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Determination of the effects of two feed supplements on *Drosophila melanogaster*

Determinação dos efeitos de dois suplementos alimentares em *Drosophila melanogaster*

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Abstract

Pleurotus spp. are considered extremely rich mushroom species from the nutritional point of view, providing immune-enhancement effects when consumed. However, few mushrooms have been tested for their phenotypic and genotypic responses in animal models to ensure the proper dosage for their use. This study aimed to evaluate the effects of two mushroom species on the reproductive capacity of *Drosophila melanogaster*. *Pleurotus citrinopileatus* Singer and *Lentinus sajor-caju* (Fr.) Fr. were provided separately, in specific concentrations, as feed supplements to the fruit flies. The total numbers of developed larvae, pupae, and adults were then measured for each treatment. Inter simple sequence repeats marker analysis was carried out to infer genotypic changes in the mushroom-fed flies. Our results suggest that *Pleurotus* spp. can cause positive changes to the flies' reproductive capacity, since *Pleurotus citrinopileatus* in particular accelerated the life cycle and revealed a higher genetic dissimilarity of the diet-supplemented flies.

Keywords: fruitflies, reproductive fitness, edible mushrooms.

Resumo

Pleurotus spp. são consideradas espécies de cogumelo extremamente ricas do ponto de vista nutricional, proporcionando reforço imunológico quando consumidas. No entanto, poucos cogumelos foram testados quanto à sua interferência fenotípica e genotípica em modelos animais para garantir a dosagem adequada para seu uso. Este estudo teve como objetivo avaliar os efeitos de duas espécies de cogumelos sobre a capacidade reprodutiva de *Drosophila melanogaster*. *Pleurotus citrinopileatus* Singer e *Lentinus sajor-caju* (Fr.) Fr. foram fornecidos separadamente, em concentrações específicas, como suplementos alimentares às moscas da fruta. O número total de larvas, pupas e adultos desenvolvidos foram então avaliados para cada tratamento. Análises de marcadores moleculares do tipo *Inter simple sequence repeats* foram realizadas para inferir mudanças genotípicas nas moscas alimentadas com os cogumelos. Nossos resultados sugerem que *Pleurotus* spp. podem causar mudanças positivas na capacidade reprodutiva das moscas, uma vez que *Pleurotus citrinopileatus*, em particular, acelerou o ciclo de vida e revelou uma maior dissimilaridade genética das moscas suplementadas com esse fungo em sua dieta.

Palavras-chave: moscas das frutas, estímulo reprodutivo, cogumelos comestíveis.

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Introduction

Several fungal species are used in human nutrition as sources of proteins and glycans, primarily because these substances are constituents of the cell wall of basidiomata (Bobek *et al.*, 1991a; Bobek *et al.*, 1991b; Zhang *et al.*, 1994; Nosal'Ová *et al.*, 2001; Hossain *et al.*, 2003; Pramanik *et al.*, 2005), thus providing favorable nutritional benefits when consumed. *Pleurotus* spp. have been characterized to display antibiotic, antiviral, and antitumor activities, where traditional medicine attributes the medicinal properties of these fungi to various substances, many of which have already been used as pharmaceuticals (Wasser *et al.*, 2000).

Pleurotus citrinopileatus Singer is considered to be an extremely rich mushroom from the nutritional point of view (Breene, 1990; Ghosh *et al.*, 1991). Research studies using animal models suggest that this mushroom species can have physiologic effects when consumed, including antitumor and immune-enhancement activities (Wang *et al.*, 2005; Shu *et al.*, 2006).

Drosophila melanogaster Meigen 1830, known as the common fruit fly, is often used as an animal model in biological and genetic studies owing to its easy maintenance under laboratory conditions, low nutritional requirements, and short life cycle, and mainly because it has metabolic reactions similar to that of mammals (Graf *et al.*, 1984; Staats *et al.*, 2018). This species uses a wide variety of substrates for reproduction (Shorrocks, 1982) that are related to feeding behavior and oviposition (Da Cunha and Magalhães, 1965; Carson, 1971; Starmer, 1981; Tidon *et al.*, 2005).

Among the food resources used for the growth of fruit flies are the yeasts that colonize fruits and flowers during the stage of decomposition (Freire-Maia and Pavan, 1949), and *D. melanogaster* females exposed to a diet rich in yeast have been observed to produce an increased number of eggs (Min and Tatar, 2005). Previous studies have identified members of the phylum Ascomycota as being important food resouces for *Drosophila* survival and fitness (Anagnostou *et al.*, 2010). However, Basidiomycota fungi are also a food resource for fruit flies, with Agaricales mushrooms being the main ones detected in the guts of *Drosophila* spp. through metagenomic strategies (Chandler *et al.*, 2012).

It remains unknown whether these food resources may be altering the reproduction rate of fruit flies, and whether feeding these flies with mushrooms may change their genotype in some way, often related to changes in fly anatomy and behavior, such as body composition, enzymatic machinery and nutrient sensing. Although these issues are far from being completely understood, there are some indications in the literature that can help guide their clarification, such feed intake, locomotor activity, fertility, aging and life span can be systematically determined in *Drosophila* in response to dietary factors (Staats *et al.*, 2018; Álvarez-Rendón *et al.*, 2018).

Woods *et al.* (1998) reported that in most vertebrate and invertebrate species, feeding thresholds are rapidly modulated during the course of a meal before systemic homeostasis is restored, indicating that feeding thresholds are partly set by signals emanating from the digestive tract (Murphy and Bloom, 2006). Studies have found that genetic responses associated with quantitative trait loci had large effects on the phenotypic plasticity of fruit flies (Gutteling *et al.*, 2007), where a part of this response was assigned to foraging genes (Kent *et al.*, 2009).

The aim of this study was to evaluate the dietary effect of two kinds of mushrooms on the reproductive capacity (both phenotypic and genotypic) of fruit flies.

Methods

The strains of *Pleurotus citrinopileatus* (hereafter abbreviated PAM) and *Lentinus sajor-caju* (Fr.) Fr. (hereafter abbreviated PSC) used were obtained from the mushroom module of the Faculdade de Ciências Agronômicas (Universidade Estadual Paulista, Botucatu, Brazil). The mycelia were stored in mineral oil (Castellani, 1967) and were reactivated by potato dextrose agar culture.

Culture media were poured into 90 × 60 mm Petri dishes (always in a laminar flow chamber) containing a 38 mm disc of fungal inoculum. The plates were incubated at 27 ± 10 °C until mycelial growth, which occurred in 7 days.

The spawn was obtained using rice grains as the growing substrate. The rice grains had previously been boiled for 15 minutes, and were drained and packed into glass jars of 8.6 × 14 cm. Following closure of the jars with foil and plastic film, they were autoclaved at 121 °C (1 atm) for 15 minutes in two stages, at intervals of 24 hours, after which they were cooled to room temperature. Under a laminar flow hood, the vials containing the spawn were inoculated with the anteriorly obtained fungal culture (primary matrix) on 10-mm-diameter discs. The vials were incubated at 28 °C to allow colonization of the grains by the fungus, thus obtaining the secondary matrix.

The fruiting bodies of the mushrooms were obtained by the Jun-Cao technique (Eira and Minhoni, 1997; Urben *et al.*, 2001), using rice straw as the substrate, which was dried at room temperature, fragmented into pieces of 7 cm, immersed in water for 24 hours, then drained to remove excess water, and finally packed into polyethylene bags with a 2 L capacity.

The polyethylene bags were closed with cotton caps and marked with the identity of each mushroom species used in this study. The substrates were subjected to heat treatment (pasteurization at 80–90 °C for 30 minutes) and then cooled to room temperature or 25 ± 1 °C. The bags with pasteurized substrate were then inoculated with 3% of the previously produced spawn.

The bags were kept at 25 ± 1 °C in a growth chamber until the appearance of basidiomata. The mushrooms were col-

lected manually and frozen in an ultrafreezer to about –50 ± 1 °C, and thereafter lyophilized and milled into a powder to be added to the *D. melanogaster* feed. The flies were donated by the GPEOSC Laboratory (Research Group in Oxidative Stress and Cell Signaling) of the Federal University of Pampa (Bagé, Rio Grande do Sul, Brazil), and were kept in a photoperiod chamber at 25 ± 1 °C during all experiments.

To verify the effect of both mushrooms on *D. melanogaster*, a base medium was prepared (Klein *et al.*, 1999) and mixed with the powder of each fungal species tested. The fly feed comprised the following ingredients: 1 kg of thick cornflour, 800 g of medium cornflour, 250 g of wheat germ, 160 g of sugar, 12 g of powdered milk, 10 g of salt, 7.5 g of soy flour, and 5 g of milled rye. Water was added to this preparation at a ratio of 1:2, and the mixture was then boiled in a beaker for 15 minutes. After cooling, different concentrations of the mushroom powder were added respectively to 15 g of the base medium. These mixtures were distributed to flasks with a cotton cover, occupying 1/3 of the flask capacity. After cooling the mixtures, each flask received 15 flies in photoperiodo chamber and was then incubated at 25 ± 1 °C for a period of 8 days.

Experiment to test the effects of PSC and PAM on the reproductive fitness of *D. melanogaster*

An experiment was conducted to determine the effects of different concentrations of each mushroom species on the reproduction capacity of the flies during 8 days.

The following amounts of the mushroom powder were added to the basal medium: 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 g. The control consisted of 15 g tubes containing basal culture medium without the mushroom supplements, using a same control for both treatments. Fifteen flies were placed in each tube and kept in a photoperiod chamber at 25 ± 1 °C for 8 days. The larvae and pupae were counted at every 24 hours during the incubation period. For each treatment, 10 replicates were made for a total of 130 flasks. The values of larvae and pupae were summed across all replicates and divided by 10 to give the average number of individuals per flask during the treatment.

The results were subjected to analysis of variance with a completely randomized design, and Tukey's test was applied to evaluate the statistical significance of the means. The statistical analyses were performed using the Statistix 8 program.

DNA extraction and PCR assays to evaluate the dissimilarity caused by the treatments

At the end of treatment, the flies were sacrificed for DNA extraction. Twenty flies per treatment, without the head, were macerated in separated microtubes with 300 µL of TirD (Tris, EDTA, and NaCl), 12 µL of sodium dodecyl sulfate (20%), and 1.5 µL of proteinase K. The microtubes were kept in a water bath at 65 °C for 20 minutes. Thereafter, 300 µL of phenol-chloroform-isoamyl alcohol was added to each tube and centrifugation was carried out for 5 minutes at 16128 RCF, after which the supernatant was removed. Then, 600 µL of absolute ethanol was added and the mixture was centrifuged for 5 minutes at 16128 RCF. After discarding the supernatant, the pellet was resuspended with 200 µL of 70% ethanol and the suspension was centrifuged at 16128 RCF for 5 minutes. Following this, the microtubes were allowed to dry for 1 hour and the resultant DNA was resuspended in 40 µL of TE (Tris, EDTA) buffer.

For the PCR assays, we used 16 inter simple sequence repeat (ISSR) markers (Table 1), which are considered ideal for studies on molecular markers variation and diversity (Wang *et al.*, 2012; Shafiei-Astani *et al.*, 2015). The amplification reaction was performed with 6.25 µL of GoTaq PCR mix (Promega), 1.25 µL of ISSR primer, 2.00 µL of DNA primer, and 2.75 µL of H_2O in a final volume of 12.75 µL. The amplification cycles were performed in a thermocycler (Eppendorf) with an initial 30-second denaturation step at 95 °C, then 30 cycles at 95 °C and 60 °C (2 min each), followed by a final enlongation step at 60 °C for 10 minutes. The reactions were made in triplicate for each sample for each treatment.

Evaluation of the PCR amplicons was done via their electrophoresis on 3% agarose gels along with a 50 bp ladder. The presence or absence of bands obtained in the ISSR analysis of flies fed with both mushroons in different concentrations, as well as of the control flies, allowed

Table 1. Primers used for amplification of ISSR regions in *Drosophila melanogaster.*

Locus	Sequence 5' - 3'
ISSR1	GAGAGAGAGAGAGAGAGAA
ISSR2	GAGAGAGAGAGAGAGAGAC
ISSR3	GAGAGAGAGAGAGAGAGAG
ISSR4	GAGAGAGAGAGAGAGAGAT
ISSR5	CTCTCTCTCTCTCTCTCTA
ISSR6	CTCTCTCTCTCTCTCTCTC
ISSR7	CTCTCTCTCTCTCTCTCTG
ISSR8	CTCTCTCTCTCTCTCTCTT
ISSR9	AGAGAGAGAGAGAGAGAGA
ISSR10	AGAGAGAGAGAGAGAGAGC
ISSR11	AGAGAGAGAGAGAGAGAGG
ISSR12	AGAGAGAGAGAGAGAGAGT
ISSR13	ATATATATATATATATATA
ISSR14	ATATATATATATATATATC
ISSR15	ATATATATATATATATATG
ISSR16	ATATATATATATATATATT

evaluation of the change in genotypic variability in each group of flies tested. For evaluation of the amplicons associated with response to treatment, an binary array (0 to absence or 1 to presence for determined amplicon) was created and a phenogram was built using the unweighted pair group method with arithmetic mean algorithm with help of the computer program NTSYSpc v. 2.1 (Rohlf, 1998). The cophenetic correlation coefficient (CP) was used for the evaluation of dissimilarity between the treatments tested.

Results

Effects of PSC and PAM on the reproductive fitness of *D. melanogaster*

Flies exposed to the different concentrations of PAM and PSC supplements exhibited variations in their reproduction period, the amounts of larvae and pupae, and especially the final number of flies. These observed variations were in relation to the respective values observed for the control untreated flies.

The flies fed PAM had different numbers of larvae, pupae, and adults at the end of the treatment compared with the control and PSC-fed flies (Tables 2 and 3).

First larvae were observed on the third day of incubation in the PAM-fed group and on the fourth day in the PSC-fed group, and the same occurred in both control tubes. Larva counting was carried out until the fifth day, owing to the large number of larvae observed in the PAM-treated group.

Pupae were observed on the fourth day in the PAM-fed group, whereas the first pupae were observed only on the seventh day in the PSC-fed and control groups.

Within the PAM-treated groups, the 1.5 g supplementation (0.1 g of basiomata/g of substrate) resulted in the highest numbers of larvae (134.9), pupae (149.0), and adult flies (52.1) (Table 2).

Treatment with PAM accelerated the reproduction cycle of the fruit flies, as anticipated, and increased the numbers of larvae and pupae, differing from that seen in the control and PSC-treated flies (Figure 1). At PAM supplementation exceeding 1.5 g per flask, a decrease in the numbers of larvae and pupae was observed, indicating that 1.5 g or 0.5 g of fungal powder/g of substrate was the best concentration to accelerate the reproductive cycle of *D. melanogaster*.

ISSR analysis of fruit flies fed the mushroom supplements

Twelve to 16 ISSR primers were used for the dissimilarity analysis by amplifying the DNA of flies in the different treatment groups. ISSR-PCR analysis of the PAM-fed flies showed alterations between the different treatments (Figure 2). The 1.5 g amount of PAM supplement, which showed the best performance in enhancing the reproductive capacity of the fruit flies, also resulted in the highest dissimilarity among the concentrations tested, based on the

Table 2. Average number of larvae, pupae and adult flies after the treatment at different concentrations of *P. citrinopileatus* (PAM) added on the *Drosophila melanogaster* diet. * Different letters in the columns indicate significative differences in the Tukey test (α = 0.05).

PAM Concentration (g)	Initial number of fruit flies	Number of larvae*	Number of pupae	Final number of fruit flies
0	15	40.7^{A}	12.5^{C}	15.0^{A}
0.5	15	93.6^{AB}	109.6^{AB}	50.6^{A}
1.0	15	110.1^{A}	144.2^{A}	49.8^{A}
1.5	15	134.9^{AB}	149.0^{A}	52.1^{A}
2.0	15	37.8^{BC}	138.5^{AB}	40.0^{A}
2.5	15	35.6^{BC}	59.1^{BC}	19.7^{A}
3.0	15	1.2^{C}	26.7^{C}	20.4^{A}

Table 3. Mean number* of the quantitative larvae, pupae and adult flies after the treatment at different concentrations *P. sajor-caju* (PSC) added on the *Drosophila melanogaster* diet. * Different letters in the columns indicate significative differencesin the Tukey test (α = 0.05).

PSC Concentration (g)	Initial number of fruit flies	Number of larvae*	Number of pupae	Final number of fruit flies
0	15	40.7^{A}	12.5^{C}	15.0^{A}
0.5	15	8.8^{A}	61.1^{A}	15.4^{A}
1.0	15	10.9^{A}	38.5^{A}	15.0^{A}
1.5	15	8.3^{A}	56.4^{A}	15.3^{A}
2.0	15	10.9^{A}	85.5^{A}	15.5^{A}
2.5	15	14.1^{A}	66.6^{A}	15.4^{A}
3.0	15	5.17^{A}	96.0^{A}	15.6^{A}

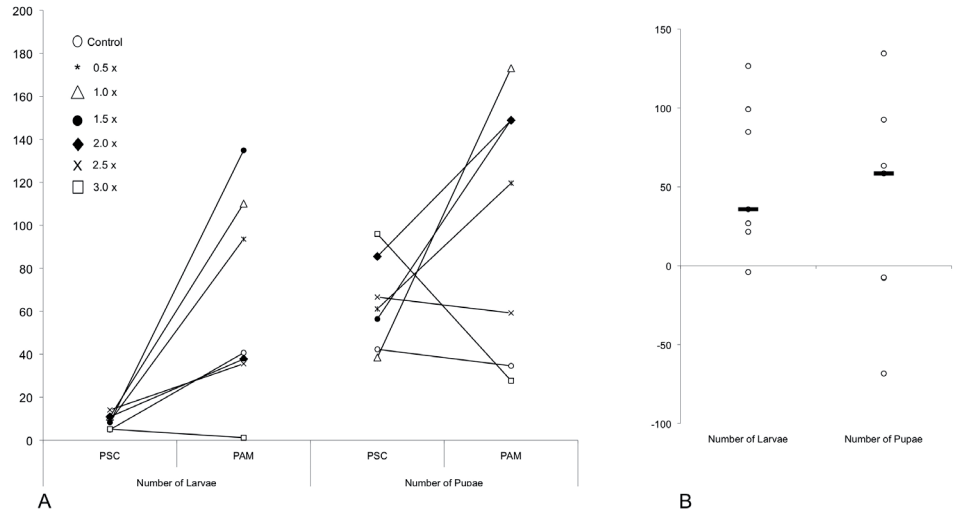

Figure 1. Effects of PSC and PAM on the reproductive fitness of *Drosophila melanogaster*: (A) Number of larvae and pupae in the distinct treatments using PSC and PAM as feed supplement. (B) Median quantity of larvae and pupae observed in different measurements (dark bar).

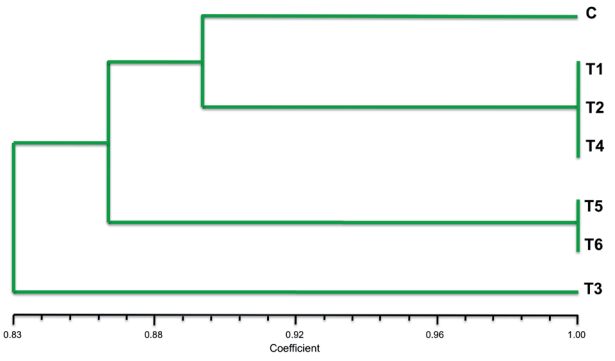

Figure 2. Dendrogram of fruit flies fed with PAM in different concentrations (C = control, T1 = 0.5 g, = T2 = 1.0 g, T3 = 1.5 g, T4 = 2.0 g, T5 = 2.5 g and T6 = 3.0 g). In the comparison it is observed that the treatment 3 (1.5 g of PAM powder) has the highest genotypic dissimilarity when compared to other treatments, indicated by the Cophenetic Correlation Coefficient (CP =0.77).

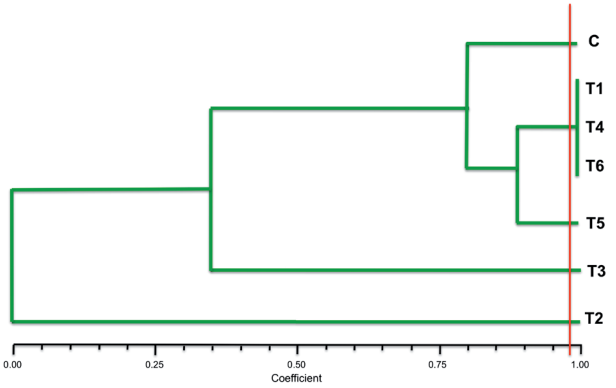

Figure 3. Dendrogram of fruit flies fed with PSC in different concentrations (C = control, T1 = 0.5 g, T2 = 1.0 g, T3 = 1.5 g, T4 = 2.0 g, T5 = 2.5 g and T6 = 3.0 g). Comparing all treatments, no genotypic dissimilarities were observed, as indicated by the red line that represents the Cophenetic Correlation Coefficient (CP =0.99).

CP value of 0.77. For the PSC-fed flies, the data from the phenotypic characterization were corroborated by the ISSR marker analysis, in that there were not observed differences between the various treatment amounts (Figure 3).

Discussion

It was observed that *D. melanogaster* individuals fed with the fungus *P. citrinopileatus* exhibited the highest development of larvae and pupae and increased number of individuals in a shorter period relative to the PSC-fed and control flies. Despite that treatment with 3.0 g of PSC also increased the number of pupae during the experimental period, the total life-cycle times of these flies were all later than those of flies treated with PAM.

Flies fed with *P. citrinopileatus* experienced a reduction in the time needed for development. The control and PSC-treated flies needed a period of 4 days for the appearance of the first larvae, while the PAM-treated flies needed only 3 days. Several factors may affect the life cycle, such as temperature, light availability, and food. To eliminate such potential interferences, the flies were fed PSC under the same conditions as those fed PAM, proving that the observed changes in the cycle are not due to environmentally induced events. On the other hand, the changes may be due to the large amount of protein that is found in the *P.*

citrinopileatus fruiting body (Musieba *et al.*, 2013), which is known to be essential to the diet of insects and adults, influencing their egg production (Plácido-Silva *et al.*, 2005).

In the normal *D. melanogaster* life cycle (Lawrence, 1992; Griffiths *et al.*, 2000), the first pupae appear at 5 days after egg deposition, as was observed in the control and PSC-treated flies. In flies treated with PAM, this stage was anticipated to occur on another day depending on the different treatments. The complete life cycle should take a period of 9–11 days (Griffiths *et al.*, 2000), but our results showed that the initial number of PAM-fed flies had increased from 15 to 52 individuals in the course of 8 days. These changes may also be due to the ~22.10% higher protein concentration in *P. citrinopileatus* than in other *Pleurotus* spp. (Musieba *et al.*, 2013; Phan *et al.*, 2014).

Despite that insects such as fruit flies—considered to be *r*-strategist species—exploit available resources temporarily and produce large numbers of offspring with each reproductive cycle (Osborne, 2000), the greater availability of mushrooms as a feed supplement in our study did not generate an exponential increase in the fitness of the treated flies, as spected for a *r*-strategist behaviour. The highest mushroom concentrations also showed no hint of any molecular markers changes in the flies, and in fact, both phenotypic (increase of oviposition behavior) and genotypic changes were observed only when the flies were fed 1.5 g of PAM. However, proportionally no increase in adults was observed at the end of the experiment in comparison to the number of eggs and larvae. Further experiments should be performed to assess whether experimental conditions, such as the size of the containers used for each replicate, may interfere with the emergence of adults because of the small space available in each experimental unit.

Chippindale *et al.* (1993) reported that dietary restrictions can greatly reduce fecundity through the early and middle parts of the life span, as well as reduce the total egg output. Other studies have shown significant increases in longevity as a result of a shift of resources, from reproductive activity to adult survival, suggesting the possibility of a genetically mediated allocation of energetic reserves in *D. melanogaster* (Holehan and Merry, 1985; Rose, 1991). In fact, it is known that both genes and neuromodulators (*e.g.*, foraging genes and allatostatin, hugin, and corazonin) can contribute to the response to changes in nutrient availability in order to adjust discrete aspects of feeding (Melcher and Prankatz, 2005; Kent *et al.,* 2009; Hergarden *et al.*, 2012). Therefore, no restrictive effect of the fungi on the flies treated in this work was revealed, as all treatments showed a higher or similar response to that of the untreated controls, at least for adults observed in the end of experiment. This result was expected because these insects naturally use mushroom resources recurrently, to the point that fruit flies are considered as pests to mushroom cultivators (Gnaneswaran and Wijayagunasekara, 1999).

The present findings suggest that changes could occur to elicit a positive effect on reproduction in fruit flies fed with PAM, whereas these same benefits are not clear when used PSC supplement. Both mushroom species are reported in the literature as being highly nutritious, presenting antioxidant and scavenging effects when consumed (Musieba *et al.*, 2013; Phan *et al.*, 2014). However, the lack of studies evaluating the effects of these mushrooms experimentally in animal models, much less their effects on fertility and fitness, prevents us from making major assumptions about the results. It may even be possible that the population fluctuations in the fruit flies are due to food availability (Beaver, 1984). The higher PAM concentrations might have caused an increase in competition between the larvae, since insects that breed in discrete unequal resources often experience intense larval competition for food. Consequently, our results for the observed adults at the end of experiments, could be explained by the intense competition generated by an overpopulation of pupae generated. However, further experiments are still needed to evaluate whether experimental conditions may be influencing the maintenance of adults. This competition has been found in natural populations of mushroom-breeding species of *Drosophila* (Jaenike, 1990). Thus, the effects observed from PAM supplementation, especially from the genotypic point of view, should be further studied to elucidate its actual relationship to the increase of reproduction in animal models.

Conclusions

Our findings suggest that using any of the fungal species as a feed supplement for *D. melanogaster* has no negative effects on the reproductive capacity of the fruit flies, and that PAM in particular accelerates the life cycle of the flies. Exposure of *D. melanogaster* to *P. citrinopileatus* altered the reproductive rate of the flies, increasing at least, the number of larvae and pupae produced. The best supplementation amount for the reproductive stimulus was 1.5 g (0.1 g of mushroom powder/g of substrate), whereas amounts greater than this could reduced the larvae fitness, suggesting that there is a maximum dose of use of this mushroom species as a food supplement. Higher concentrations inhibited the positive changes in reproductive capacity, equaling the reproductive rates observed in flies not fed the mushroom supplement.

These results highlight the potential of some mushroom species as stimulants of reproduction in the fruit fly animal model under laboratory conditions.

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