant differences in mean length for species or sex for SCo in antennae or legs (Table 4 and 5).

Sensilla Cavity (SCa)

The SCa are caracterizad as cuticular pores, found on the surfaces of the antennae and legs have various sizes and locations. In the antennae a greater number of smaller pores occurs in the 6th flagellomere, whereas single large pores also occur at the base of all flagellomeres. In the legs also single large pores occurred closer to the base of the segments and smaller pores in the spur. No difference in the mean diameter for SCa was observed in the legs, however differences between sex (P <0,05) was observed for antennae in *S. zeamais* (Table 4 and 5).

Tenent setae (TS)

TS were covering the ventral surface of the pulvillus for all legs (forleg, midleg and hindleg), sex and species. The TS was characterized as long bristles with spatula-like tip. The mean length of the TS was different between species when present in female Midleg (Table 5).

3.3.3. Density and abundance of sensilla types

For *S. zeamais* and *S. oryzae* no structural sexual dimorphism was observed. However, these species showed differences in the density of some types of antenna sensilla. Males of *S. zeamais* had a higher number of SC3 type sensilla than females (F = 11.189; P = 0.001), but did not differ from *S. oryzae* species (P> 0.05) (Figure 5), whereas ST1 sensilla appeared with higher density in males of the S. oryzae species (P = 0.010), figure 6. Differences in density between especies were also observed for SB1 sensillas of different segments (3rd Flagellomere: F=6.941, P = 0.007, 5th 1196 Flagellomere: F = 6.417, P = 0.009; 6th Flagellomere F = 8.969, P = 0.003), 1198 figure 7. For the other types of sensilla there was no significant difference in the number or density of sensilla in the antennas between sex or species, figures 6 and 7.

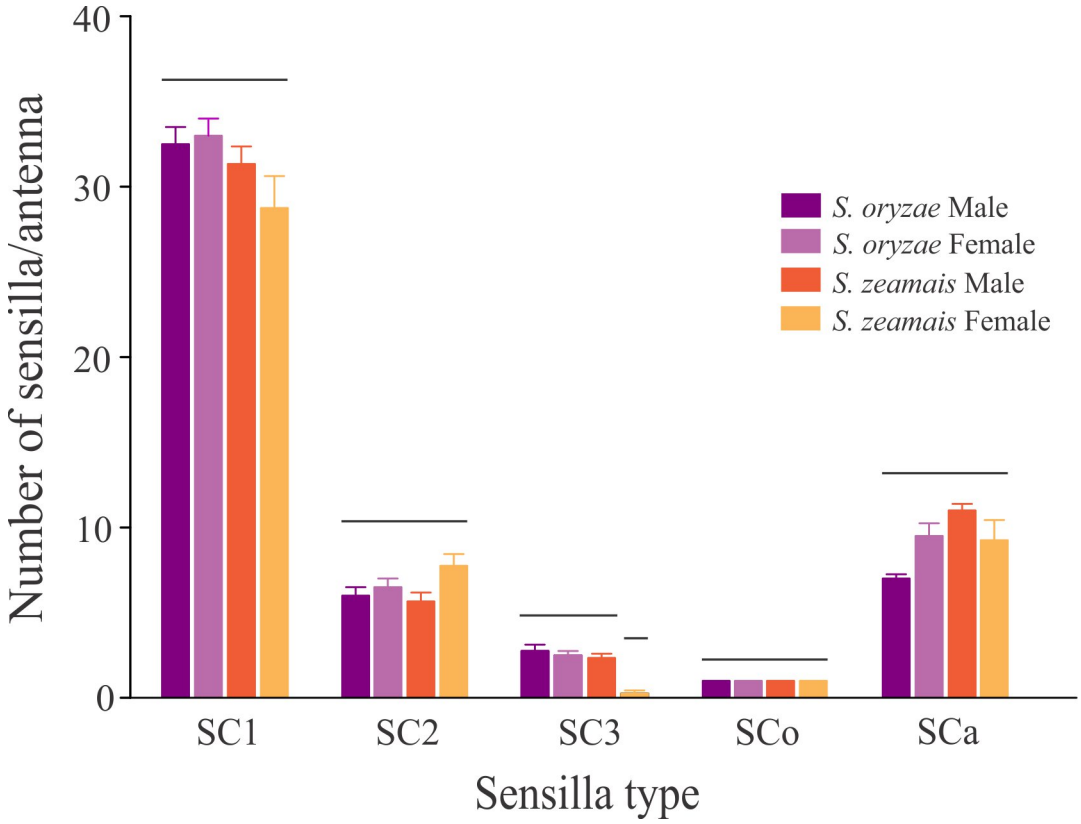

Figure 3.5: Abundance of sensilla types SC1, SC2, SC3, SCo and SCa present in the male and female antenna of *S. oryzae* and *S. zemais*. SC1: sensilla chaetica type 1; SC2: sensilla chaetica type 2; ST1: sensilla chaetica type 3; SCo: sensilla coelonic; SCa: sensilla cavity. Bars at different heights represents mean significantly different for sex and species at 5% level by Tukey test.

Differences in the density were observed to two types of legs sensilla, SC1 and SC5 (Figure 8). The SC1 sensilla appeared in highest total numberor *S. zeamais* to forleg (F = 12.398; P <0.001) and Hindleg (F = 12,398; P = 0.041), and the total number of SC1 in the previous leg differed between males of the studied species (F = 6.008; P <0.001). The density of SC5 sensilla was statistically higher for *S. zeamais* in the different legs, forleg, midleg and hindleg (F = 7.584; P <0.001; P = 0.022; P = 0.047, respectively). In the forleg, there was also a difference in density of SC5 sensilla between sex for *S. zeamais* (F = 7.584; P = 0.047). Higher TS densities were observed for *S. zeamais* with significant difference for forleg (P = 0.001). For the other types of sensilla there is no difference in the number or density of sensilla in the legs, since they occur in a fixed number of sensilla (SC6 = 2; ST3 = 2; ST4 = 1; ST6 = 2/leg), or can not be counted (ST5), or because statistical differences were not observed, such as for type SB1 (F = 0.677; P = 0.748), 1215 figure 9.

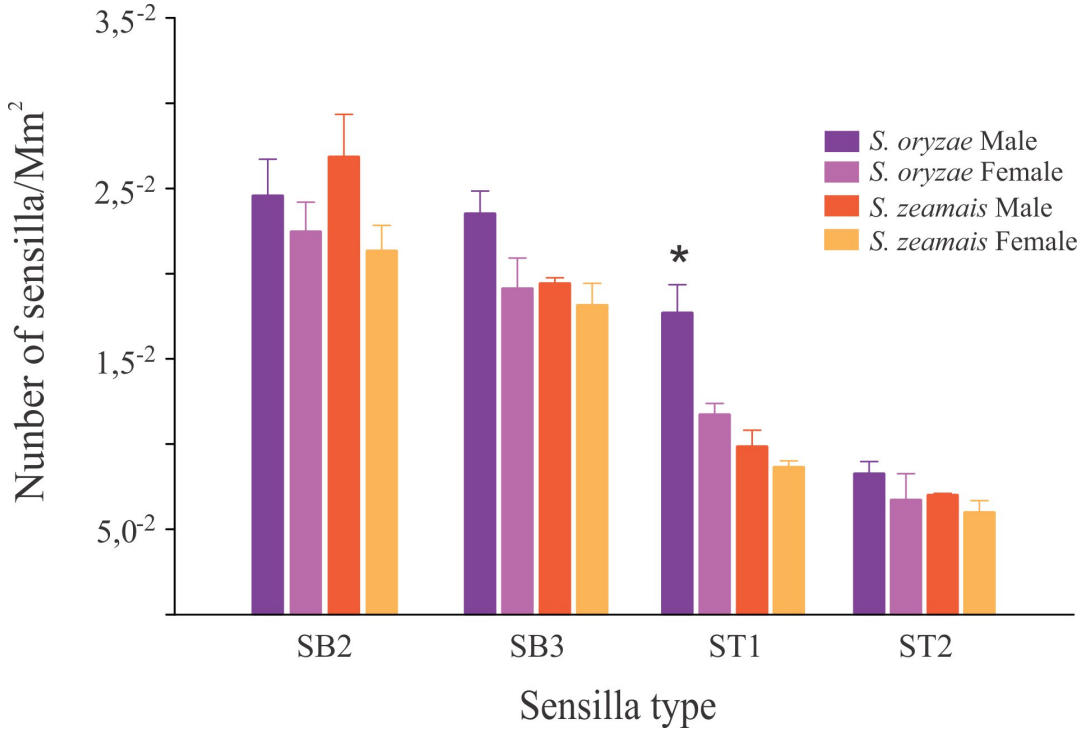

Figure 3.6: Abundance of sensilla types SB2, SB3, ST1 and ST2 present in the male and female antennae of *S. oryzae* and *S. zemais*. SB2: sensilla basiconica type 2; SB3: sensilla basiconica type 3; ST1: sensilla trichodea type 1; ST2: sensilla trichodea type 3. * Mean significantly different for species at 5% level by Tukey test.

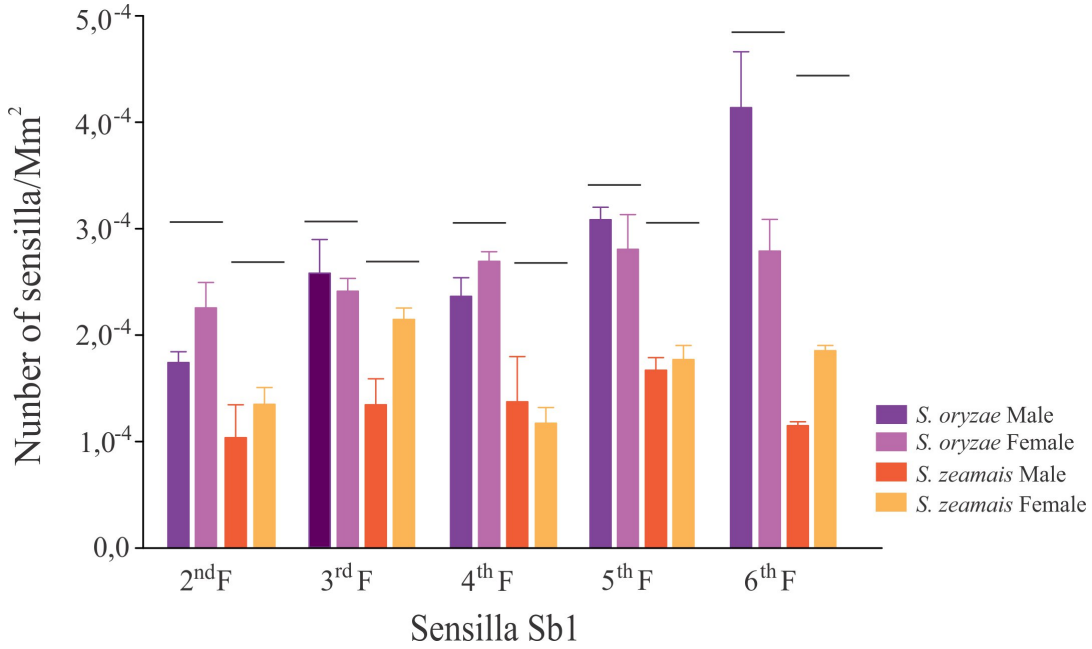

Figure 3.7: Abundance of sensilla SB1 (sensilla basiconic type 1) present in the different segments of the male and female antennae of *S. oryzae* and *S. zemais* 2*nd* F: 2*nd* flagellomere; 3*rd* F: 3*rd* flagellomere; 4*th* F: 4*th* flagellomere; 5*th* F: 5*th* flagellomere; 6*th* F: 6*th* flagellomere. Bars at different heights represents mean significantly different for species at 5% level by Tukey test.

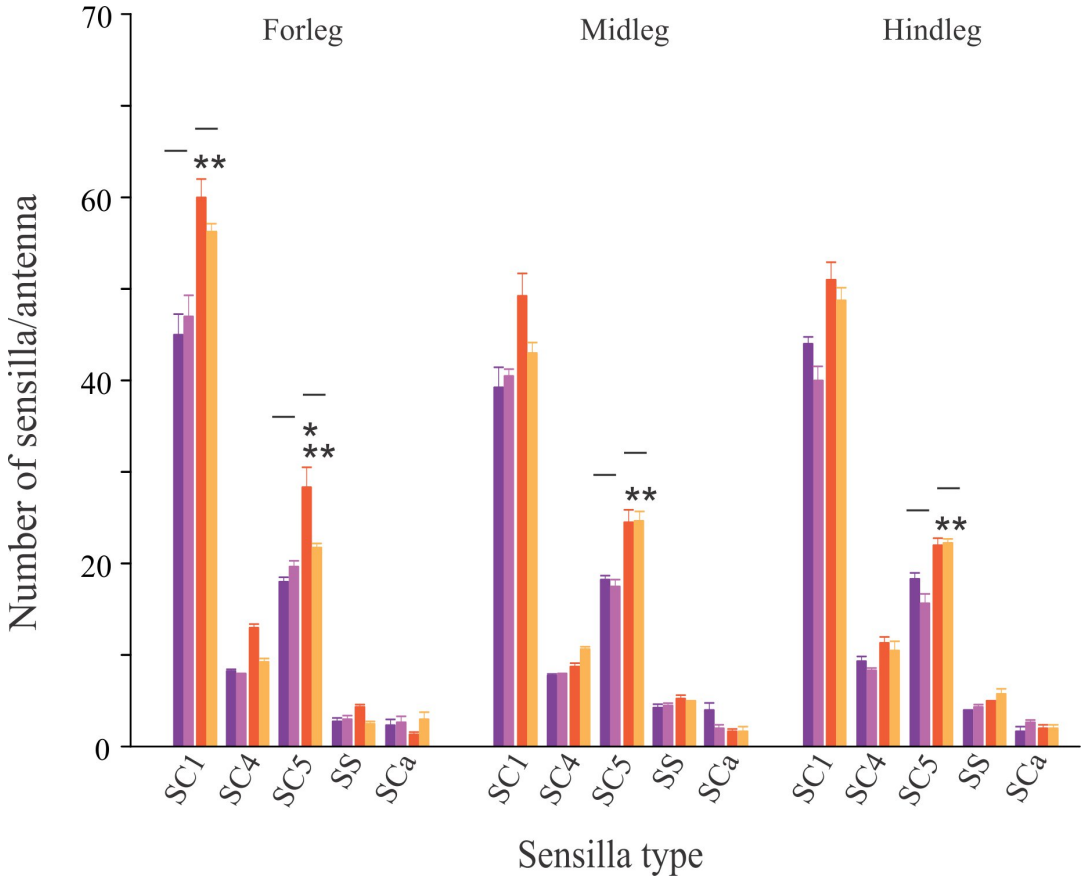

Figure 3.8: Abundance of sensilla types SC1, SC4, SC5, SS and SCa present in the legs (forleg, midleg ande hindleg) of males and females of *S. oryzae* and *S. zemais*. SC1: sensilla chaetica type 1; SC4: sensilla chaetica type 4; SC5: sensilla chaetica type 5; SS: sensilla squamiformia; SCa: sensilla cavity. Bars at different heights represents mean significantly different for species at 5% level by Tukey test. * Mean significantly different for sex at 5% level by test. ** Mean significantly different for the same sex between species at 5% level by the Tukey test.

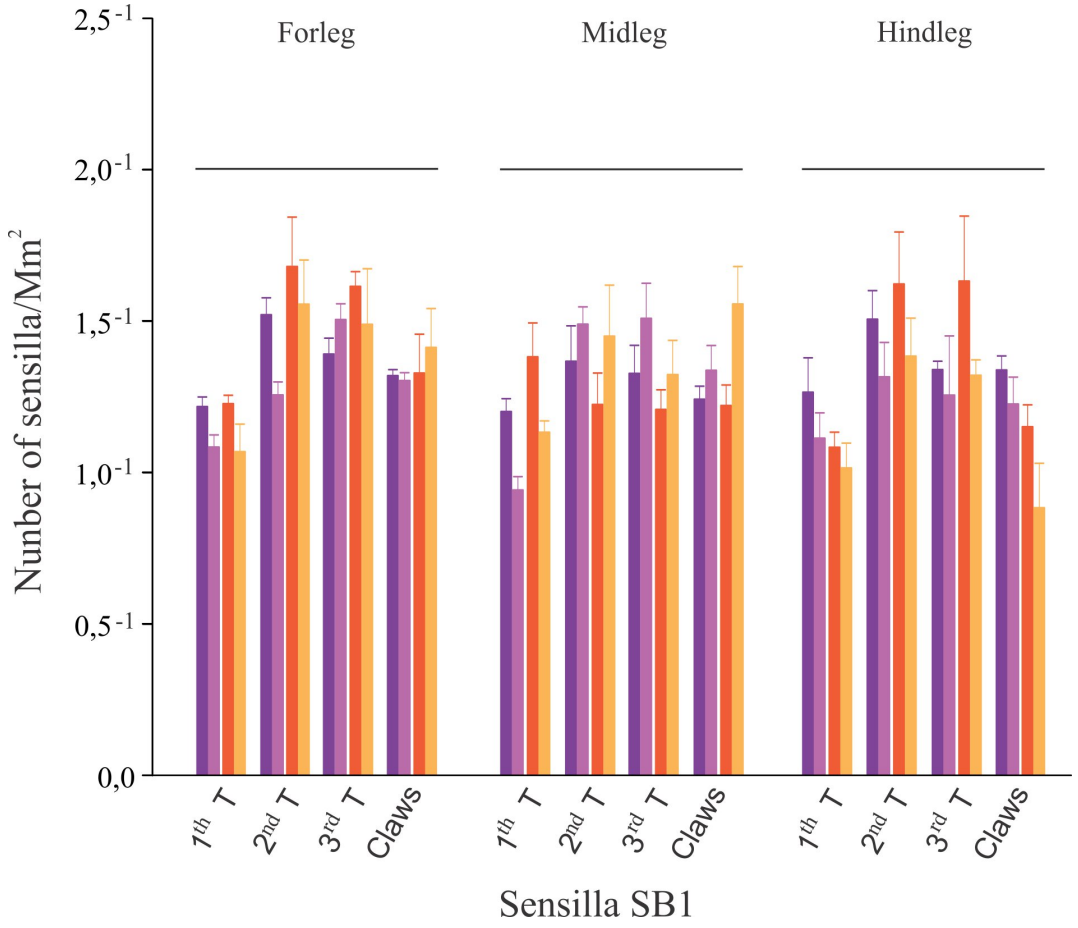

Figure 3.9: Abundance of sensilla SB1 (sensilla basiconic type 1) present in the different segments of males and females legs (forleg, midleg and hindleg) of *S. oryzae* and *S. zemais*. 1th T: 1th Tarsomere; 2nd T: 2nd Tarsomere; 3th T: 3th Tarsomere; Cl: Claws. Bars at the same height represent that the means do not differ for sex and species at the 5% level by the Tukey test.

3.4. DISCUSSION

This is the first study describing legs sensilla types and their distribution in *S. zeamais* and *S. oryzae*, as well as the description of the antennal sensillum types (subtypes) and distribution in *S. zeamais*, and the comparison of all these sensilla between species. The antennal organization of *S. zeamais* and *S. oryzae* are similar to the antennae of other coleopterans species (Alabi et al., 2014; Ali et al., 2016; Chen et al., 2010; Fouda et al., 2016). Composed by scape, pedicel and flagellum with 6 flagellomeres that end a club (6th flagellomere), the sexual dimorphism was not documented to *S. oryzae* and *S. zeamais*.

Based on shape, morphology external, size, cuticular attachment and distribution a complex arrangement of multiple sensilla was revealed (sixtypes of sensilla and fourteen subtypes. Antennae sensilla were predominantly characterized by sensilla chaetica (subtype 1, 2 and 3), trichodea (subtype 1 and 2), basiconica (subtype 1, 2 and 3), coeloconica and cavity, while the legs comprised these sensilla types (sensilla chaetica (subtype 1, 4, 5 and 6)), trichodea (subtype 3, 4, 5 and 6), basiconica (subtype 1), coeloconica and cavity) and more squamiformia sensilla, which were observed for males and females of *S. zeamais* and *S. oryzae*. Whereas many sensilla occur in the antennae and legs in different forms (e.i., hair, pegs, pits or cones) and based on observations of SEM these types of sensilla, recorded in this study, are in great conformity with those reported for other beetle's species (Alabi et al., 2014; Chen et al., 2010; Fouda et al., 2016; Hu et al., 2009; Yi et al., 2016).

Based in the ultrastructure those sensilla described in other insect species, these sensilla are organs involved in the perception of various stimuli (e.i., olfactory (ST, SB), gustatory (SC, SB) and tactile as well as thermo- and/or hygroreception (SCo, SCa) (Altner and Prillinger, 1980; Hu et al., 2009; Zacharuk, 1980). The highest density and a greater diversity of anntenal sensilla types was found on the antennal club (6th flagellomere), seven types of sensilla from a total of ten sensilla types are founded, corroborating with other observations made to Sitophilus spp. (Alabi et al., 2014; Fouda et al., 2016), as well as for other groups of insects (citações). This distribution pattern of sensilla types provides the importance of distal antennal segments in environmental perception and communication, with relevance in olfactory/gustatory behavioral studies in different species Fouda et al. (2016); Ravaiano et al. (2014); Sen and Mitchell (1995).

The SC types are variable in shape and length and were located inside deep pit, cuticle sunk or arising from raised cuticle, as observed in other studies (citações). Some of the SC types here founded were similar to those found in Sitophilus spp. (Alabi et al., 2014; Fouda et al., 2016), Dendroctonus valens (Coleoptera: Curculionidae: Seolytinae) (Chen et al., 2010) and Paussus favieri (Coleoptera: Curculionidae: Paussini) (Di Giulio et al., 2012). This type of sensilla are believed to have a dual function of mechanoreception and contact chemoreception (Jourdan et al., 1995), or only a mechanoreception (Gao et al., 2007; Ochieng et al., 2000) or only 1262 the chemoreception function (Merivee et al., 1999). Although the chaetic 1263 sensilla, when found in antennas, may be more related to the gustatory function by chemoreception (Ali et al., 2016; Hu et al., 2009; Merivee et al., 1999, 1998), however, for (Dyer and Seabrook, 1978), these types of sensilla in basal regions of antennas can act as mechanoreceptors allowing the insect to determine the possibility of the antenna by contact with the substrate or functioning as wind speed receivers. Thus, we may suggest that antennal SC1 in *S. oryzae* and *S. zeamais* may also be associated with machanoreception function. As it happens also for legs, where SC seems to be related to both chemoreception and mechanoreception (Dey et al.,2 1995; Zhang et al., 2012), probably acting to obtain information andperception of the substrate (Zhang et al., 2012), which would also justify he occurrence of SC1 in parts (antenna and leg). For chaetica sensilla only the SC3 was a type that presented difference on the abundance in antennae. For *S. oryzae* male and female, and *S. zeamais* male this type occurred distantly on the club. However, for the S. zeamais female SC3 had a low occurrence ($P < 0.005$). According to (Fouda et al., 2016), in S. granarius the SC3 type was described only for male. For the legs, types SC1 and SC5 showed differences between species regarding the total number of sensillae per type of leg. Differences for SC1 occurred only for forleg ($F = 13.156$; $P < 0.001$) wile differences for SC5 occurred for forleg, midleg and hindleg ($F = 7.584$; $P < 0.001$).

Described in antennae and legs of the different insects, the ST are characterized by shape of hair or bristles and may have chemo- or mechanoreception function according to the location and group of insects (Hu et al., 2009; Keil, 1999). The perception of mechanosensory stimuliand and the presence of mechanoreceptive ST has been reported in antennas of different families of parasitoid wasps, such as Trichogrammatidae (Amornsak et al., 1998), (Isidoro et al., 1996), Pteromalidae (Onagbola and Fadamiro, 2008; Pettersson et al., 2001), Braconidae (Roux et al.,

2005), Ichneumonidae (Zhang et al., 2015). However, based on the morphology and distribution in *S. oryzae* and *S. zeamais* antennae and compared to other coleopterans that have proven antennal ST (Faucheux, 1994; Fouda et al., 2016; Hu et al., 2009; Isidoro and Solinas, 2016; Kim and Yamasaki, 1996; Lopes et al., 2002; Saïd et al., 2003; Yi et al., 2016) a chemoreceptive/olfactory function is suggested for ST type, agreeing with (Keil, 1999) reported that antennal trichoid sensilla as olfactory receptors. In some insects the ST of males also respond a sex pheromone related compounds (Faucheux et al., 2006; Hallberg et al.,01 1994; Hu et al., 2009; Merivee et al., 1999; Zacharuk, 1985). The sensilla trichoid type 1 (ST1) was more present in *S. oryzae* than *S. zeamais* ($F = 17.363$; $P = 0.001$) and with higher occurrence in males than in females ($P = 0.010$). Thus, differences in the number of these ST1 on *S. oryzae* male antennae suggest that they possibly act as receptors for sex pheromone or other important functions for the species. In contrast, ST present in the legs/tarsus are closely related to mechanoreception (Faucheux, 1991; Zhang et al., 2012), which occur mainly in females who need information about suitable site to lay eggs, that is evidenced, in some cases, by thesexual dimorphism in the distribution of sensilas in tarsu due oviposition behavior (Faucheux, 1991). However, there is no apparent sexual difference for ST in *S. oryzae* and *S. zeamais* legs ($P > 0.05$).

The SB are distinguished by their cone-like appearance (or finger) that may be slightly curved. Two SB types found (SB2 and SB3) are similar to those found in other studies with Sitophilus spp. (Alabi et al., 2014; Fouda et al., 2016) and other Callosobrusus spp. (Coleoptera: Bruchidae) (Hu 1317 et al., 2009), but the SB1 type has not been described in previous studies. 1318 Based on studies with S. granarius antennae and other species of coleopteran, the SB has the function of chemoreception involved in the detection of sex pheromones, during courtship, in conjunction with detection of host plant odors, during host recognition (Ali et al., 2016; Chapman, 1982; Fouda et al., 2016; Hu et al., 2009; Zacharuk, 1985). The SB1 was shown as a group of small spines and are similar the Böhm Bristles (BB) sensilla of the Callosobruchus spp. antennae (Hu et al., 2009), Hair Plate Sensilla of others coleopterans and trichoid antennal sensilla of some hymenopteran wasps (Amornsak et al., 1998; Onagbola and Fadamiro, 2008; Isaac et al., 2015). Considering the location and concentration of the SB1 in the antennae, on the basal region of the flagellomeros (intersegmental), and in the legs, surrounding all the tarsomeros, is possible to suggest that these sensilla might be mechanoreceptors to perceive antenna movements and positions, as

described for many insects (Hu et al., 2009; Merivee et al., 2002; Schneider, 1964; Zacharuk, 1985). The SB1 presented differences in densities between species when present in antennas for all segments that are present: 2nd ($F = 7.899$; $P = 0.015$); 3rd ($F = 21.636$; $P < 0.001$), 5th ($F = 20.969$; $P < 0.001$) and 6th ($F = 14.907$; $P = 0.002$), but no difference in SB1 density was observed in the legs.

The SCo are commonly characterized as small pegs/cones present in chambers sunken of the cuticle surface, however for different insects SCo have highly variable external shape (Altner and Loftus, 1985; Altner et al., 1981; Ravaiano et al., 2014; Ruchty et al., 2009; Schneider and Römer, 2016). Several studies ensure that SCo are directly involved in temperature and humidity sensing, but olfactory functions may be associated (Altner and Loftus, 1985; Altner et al., 1981; Ruchty et al., 2009; Zhang et al., 2015; Schneider and Römer, 2016). For (Ali et al., 2016), in S. granarius, based on the internal morphology (e.i., dendritic branches and cuticular pore) and distribution, the SCo could also be associated with the perception of humidity, temperature, heat and CO2.

The SCa appers as cuticular pores with variation in diameter and shape for different insects (Chen et al., 2010; Hu et al., 2009; Merivee et al., 2002). (Chen et al., 2010) reported the presence of openings of the antennal glands on sensilla socket of the Dendroctonus valens and points out that differences in size and position of these pores indicates differences in the function of particular cuticular glands. The SCa must probably function as chemo-, thermo- and hygroreceptors (Zacharuk, 1985). However, enzymatic activity with degradation of pheromones molecules was attributed to SCa (Dickens 1357 et al., 1993; PRESTWICH, 1987; Taylor et al., 1981; Vogt and Riddiford, 1981).

Sensillae squamiformia (SS), generally similar to an elongated scale with a sharp end, are commonly present in lepidopteran insects (Faucheux et al., 2011; Faucheux, 1999; Ma et al., 2017), however different shape and distribution of these sensilla type are report in studies with others insects, as coleopterans (Alabi et al., 2014; Gao et al., 2007; Hix et al., 2003). These sensilla, based in their morphology and distribution, are inferred to have a mechanoreceptive function (Schneider, 1964).

In conclusion, the present study has identified and characterized the distribution of different sensilla types on the antennae and legs for male and females of *S. zeamais* and *S. oryzae* using SEM. This study is the first to describe the morpholoy and distribution of the sensilla legs in Sitophilus spp., as well as, the pioneer to characterize

the antennal sensilas of *S. zeamais.* Six sensilla types, covering eighteen sensilla subtypes, were found in antennas e legs parts evidencing that antennae and legs possess sensitive structures that play pivotal roles in the environmental perception (e.g., locating mates and host plants, choice of food substrate and place for oviposition). A more extensive study of the functional morphology in antennae and legs parts using transmission electron microscopy (TEM)will likely confirm the functions of the different sensilla identified in this study. There are no obvious differences between the sexes and species regarding the morphology of the antennas and legs and the types and location of sensilla. However, important differences were observed for size and abundance. The morphological differences found in the sensilla of the different species de Sitophilus spp., might be supply evidence to the differences responses and behaviors of these species. Sexual differences in the numbers and distribution of various sensilla were also evaluated which establishes a foundation for future physiological, behavioral and molecular studies aimed at elucidating potential differences in olfaction between both sexes of Sitophilus spp. Such studies could clarify the functional roles of various insect sensillae related to the perception of pheromones and volatiles of hosts and non-hosts, as well as the underlying mechanisms that could elucidate insect differential responses to common used control tools and possibly the development of new control methods.

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4. CHAPTER 4

Effect of diatomaceous earth applied to bean grains on life parameters of the bruchid *Acanthoscelides obtectus* (Coleoptera: Crysomelidae)

ABSTRACT: Bean grains losses caused by Acanthoscelis obtectus (Coleoptera: Chrysomelidae) during postharvest storage are of paramount economic importance in Brazil, so their control is necessary. Diatomaceous earth (DE) is an inert powder that can be used as an alternative control again stinsect pests of grains and stored produce. The aim of this study was to evaluate the effects of DE (Insect formulation) as a potential tool in the control of *A. obtectus*. Mortality, survival, reproductive capacity (instantaneous growth rate-ri) and grain consumption (grain loss) tests were performed with different doses of DE (0.0, 0.01, 0.05, 0.2, 0.5 and 1.0 g DE / kg of red bean grains. Mortality was assessed 3, 11, 17, 25 and 33 after the onset of insect exposure to DE, with higher mortality rates (70-100%) only occurring for all doses tested in the longest exposure period ($P < 0.05$). For the survival analysis also all tested concentrations differed from the control ($P < 0.05$) presenting shorter average survival time, which did not differ from each other ($P > 0.05$). There was also a significant reduction in intantaneum population growth and grain loss ($P < 0.05$) for the highest concentrations tested (0.2, 0.5 and 1.0 g DE / kg of grains), which did not differ from each other ($P > 0.05$). Our results show that DE had insecticidal effect on *A. obtectus*, however the formulation tested was not efficient for bean weevil control, as a satisfactory control rates (above 80% mortality) were only observed around 30 days after the exposure of insects to DE. Thus, further studies are needed on the specificity of DE action, as well as the evaluation of new and different DE formulations that are efficient in the control of *A. obtectus* and can be used in storage units.

Key words: Weevil bean. Grain stored. Alternative control. Inert dusts. DE.

4.1. INTRODUCTION

The bean weevil, *Acanthoscelides obtectus* Say (Coleoptera: Chrysomelidae), is a main bean pest and other legumes of the Fabaceae family. Widely distributed worldwide this weevil have the primary host the common bean (Phaseolus vulgaris) and the consequence of its damage is quantitative and qualitative losses in the nutritional properties and, consequently, the depreciation of the market value of the stored legumes (Caswell, 1981). Adult weevils do not feed, but they can lay their eggs in field pods or stored seeds, from which small larvae emerge and bore into and feed on the seeds, causing quantitative and qualitative losses (Caswell, 1981).

In stored products, various control methods have been applied to insect pests (e.g., chemical control, physical control, biological control etc.). Synthetic insecticides are commonly used to reduce insect pest damage and maintain grain quality (Gahukar, 1994) adicionar cit, however, although effective, their long-term indiscriminate use has been questioned as it may affect and/or disrupt the biological control of natural enemies causing the emergence of new pest species and sometimes result in the development of resistance (ARPD, 2019; Guedes et al., 1995; Lorini et al., 2007; Obeng-Ofori, 2010; Pimentel et al., 2010). Also, due to intense use and often misuse, there are also serious concerns about the environmental degradation of synthetic insecticides that harm the environment and leave food residues that can be detrimental to human health (Gilliom et al., 2006; Handford et al., 2015). However, the development of insect pest resistance to insecticides is currently the mainfactor that encourages the development of new insecticides (Sparks, 2013) and encourages the search for alternative control methods (Subramanyam and Hagstrum, 1995). Promising strategies for controlling *A. obtectus* include the use of natural enemies, plant extracts, essential oils and inert powders (Adarkwah et al., 2017; Berger et al., 2017; Jovanovi´c et al., 2007; Jumbo et al., 2014; Stathers et al., 2004; Wille et al., 2019).

The diatomaceous earth, the most importantly inert dusts, can be used as a safe alternative to the chemical control of stored grain pest insects, and has been successfully incorporated into the IPM program in several countries, proving to be effective against over 15 pest insect species(Athanassiou et al., 2005; Fields et al., 2003; Koruni´c, 2013; Mewis and Ulrichs, 2001; Nwaubani et al., 2014; Prasantha, 2003; Quarles et al., 1996; Shah and Khan, 2014; Stathers et al., 2004; Subramanyam

and Roesli, 1457 2000). The DE is an inert powder obtained from fossilized silica remains of unicellular and microscopic diatom algae that have a fine carapace composed mainly of silica (Korunic, 1998; Quarles et al., 1996). DEs havean insecticidal effect mainly due to silica's ability to absorb cuticular lipids and also abrades the insect cuticles causing desiccation death (Ebeling, 1971).

As a natural insecticide, DE also stands out for its low mammalian toxicity (Fields, 1998), low level of breakdown (long duration of efficacy) (Fields and Korunic, 2000; Fields et al., 2001; Fields, 1998; Golob, 1997; Korunic, 1998; KoruniC, 1997; Subramanyam and Roesli, 2000), limited chances of developing insect pest resistance (Fields et al., 2001) and being easily removed during processing (Koruni´c, 2013). However, its use remains controversial, becase its efficiency for insect control is varies depending of the insect (e.g., species, population, growth stage, etc.), of the origin of the DE deposit (e.g., type of formulation), of the environmental conditions (e.g. method of application, temperature and humidity) and of the grain conditions (e.g., grain moisture and origin) (Adarkwah et al., 2012; Aldryhim, 1993; Banks and Fields, 1995; Prasanthaet al., 2002; Chanbang et al., 2007; Demissie et al., 2008; Fields and 6 Korunic, 2000; Kavallieratos et al., 2012, 2005; Malia et al., 2016; Nwaubani et al., 2014; Rigaux et al., 2001; Wille et al., 2019). In this context, the present study aimed to evaluate the action of a DE formulation (Insecto R) on the mortality / survival rate, grain loss and progeny production of *A. obtectus.*

4.2. MATERIAL AND METHODS

4.2.1. Overall experimental conditions: Insects and DE formulation

Adult bean weevils, not sexed, were collected randomly and used as needed in all the experiments carried out. The populations were maintained in glass containers in rearing facilities under controlled conditions (25 ±2 C. 70 ±10% relative humidity. 14 h: 10 h lighting regime [D: L]) and reared on insecticide-free red bean grains at theDepartamento de Entomologia da Universidade Federal de Viçosa, Viçosa, Minas Gerais, Brazil. Diatomaceous earth Insecto® (Natural Insecto 1488 Products, Orange, CA, USA) was used for the bioassays with register and recommendation to control stored product pests in Brazil at the dose of 1g/kg (Química, 2015). Insecto R is a formulation of marine origin containing 86.7% SiO2 and particle size distribution

between 0.82 and 52.33 µm, but centering between 9 and 30µm (Subramanyam, 1993). The DE treatments were applied to whole red bean grains using a glass container to assist the manual homegeinization during 1 min.

4.2.2. Time-mortality (survival), grain consumption (grain loss) and progeny production

To verify the efficacy of DE against *A. obtectus*, mortality and survival tests were performed using the dosages of 0.01, 0.05, 0.2, 0.5 and 1.0 g o DE / kg of red bean grains, and untreated grains to the control. Fifth replicates consisting of one plastic Petri dishes (9 cm x 2 cm) each containing 30 g of grains, received 20 unsexed adult insects (up to 3 days old) and the mortality was daily assessed within 3, 11, 17, 25 e 33 days 1 after the beginning of the exposure. Survival tests were performed under the same conditions as mortality tests; however, 5 repetitions per treatment were performed and mortality was assessed every 2 days after the beginning of exposure. After monitoring mortality and survival, the grains were sifted and stored for later evaluation of grain loss and instantaneous growth rate (ri) (progeny production) at the end of 50 day safter the onset of exposure.

4.2.3. Statistical analysis

The survival data was analyzed used Log-Rank test to obtain survival curves and the treatments were compared using and Kaplan-Meier Survival Analysis (SSI, 2008). The others data were subjected to analysis of variance (ANOVA) and all means comparisons, whenever necessary, were performed by applying Tukey test at 5% probability using SigmaPlot program (SSI, 2008).

4.3. RESULTS

Mortality of *A. obtectus* after the 17-day exposure period increased significantly compared with independent concentration control ($F = 18.935$; $P < 0.001$). The increased period of insect exposure resulted in significant increases in *A. obtectus* mortality rate ($P < 0.05$). Significant differences between treatments occurred for 25 day-exposure, with control treatment differing from all treatments with DE ($P < 0.05$),

and for 33days-exposure with the highest concentrations (0.2, 0.5, and 1.0 g) differing from other treatments with mortality rates above than 90%. The lowest concentration tested reached the lowest mortality rate (70.8%) which did not differ from the control (Figure 1).

The survival of *A. obtectus* decreased significantly compared to the control (DF = 5; P <0.001) when they had to colonize the DE-treated grainmasses. The lowest concentration tested (0.01 g), with a median survival time of 33 days, had the lowest effect on insects and differed statistically from the other treatments (P <0.001) and the highest concentrations tested (0.5 and 1.0 g) showed median survival time of 30 days and did not differ from each other (P> 0.05) (Figure 2).

After the 50-day exposure period, the results showed that theapplication of DE results in a significant decrease (F = 16,104; P <0.001) in the population growth rate (ri) compared to the control. Increases inDE-concentration for 0.2, 0.5 and 1.0 g resulted in differences in growthate (P> 0.05), while treatments with lower DE contraction (0.01 and 0.05g) did not differ from control (P> 0.05) (Figure 3).

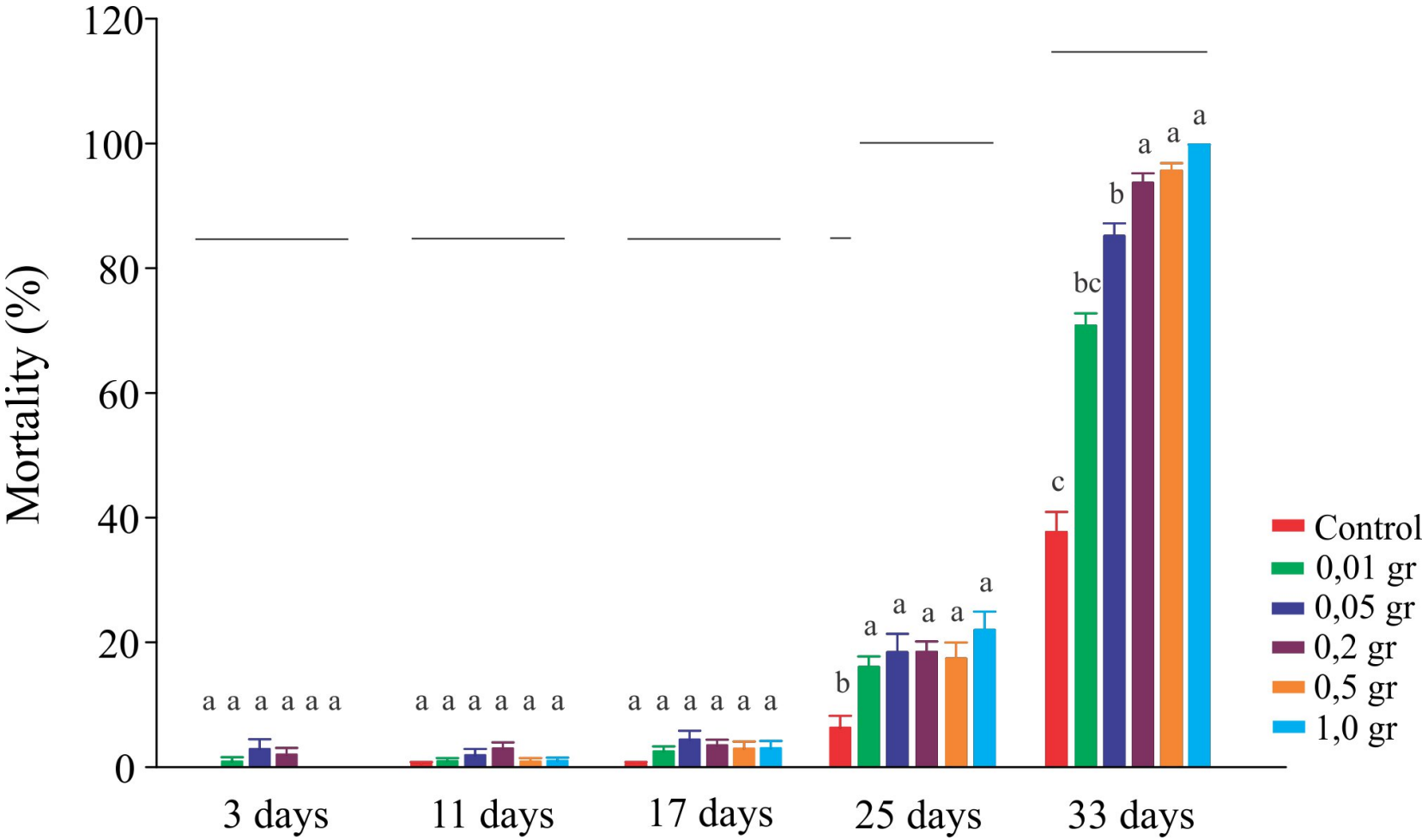

Figure 4.1: Mortality of adults insects of *A. obtectus* after 3, 11, 17, 25 and 33 days of exposure to DE at 0, 0.01, 0.05, 0.2, 0.5 and 1.0 g/kg of red bean grains. Different vertical bars indicate significant differences (Tukey test at 5% of probability). Different lower case letters indicate significant difference for treatment as an evaluation time (Tukey test at 5% probability).

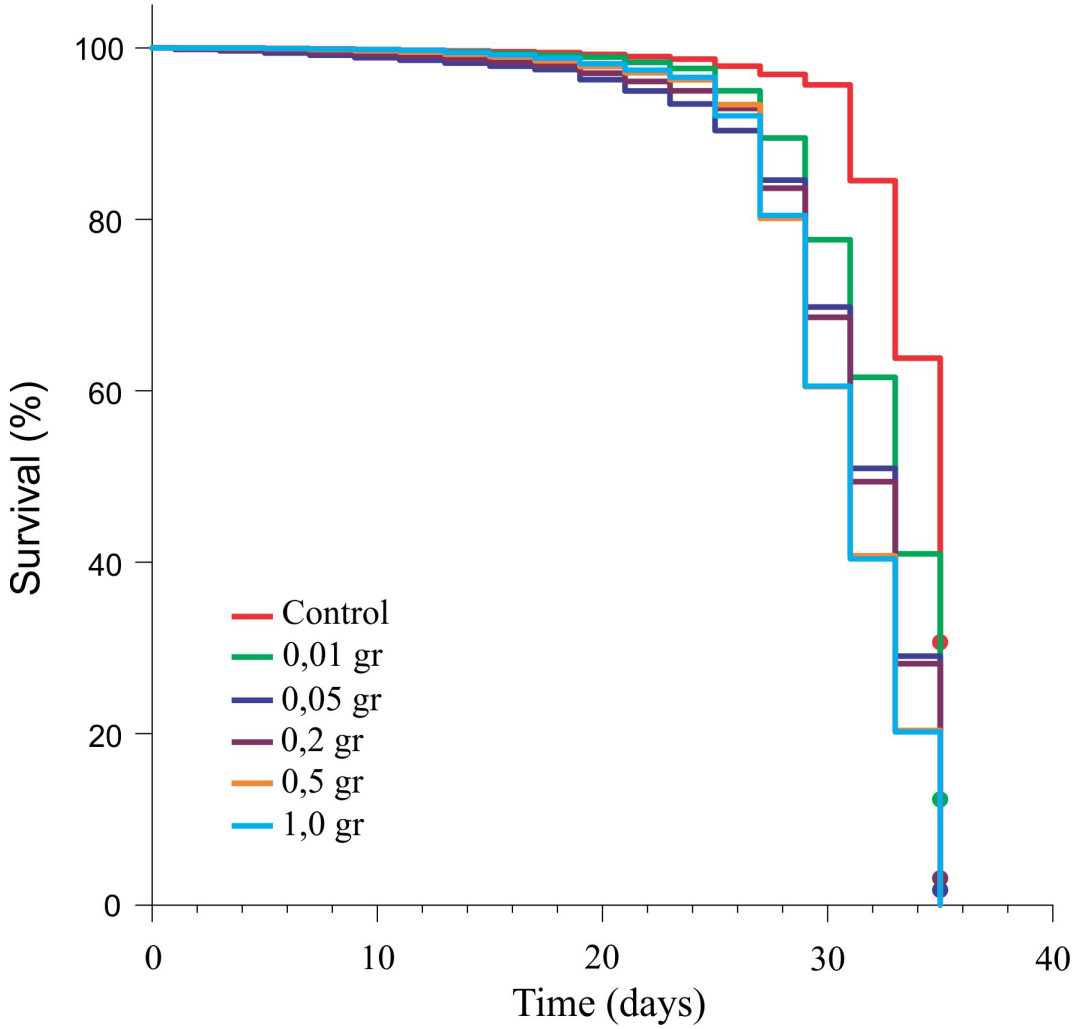

Figure 4.2: Survival of *A. obtectus* on exposure to DE dosages at 0, 0.01, 0.05, 0.2, 0.5 and 1.0 g/kg of red bean grains.

Grain consumption (grain loss) decreased significantly after 50 days of exposure as a function of treatment (F = 9.573; P <0.001). Concentrations of 0.2, 0.5 and 1.0 g provided the largest reductions in grain loss (24.34, 21.86 and 22.10%, respectively), which differed from control and other treatments (P <0.05), but did not differ from each other (P> 0.05). The lowest concentrations tested (0.01 and 0.05 g) did not differ from the 1543 control (P> 0.05) (Figure 4).

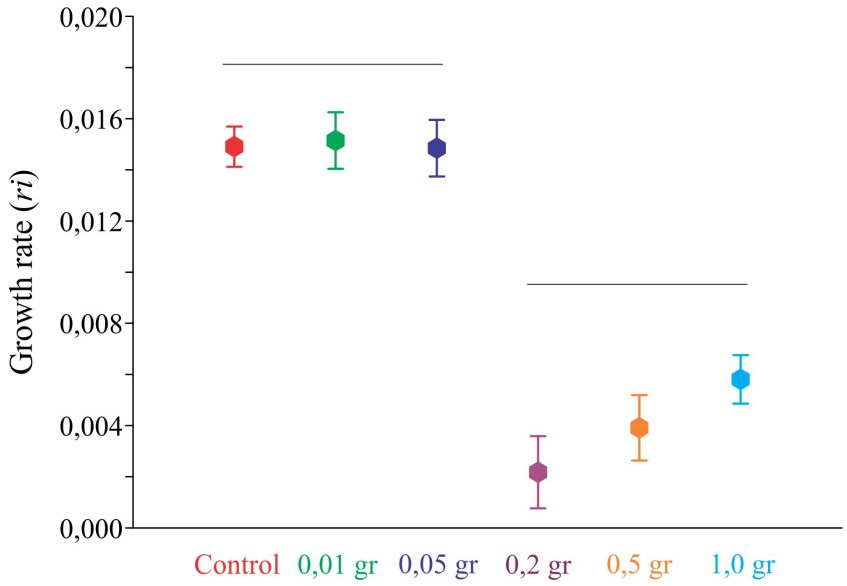

Figure 4.3: Population growth (ri) of *A. obtectus* after 50 days of exposure to DE dosages at 0, 0.01, 0.05, 0.2, 0.5 and 1.0 g/kg of red bean grains. Different vertical bars indicate significant differences (Tukey test at 5% of probability).

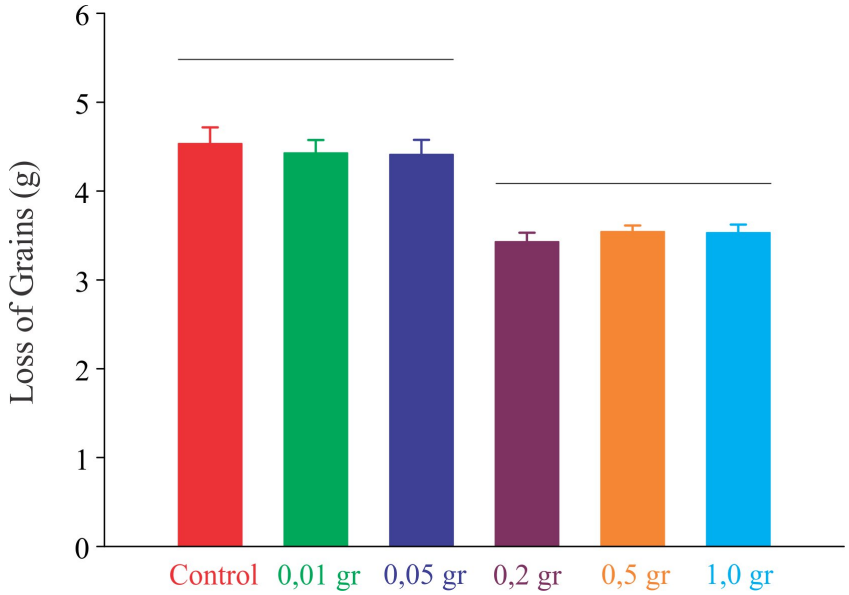

Figure 4.4: Grains loss for *A. obtectus* after 50 days of exposure to DE dosages at 0, 0.01, 0.05, 0.2, 0.5 and 1.0 g/kg of red bean grains. Different vertical bars indicate significant differences (Tukey test at 5% of probability).

4.4. DISCUSSION

The DE had effect against *Acanthoscelides obtectus* depending of the dose and exposure time in which mortality increases, reduction in instantaneous population growth rate and reduction in consumption (loss of grain) could be observed at the highest doses tested (0.2, 0.5 and 1.0 g DE / kg of grains). However, the DE Insecto R formulation, despite showing insecticidal effect against *A. obtectus*, was not efficient for bean weevil control, as satisfactory control rates (above 80% mortality) were only observed around 30 exposure days of the insects to DE.

The effectiveness of DE depends mainly on the quantity and quality of amorphous silica content such as average particle size, high oil sorption capacity, extensive active surface area and the presence of little or no impurity (Mewis and Ulrichs, 2001). Among the various factors that may affect the insecticidal activity of DE formulations in pest control are the insect (e.g., arthropod / insect species, growth stage/age, etc.), origin of DE (e.g., formulation type, silica content, particle size, etc.), environmental and grain conditions, etc. (Aldryhim, 1993; Alkan et al., 2019; Chanbanget al., 2007; Fields and Korunic, 2000; Rojht et al., 2010; Malia et al., 2016; Prasantha et al., 2002; Rigaux et al., 2001; Shah and Khan, 2014).

In general, reduced DE particle sizes may provide greater attachment of these particles to the insect body due to increased surface area increasing, consequently, the insecticidal activity with increased mortality rates (Alkan et al., 2019; KoruniC´ 1997; Vayias et al., 2009). Alkan et al. (2019) investigating the effect of particle size on the efficiency of 3 types of E in the control of *A. obtectus* observed that formulations with higher silica content (SiO2) and smaller particle size had a high mortality effect causing high mortality rates (approx. 100%) for low dosages (<0.4 g / kg) and a short exposure period (4 days) and in contrast found that the 1 formulation with larger particle required increases in dose and/ or exposure time (24 days or more) to achieve a high mortality rate. Variable concentrations for different DE formulations are used to control different insect species (Koruni´c, 2016; Shah and Khan, 2014) and variations in the control rate of *A. obtectus* as a function of the applied concentrations and exposure time were also observed in other studies (Prasantha et al., 2015, 2002; Wille et al., 2019).

The morphological and behavioral characteristics of the insect species are other factors that may affect the activity of DE (Adarkwah et al., 2012; Alkan et al., 2019; Bartlett, 1951; Carlson and Ball, 1962; Fields and Korunic, 2000; Hidayat et al., 1996; Malia et al., 2016; Mewis and Reichmuth, 1998; Rigaux et al., 2001; Shah and Khan, 2014). Some insects have greater sensitivity to DE due to their anatomy and physiology as the surface area volume, the number and distribution of appendages and body hair, the cuticle thickness, the insect biological period (age) and the insect behavior (Bartlett, 1951; De Paula et al., 2002; Mewis and Reichmuth, 1998; Subramanyam et al., 1998).

The effect of physiological age on *A. obtectus* mortality is important because the adult shelf life of beans weevil is 7 to 14 days (Caswell, 1981; Sönmez et al., 2016) and when conducting studies about the effectiveness of DE in insects, the age of the insect should be standardized in terms for reduced the variability, since it is known that the action of DE may be slower compared to other control methods (e.g., chemical control) (Fields, 1998). Thus, age limitation was used in this study to try to gain more certainty about the effect of DE tested here, in which mixed-sex adults were also randomly selected to avoid possible gender differences, especially regarding insect size (Caswell, 1981). The *A. obtectus* lifestyle and behavior may also have compromised the effect of DE. For example, bean weevils are intense in flight and do not feed in adulthood (Caswell, 1981), which could have increased the chances of the

insect avoiding contact with DE particles. Another factor to be considered regarding the low mortality of *A. obtectus* is the type and the conditions of the grains (Aldryhim, 1993), since bean grains have smooth peel which impairs the adhesion of DE particles to the grain surface from which they give off easily.

Our results suggest that the diatomaceous earth, Insect R formulation, despite its insecticidal effect it was not efficient for the control of *A. obtectus* under storage conditions. Thus, further studies on the specificity of DE action are needed, as well as the evaluation of new and different formulations of DE that are effective against this pest.

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GENERAL CONCLUSIONS

This thesis was conducted with the objective of better investigating the action of diatomaceous earth (DE) which is an environmentally safe tool to be used in stored grain pest management programs. We focus our tests not only on the lethal action of DE, but mainly on the different responses generated, at species and sex level, from the sublethal exposure of *Sitophilus zeamais* and *Sithopilus oryzae*.

Our experiments have revealed that for Sitophilus spp. the *S. zeamais* specie is most affected when exposed to DE showing not only higher mortality rate, but also impaired walking, grain colonization (treated grain repellency), reproductive capacity (e.g., oviposition and number progeny). Thus, we argue that behavioral and morphological differences could be more directly related to greater *S. oryzae* tolerance to DE, whereas physiological resistance would be unlikely to occur in this scenario.

We were also able to show significant differences between males and females when they were sublethally exposed to DE and, through a detailed analysis of the leg and antenna sensilla, we found that some types of sensilla may not only be smaller or less abundant in a given species, but also between sex. We also found that although the DE formulation tested achieved high control rates for Sitophilus over a satisfactory period of time, for the *Acanthoscelides obtectus* species the same formulation did not show effective control, highlighting the variation of the DE action between different insect groups.

Finally, given the many advantages that DE has as a natural insecticide, its effect on the most susceptible species and the possibility of being combined with other techniques, DE remains promising in Integrated Pest Management for weevil and other stored grain pest insects control.