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INFLUENCE OF SHIITAKE MUSHROOM *Lentinula edodes* ON REPRODUCTION OF *Drosophila melanogaster*

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Shiitake mushroom Lentinula edodes *is an edible basidiomycete cultivated worldwide, with high nutritious value and diverse biological activity. There has been an increase in its use as food supplement. Influence of shiitake mushroom extract on the reproductive function and development in an object fruit fly* Drosophila melanogaster *is described in this research. Fruit flies were maintained on banana medium with or without supplementation of shiitake mushroom`s extract, standardized per amount of crude polysaccharides. Shiitake extract supplement, 0.030% and 0.015% crude polysaccharides per volume, induced a statistically significant increase in total number of pupae and flies, and promoted pupae viability. Adult drosophilae males, which received shiitake extract supplement for seven days, had a statistically significant reduction in copulation latency, while thirty day exposure to extract promoted a statistically higher rate of mated flies. Females showed an increase in number of mated flies and reduction in copulation latency post seven day feeding period and had no significant effect on mating ability and fertility post thirty days. Further studies are planned to identify the biologically active components of shiitake mushroom hot water extract and to characterize their effects on reproductive function.*

Key words: *shiitake mushroom, water extract, fruit fly, reproduction.*

INTRODUCTION

Shiitake *Lentinula edodes* (Berk.) Pegler is an edible mushroom cultivated worldwide with medicinal properties (Mizuno, 1995; Chang *et al*., 1999; Hobbs, 2000).

In traditional Orient medicine it is used to promote good health and vitality and to increase the body's adaptive capabilities (Mizuno, 1995; Chang *et al*., 1999; Yap and Ng, 2005). It also has anti-inflammatory, antitumor, antibacterial, antiviral, blood pressure and cholesterol level regulating, anti-diabetic, hepatoprotective and sexual function improving applications (Chang *et al*., 1999; Wasser and Weis, 1999; Stamets, 2002).

Some shiitake components have been studied in details and their pharmacological activities have been shown. Most studies of active substances, incl. polysaccharides, are performed to investigate immune system modulating, antioxidative, antimutagenic and cholesterol reducing effects in various biological systems *in vivo* and *in vitro* (Chihara *et al*., 1970; Hobbs, 2000; Yap and Ng, 2005; Lee *et al*., 2009 b). In comparison with other effects, there is little information of the influence of shiitake on animal reproduction and development (Cozens *et al*., 1981a; Cozens *et al*., 1981b; Cozens *et al*., 1981c; Zorenko *et al*., 2003).

In studies on biological active substances of shiitake as models standard laboratory animals, such as mice, rats, and rabbits have been used (Chihara *et al*., 1970; Cozens *et al*., 1981a; 1981b). The biological activity of shiitake was shown in a Social vole model (Matjuskova *et al*., 2001). Fruit fly *Drosophila melanogaster* is one of the main organisms used for study in various fields of biology, including researches on biological substances. It is used for screening potential medicinal properties of chemicals (Tickoo and Russell, 2002) and botanicals (Li *et al*., 1993; Taira *et al*., 2005; Jafari *et al*., 2007; Bahadorani and Hilliker, 2008; Zhao *et al*., 2008; Jafari *et al*., 2008; Матюшкова и др., 2010), as well as for evaluating toxicity of various compounds (Goldstein and Babich, 1989; Affleck and Walker, 2008). Evolutionary conservation has been identified between fly and human genomes, and there are similarities in sex determination, gametogenesis, embriogenesis, aging process, host defense and other processes (Tickoo and Russell, 2002; Helfand and Rogina, 2003; Zhang *et al*., 2004; Haag and Doty, 2005; Mutova and Cooley, 2005; Lemaitre and Hoffmann, 2007).

We suggest that fruit fly is a well suited model organism for studying the spectra of biological activity of shiitake mushroom. There are several reasons for using drosophila in laboratory studies, such as, well described biology and protocols, short life cycle, low costs, relative easy maintenance and biological similarities which they share with higher animals. In Latvian legislation there are no regulations on in-

vertebrate, including insects, use in laboratory procedures (Anonīms, 2010).

The present study aimed to evaluate the effects of shiitake mushroom hot water extract on *D. melanogaster* pupa and fly development and on reproductive function, including fly fertility and mating ability in terms of copulation latency, duration of copulation, remating and number of mating flies.

MATERIALS AND METHODS

Preparation of aqueous extract of shiitake mushroom. Fresh fruiting bodies (200 g) of *L. edodes* strain DSM3565 (German Collection of Microorganisms and Cell Cultures) were homogenised and extracted with distilled boiling water (1 L) for 15 h at 80 °C. Supernatant was obtained by centrifugation; insoluble matter was removed. The amount of crude polysaccharides in hot water extract was determined by ethanol precipitation (Chihara *et al*., 1970). Approximate crude polysaccharide content in shiitake fruiting body extract was 1.7 gram per litre. The extract was pasteurised, lyophilised and stored at 4 °C until use in current experiments.

Rearing of flies. The line of Normal (wild type) *D. melnogaster* used for study was obtained from the Institute of Biology, University of Latvia. To ensure genetic homogeneity of the drosophila population an inbreeding procedure had been employed. All flies were maintained on banana medium (Demerc and Kaufman, 1996) in standard 50 ml plastics vials at 23 ± 1 °C, 12 : 12h light: dark cycle and 50% relative humidity. Before use, a suspension of dry baker's yeast (0.5 mg per 25 ml) was added on the medium surface. Virgins were collected by diethyl ether separating males and females within 12 h post eclosion. Adult flies were maintained in batches of 20 flies per vial.

A water solution (E) of shiitake lyophilized extract was added to banana medium or to yeast suspension maintaining a standard crude polysaccharides content. There were three treatment groups: control group (Control) — flies maintained without extract, and two groups exposed to shiitake extract supplement (E 0.015%; E 0.030%) — 0.015%, 0.030% polysaccharides per volume of medium or baker's yeast suspension, respectively.

Development test. The influence of shiitake on development was estimated by pupa and fly yield as well as by pupae viability. One male and two females were transferred to vials containing banana medium with or without shiitake extract supplement. Flies were allowed to mate and they laid eggs for seven days, then they were discarded from vials. Larvae were allowed to feed, develop and undergo a metamorphosis. First-generation pupae and flies were counted for seven successive days after their emergence (Goldstein and Babich, 1989; Pendleton *et al*., 2000). Pupa to imago viability was determined as percentage of viable flies produced by pupae (Tantawy and El-Helw, 1970). Replication was 6 to 11 vials per group and the experiment was performed three times.

A mating ability test. Mating ability test was performed for flies that received shiitake supplement as imago. Five days old virgin females and males were fed separately with shiitake supplement added to yeast suspension for seven and thirty day periods. Flies were moved to fresh medium every seven days. Approximately twenty-four hours before the test flies were set-up singly on medium without supplement. Ten-days-old virgin mates from a Normal line population (two females or males) were exposed to experimental group flies in mating vials.

Mating behaviour patterns were recorded during a 120-minute observation period. Copulation latency (mating speed), duration of copulation, male remating time and duration of second copulation as well as number of mating flies were determined (Mac Bean and Parson, 1967; Singh and Singh, 2000). Twenty mating vials were set up for each group.

Fertility test. After the mating ability test, flies were allowed to mate and lay eggs for two days and then discarded. Fertility was determined as F_1 fly number per vial (Tantawy and El-Helw, 1970). Drosophilae development from egg to adult fly in laboratory conditions lasted approximately twelve days (data not shown). Therefore, offspring number was determined at the eighteenth day post laying to ensure that only for F1 progeny were counted. Offspring number was determined in control and shiitake supplement fed groups after a 30-day exposure. Twenty pairs were examined for each group.

Statistical analysis. Data were processed by Microsoft Office Excel 2003. For development test, fertility test and for mating behavior patterns, data on copulation latency, remating time, duration of first and second copulation, are presented as mean ± standard error of mean (SE) and standard deviation (SD); in the mating ability test, the number of mating flies are expressed as percentage of total fly number per group. Statistical analysis was performed using SPSS 18 for Windows. Significant differences between experimental groups and control were determined by one-way analysis of variance (Least Significant Difference test, LSD) and the Chi-square test.

RESULTS

Effect of shiitake hot water extract on fly yield and viability. Shiitake extract supplement caused an increase in pupae and fly yield and pupae viability. The group receiving extract in banana medium (0.030% polysaccharide per volume) had a statistically significantly higher mean number of pupae and flies, and pupae viability than the control group — 1.2, 1.5 and 1.3 times, respectively. In extract supplement (0.015% polysaccharide per volume) the differences with the control in fly yield and pupa to imago viability were not significant (Table 1).

Table 1

YIELD AND VIABILITY OF *D. melanogaster* EXPOSED TO SHIITAKE SUPPLEMENT

Group	N	Number of pupae		Number of flies		Pupae viability %	
		mean ± SEM	SD	mean ± SEM	SD	mean ± SEM	SD
Control	28	46.54 ± 3.48	18.42	27.04± 3.83	20.25	52.15± 4.29	22.68
E 0.015%	27	56.59 ± 3.65*	18.95	36.19± 3.99	20.73	60.80± 3.70	19.22
E 0.030%	27	56.70 ± 3.12*	16.23	39.44± 4.14*	21.50	66.05±3.43*	17.85

Control – no supplement; E 0.015%, E 0.030% – extract supplement in respect to its crude polysaccharides content (0.015%, 0.030% – crude polysaccharides per volume of banana medium). SEM, standard error of mean, SD, standard deviation, N, number of vials. The significance of the difference between means was determinate by one-way analysis of variance (LSD test) (*$P < 0.05$).

Effect of shiitake hot water extract on fly mating ability. Fly mating ability increased with a shiitake extract supplement diet (Table 2, Fig. 1 and Fig. 2). After seven-day exposure to shiitake extract supplement in both concentrations (0.015% and 0.030% of crude polysaccharides respectively), the number of mated females significantly increased in comparison to the control (Table 2). Males showed a statistically significant effect on 0.015% diet after thirty days of feeding (Table 2).

Shiitake extract supplement received for seven days, in comparison to control, reduced copulation latency in males (Fig. 1 A) and females (Fig. 2), and induced a decrease of male remating time (Fig. 1 B). An effect of a post seven-day feeding period was statistically significant only for copulation latency in males in group E 0.030% and females in group E 0.015%. The supplement had no visible effect on duration of copulation (Fig. 1 A, B, Fig. 2). There was no statistically significant difference in copulation latency, remating time and duration of copulations between shiitake extract supplement fed groups and the control after thirty days of feeding (data not shown).

Effect of shiitake hot water extract on fly fertility. There was no statistically significant increase in mean number of progeny per fertile males fed with shiitake extract supplement for thirty days, and shiitake showed no remarkable effect also on female fertility (Table 3).

Table 2

MATING ABILITY OF *D. melanogaster* MALES AND FEMALES EXPOSED TO SHIITAKE EXTRACT SUPPLEMENT FOR DIFFERENT TIME PERIODS

Exposure time	Group	Percentage of mated flies	Percentage of remated flies
7 days			
	Males (♂x♀♀)		
	Control	100.0	68.8
	E 0.015%	93.8	62.5
	E 0.030%	93.8	62.5
	Females (♂♂x♀)		
	Control	29.4	nd
	E 0.015%	70.6**	nd
	E 0.030%	88.9**	nd
30 days			
	Males (♂x♀♀)		
	Control	20.0	5.0
	E 0.015%	50.0**	10.0
	E 0.03%	35.0	0.0
	Females (♂♂x♀)		
	Control	45.0	nd
	E 0.015%	60.0	nd
	E 0.030%	50.0	nd

Control – no supplement; E 0.015%, E 0.030% – extract supplement in respect to its crude polysaccharides content (0.015%, 0.030% – crude polysaccharides per volume of baker' yeast suspension). Per each group were performed twenty vials. Remating was not determined for female groups (nd). The significance of the difference between experimental groups and control was determinate by Chi-square test (** $P < 0.01$).

DISCUSSION

The present study demonstrates that shiitake mushroom hot water extract has an effect on *D. melanogaster* reproduction and development. There was an increase in pupae, fly yield and pupa to imago viability when larvae passed through metamorphosis on shiitake extract supplemented medium. Drosophilae exposure to a shiitake supplement at the imago stage improved mating ability and fertility. The effects depended on supplement concentration, exposure time and, in

Table 3

FERTILITY OF *D. melanogaster* MALES AND FEMALES AFTER THIRTY DAY EXPOSURE TO SHIITAKE EXTRACT SUPPLEMENT

Group	N	Number of fertile flies	Number of offspring flies (mean±SEM)	SD
Males (♂x♀♀)				
Control	19	16	46.19 ± 6.56	26.25
E 0.015%	20	16	57.44 ± 5.26	21.02
E 0.03%	20	18	54.06 ± 5.91	25.08
Females (♂♂x♀)				
Control	19	13	27.92 ± 4.10	14.79
E 0.015%	20	16	24.29 ± 3.58	14.33
E 0.030%	19	15	23.60 ± 3.07	11.90

Control – no supplement; E 0.015%, E 0.030% – extract supplement in respect to its crude polysaccharides content (0.015%, 0.030% – crude polysaccharides per volume of baker' yeast suspension). SEM, standard error of mean, SD, standard deviation, N, number of fly couples. The significance of the difference between means was determinate by one-way analysis of variance (LSD test).

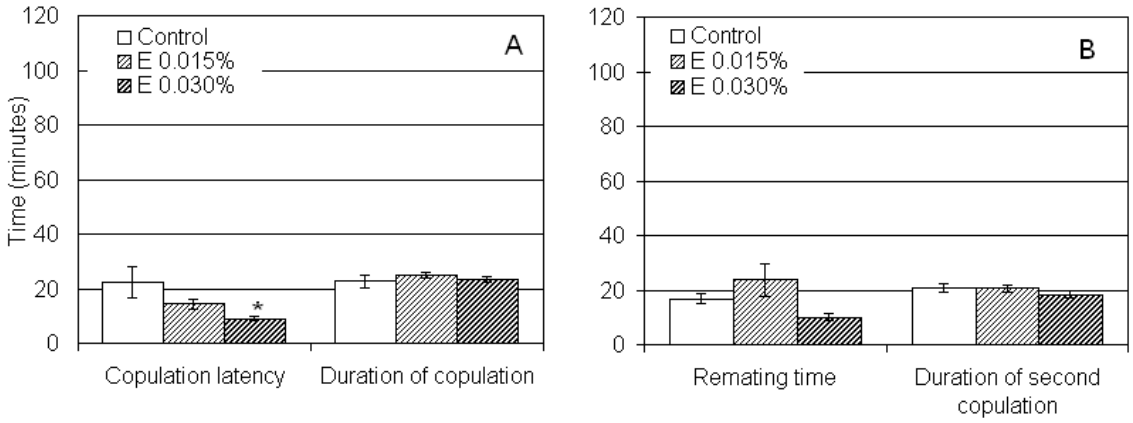

Fig. 1. Mating (A) and remating (B) ability of *D. melanogaster* males exposed to shiitake extract supplement for seven days. Control – no supplement; E 0.015%, E 0.030% – extract supplement in respect to its crude polysaccharides content (0.015%, 0.030% – crude polysaccharides per volume of baker' yeast suspension). Data is expressed as mean number ± standard error of mean of twenty vials. The significance of the difference between means was determinate by one-way analysis of variance (LSD test) (*$P < 0.05$).

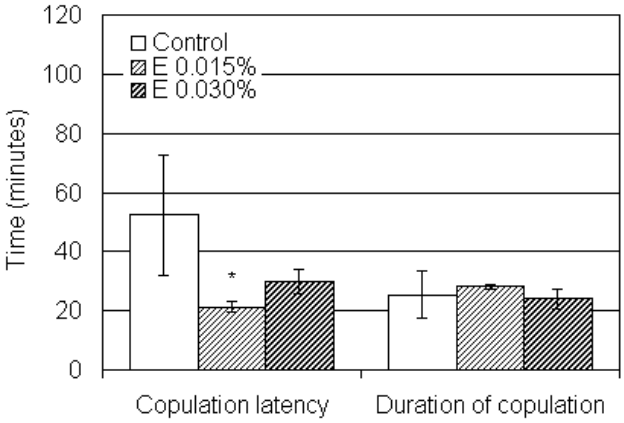

Fig. 2. Mating ability of *D. melanogaster* females exposed to shiitake extract supplement for seven days. Control – no supplement; E 0.015%, E 0.030% – extract supplement in respect to its crude polysaccharides content (0.015%, 0.030% – crude polysaccharides per volume of baker' yeast suspension). Data is expressed as mean number ± standard error of mean of twenty vials. The significance of the difference between means was determinate by one-way analysis of variance (LSD test) (*$P < 0.05$).

some cases, on drosophilae sex. Increase in fly yields and pupae viability was more remarkable at higher shiitake extract concentration, 0.030% polysaccharide per volume of medium. Mating ability and fertility responses to supplement mostly were similar in both concentrations. The differences in responses might be explained by a difference in supplement intake, at larval and imago stages, respectively. Fly mating ability and fertility declines with age (Grotewiel *et al*., 2005). Our results differed in some respects with theory on female mating ability, as in the control group it was low at young age and did not decline with age. We used the fertility test only post thirty-day exposure period, as we supposed that differences between control and supplement fed groups would differ more remarkable in old age drosophilae than in young flies.

Our results are similar to those using other model organisms, such as Social vole *Microtus socialis* (Zorenko *et al*., 2003) and rats (Matjuskova *et al*., unpublished data). There were improvements in Social vole fertility and offspring ontogenesis and rat male mating ability when animals were fed with shiitake hot water extracts (Zorenko *et al*., 2003; Matjuskova *et al*., unpublished data).

Shiitake mushroom is known to affect sexual function (Chang *et al*., 1999; Wasser and Weis, 1999; Stamets, 2002) and this has been suggested also for mushrooms *Cordyceps sinensis* (Stamets, 2002) and *Ganoderma lucidum* (Wasser and Weis, 1999). In literature there are little data on botanical, including mushroom, substance effects on *D. melanogaster* mating ability; one of the few researches in this area is about *Coriolus versicolor* (Li *et al*., 1993). Li *et al*., (1993) observed that fruit fly mating frequency, progeny number and female mean life span were increased by exposure to *C. versicolor* polysaccharides.

The biological activity of zinc, eritadenine and immune system modulating polysaccharides, especially lentinan (β-1,3-D glucan), have been suggested as substances that might explain the shiitake effect on sexual function (Flynn, 1991; Chang *et al*., 1999). Hot water extraction is one of the main methods used for obtaining material rich in high molecular weight polysaccharides from different natural sources, including shiitake mushroom (Miles and Chang, 1997; Zhang *et al*., 2007; Lee *et al*., 2009b). With this procedure it is possible to obtain different types of biologically active polysaccharides, such as non-protein bound β-glucans, including lentinan, heteropolysaccharides and protein-bound polysaccharides (Chihara *et al*., 1970; Zhang, *et al*., 2007; Lee *et al*., 2009a). Mushroom polysaccharides are well known as immunomodulators (Smith *et al*., 2002; Yap and Ng, 2005, Lee *et al*., 2009b). Host defense triggering by β-glucans has been described in vertebrates and in invertebrates (Vetvicka, Sima, 2004; Novack, Vetvicka, 2009). In organisms, physiological system activities are integrated. The immune system interacts with many processes in organisms, including reproductive function. This relationship also has been noted in invertebrates (Lutton and Callard, 2006). It is known that *D. melanogaster* and vertebrates, including human, share similarities in intracellular

signaling pathways, humoral and cellular responses of innate immunity (Lemaitre and Hoffmann, 2007).

In conclusion, it could be suggested that there are some general mechanisms by which shiitake influences animal reproductive function and that one of the substances mediating this effect might be polysaccharides. The studies are being continued to determine shiitake polysaccharide effects on fruit fly life span and fertility, in terms of gamete production and it viability.

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ŠITAKĒ SĒNES *Lentinula edodes* IETEKME UZ *Drosophila melanogaster* REPRODUKTIVITĀTI

Šitakē sēne *Lentinula edodes* ir plaši kultivēta ēdamā bazidiomicēte ar augstu uzturvērtību un plašu bioloģiskās aktivitātes spektru, kas arvien plašāk tiek lietota kā pārtikas piedeva. Šajā darbā iegūti dati par *L. edodes* ekstrakta iedarbību uz modeļobjekta augļu mušas *D. melanogaster* vairošanās funkciju un attīstību. Šitakē karsta ūdens ekstrakts, standartizēts pēc polisaharīdu satura, tika izmantots kā barības piedeva, audzējot mušas banānu barotnēs. Lietojot kā barības piedevu ekstraktu ar polisaharīdu koncentrācijām 0.030% un 0.015%, novēroja statistiski būtisku kūniņu un mušu skaita palielināšanos, kā arī pozitīvu ietekmi uz kūniņu dzīvotspēju. Drozofilu tēviņiem, kas šitakē ekstrakta piedevu saņēma septiņas dienas, statistiski būtiski samazinājās kopulācijas latentais periods, bet pēc 30 dienu ilgstošas lietošanas statistiski būtiski palielinājās sakopulējošo mušu skaits. Mātītēm pēc septiņām dienām samazinājās kopulācijas latentais periods un palielinājās sakopulējušo mušu skaits. Pēc 30 dienu barošanās perioda mātītēm nenovēroja pārošanās spējas un auglības izmaiņas. Turpmākajos pētījumos plānots noteikt bioloģiski aktīvos savienojumus šitakē sēnes karsta ūdens ekstrakta frakcijās un raksturot to ietekmi uz vairošanās funkciju.