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Effects of reserpine on reproduction and serotonin immunoreactivity in the stable fly *Stomoxys calcitrans* (L.) ☆

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Abstract

Biogenic amines are known to play critical roles in key insect behaviors such as feeding and reproduction. This study documents the effects of reserpine on mating and egg-laying behaviors of the stable fly, *Stomoxys calcitrans* (L.) (Diptera: Muscidae), which is one of the most significant biting fly pests affecting cattle. Two sperm staining techniques were adapted successfully to reveal the morphology of stable fly sperm, for the first time, and determine successful mating in females through the assessment of sperm transfer. This approach was also applied to assess sperm transfer by males treated with different doses of reserpine. Mating or sperm transfer did not occur in flies during the first 3 days after emergence. Thereafter, the percentage of females that mated increased with age. Reserpine treatment of males reduced sperm transfer in a dose-dependent manner. Older males were more sensitive to reserpine treatment than younger flies. Reserpine treatment of 5 days old females reduced the number of eggs laid, but had no effect on egg-hatching rates. Results of immunoreactivity (IR) experiments indicated that serotonin in the neuronal processes innervating male testes was completely depleted by reserpine within 5 h after treatment. This effect was transient as the serotonin immunoreactive signal was recovered in 33.3% of the males at 1 day post-treatment and in 94.4% of the flies at 3 days post-treatment. The results of this study concur with previous findings in other insect species and extend our knowledge of the critical roles biogenic amines play in mating and oviposition behaviors of the stable fly. The work could provide a foundation to further characterize the specific roles of individual biogenic amines and their receptors in stable fly reproduction.

Keywords

Stable fly; Mating; Oviposition; Sperm transfer; Serotonin immunohistochemistry

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1. Introduction

Serotonin, octopamine, and other biogenic amines function as neurotransmitters, neuromodulators, and neurohormones to control and modulate many behaviors in insects (Evans, 1980; Blenau and Baumann, 2001). The physiological roles of biogenic amines in insect reproductive behaviors, specifically the egg-laying behavior, have been well documented in several insect species (Lange et al., 1984; Orchard and Lange, 1986; Clark and Lange, 2000, 2003; Lange, 2004; Lee et al., 2003). Octopaminergic neurons are found to innervate the ovaries and oviduct in *Drosophila melanogaster* (Monastirioti, 2003), and octopamine receptors are required for ovulation in the locust, *Locusta migratoria*, and *D. melanogaster* (Orchard and Lange, 1986; Lee et al., 2003). Physiological studies indicate muscle contractions in the female reproductive system of the locust are regulated by octopamine, serotonin, and tyramine (Lange et al., 1984; Orchard and Lange, 1986; Clark and Lange, 2002, 2003; Donini and Lange, 2004; Lange, 2004; da Silva and Lange, 2008). Injection of octopamine, serotonin and tyramine in the bean weevil species, *Callosobruchus chinensis* and *Callosobruchus maculatus*, increased the number of eggs laid by females (Yamane and Miyatake, 2010). Lee et al. (2001) reported that infertility of the fruitless-mutant *Drosophila* males correlated with defective serotonergic innervation of reproductive organs. In many studies, the functional roles of biogenic amines were investigated by depleting the biogenic amines using chemical agents, such as reserpine.

Reserpine is an amine-depleting compound that has been used to elucidate the functions of biogenic amines in insects. Reserpine selectively depleted octopamine, dopamine and serotonin in the nervous system of the cockroach, *Periplaneta americana* (Sloley and Owen, 1982; Sloley and Orikasa, 1988). The amine-depleting effect of reserpine has also been demonstrated in beetles, blow flies, honeybees, crickets, and butterflies (Masner et al., 1970; Brookhart et al., 1987; Menzel et al., 1999; Stevenson et al., 2000; Vieira et al., 2007). Reserpine was reported to act as a chemosterilant when fed to the Mediterranean fruit fly, *Ceratitis capitata*, where fertility and fecundity were interrupted (Cohen, 1983). The inhibitory effect of reserpine on female oviposition or fecundity has also been reported in the house fly, *Musca domestica*, and the southern house mosquito, *Culex quinquefasciatus* (Hays et al., 1969; Tennakoon et al., 1994).

Most of the currently available pesticides are nerve poisons that selectively attack a molecular target, such as the sodium channel and acetylcholinesterase (AChE), in the pest species and few of these alter the function of biogenic amines or their receptors. Amitraz is a formamidine acaricide that kills ticks and mites by irreversibly binding to the octopamine receptor, which leads to over excitation of the nervous system and eventual death (Evans and Gee, 1980). Amitraz also blocks female oviposition at sublethal doses in ticks (Booth, 1989). Pymetrozine is a novel insecticide that has been used to control whiteflies and aphids (Harrewijn and Kayser, 1997). Pymetrozine is believed to act on the serotonergic neuronal pathway that controls insect feeding behavior (Kaufmann et al., 2004). Given the serious problems associated with insecticide resistance in many of the economically important insect pests, new insecticides with novel modes of action are desperately needed in order to control resistant pests. Biogenic amine receptors are candidate targets to develop the next

generation of insecticides (Harrow et al., 1991; Ismail et al., 1996) for the control of economically damaging agricultural pests, including the stable fly.

The stable fly, *Stomoxys calcitrans* (L.) (Diptera: Muscidae), is one of the most economically important pests of livestock, particularly cattle in North America (Stork, 1979; Campbell et al., 1987; Taylor et al., 2012). Economic damage of the stable fly to cattle production in the United States is estimated at over two billion U.S. dollars annually (Taylor et al., 2012). The stable fly is difficult to control due to its feeding and reproductive behaviors. In addition, stable fly populations developed resistance to insecticides (Cilek and Greene, 1994; Marcon et al., 1997; Pitzer et al., 2010). It is necessary to understand the molecular and physiological mechanisms of key behaviors including feeding and reproduction in order to develop novel insecticides or behavioral disruptors for effective control of the stable fly (Bram, 1992).

Previous studies characterized the morphology and muscular organization of the male and female reproductive systems in the stable fly (Meola, 1988; Cook and Peterson, 1989). A physiological and pharmacological study on oviduct muscle demonstrated that octopamine inhibited oviduct muscle contraction in the stable fly (Cook and Wagner, 1992). The presence of serotonergic neurons in the central nervous system and the feeding system in the adult and larval stages of the stable fly was reported (Liu et al., 2011). Innervation of testes by serotonergic and octopaminergic neurons in adult male flies, and innervation of the ovary and oviduct only by octopaminergic neurons in female flies was observed (Li et al., unpublished). Therefore, the objective of the present study was to determine the functional roles of biogenic amines in reproductive behaviors of both male and female stable flies by using reserpine as a depleting agent of biogenic amines. The morphology of stable fly sperm was determined using two sperm staining techniques and it is reported here for the first time. These techniques were also used to determine rates of sperm transfer or successful mating by examining sperm in the testes of males and the spermatheca of females. The amine-depleting effect of reserpine was conclusively confirmed by a serotonin immunohistochemical technique.

2. Materials and methods

2.1. Insects

Adult stable flies used in this study were obtained from a laboratory colony maintained at the USDA, ARS, Knipling-Bushland U.S. Livestock Insects Research Laboratory, Kerrville, TX. Male and female flies were held in a screen cage maintained at 28 ± 1 °C, $60 \pm 1\%$ humidity, under photoperiod of 12:12 (L:D). Flies were fed daily by placing a cotton pad soaked with citrated bovine blood on the top of the screen. The citrated bovine blood contained sodium citrate (6.6 g/l), kanamycin sulfate (264 mg/l), and nystatin (264,000 units/l). Larvae were reared on Purina Fly Larva Media (PharmaServ, Gramingham, MA) covered with peanut hull pellets from a local livestock feed mill.

2.2. Generation of baseline data on blood feeding, oviposition, and adult fly mortality

Initial experiments were conducted to generate baseline data on blood feeding, oviposition, and adult fly mortality to set parameters for subsequent bioassays with reserpine.

Approximately 1000 adult flies emerging within a period of 24 h were set up in a cage for blood feeding bioassays. On the day of adult emergence (day 0), three groups of 10 males and 10 females were randomly chosen from the cage; each group represented one replicate. The flies in each group were transferred into one 20 ml glass vial to obtain pre-feeding fly weight using a precision analytical balance (A&D Company Ltd, Japan).

Flies sampled on a specific day were starved for 4 h prior to feeding. The flies were then transferred to a small feeding cage ($5.5 \times 3.0 \times 7.5$ cm) and a piece of socked blood pad was placed on top of the cage to allow flies to feed for 2 h. The post-feeding weight was obtained for each group of flies. The mean blood meal size (in mg) per fly was calculated. Flies were then returned to the holding cage and fed blood once a day for the duration of the experiment. Subsequently, adult flies were sampled at 2, 4, 6, 8 and 10 days post-emergence. For every sampling date, three groups, each containing 10 males and 10 females, were established.

Cumulative mortality of male and female flies as well as daily female oviposition was determined in another experiment. Ten newly emerged female and ten male flies were put together in a small cage ($5.5 \times 3.0 \times 7.5$ cm) after adult fly emergence. A total of five replicates were prepared. Paired flies in the small cages were fed daily using cow blood pad. Mortality of male and females flies was recorded daily for 22 days and dead flies were removed from the cages and counted. Eggs on the blood pad were also counted when blood pads were replaced. Cumulative mortality curves over time were generated for both male and females flies. The daily mean number of eggs laid per female was also calculated.

A second feeding experiment was conducted to determine the blood meal size for each sex. When adults reached the desired age (2 or 5 days old), a group of 30 flies of each sex was blood fed as previously described. Experiments were repeated three times to obtain the mean blood meal size for males and females of both age groups.

2.3. Sperm staining techniques for determination of sperm transfer

Two previously reported tissue staining techniques (Bertin et al., 2010; Twig and Yuval, 2005) were utilized in this study to determine mating status and sperm transfer success of male and female stable flies. Five days old flies were used to test and validate the sperm staining techniques. Approximately 15–20 female and male flies were removed from the colony. At least five virgin females and five males were selected by separating pupae before adult emergence. Using unmated flies ensured that no sperm was detected in the spermathecae of unmated females while sperm was observed in testes of all male flies.

The first technique involved the use of the Live/DeadTM Sperm Viability Dyes (Invitrogen, Eugene, OR, USA), which allowed visualization of sperm in the testes of male flies and spermathecae of female flies. Flies were chilled at 4 °C for 30 min before being pinned down in a Petri dish lined with black Silgard[®] silicone at the bottom. A volume of 10 ml cold stable fly physiological saline was added to the Petri dish before dissections started

under a dissection microscope (Wild Heerbrugg Switzerland). The stable fly saline contained 105 mM NaCl, 5 mM KNO₃, 5 mM CaCl₂, 3 mM MgSO₄ * 7H₂O, 10 mM L-histidine, 28 mM glucose (pH 6.8) (Cook, 1992). The “Component A” solution was freshly prepared by first adding 2 µl of SYBR 14 solution to 18 µl DMSO in a 2 ml Eppendorf tube. The solution was completed by adding 80 µl of the stable fly saline to the tube. Dissected fly tissues (testes and spermathecae) were transferred to a standard microscope slides (Platinum Line, Germany). A drop of the “component A” solution was added to the slide, after which tissues were ruptured using the tips of a pair of fine forceps. The tissue slides were incubated at 36 °C for 5–10 min. A drop of “component B” was then added to the tissues, and then the preparation was incubated 5–10 min at 36 °C. The “component B” solution was also prepared freshly before use by adding 1 µl of propidium iodide to 200 µl of the stable fly saline in 2 ml Eppendorf tube. After a cover slip was placed over the tissues, specimens on slides were examined under an Olympus BX60 fluorescence microscope (Olympus America Inc. Center Valley, PA. USA). Live (green) and dead (red) sperm can be differentiated qualitatively, and the images of sperm associated with testes and spermathecae were acquired using an Olympus DP70 digital camera attached to the microscope.

Sperm samples were prepared using a simpler one-step method that utilized a blue-fluorescent nucleic acid stain, 4',6-diamidino-2-phenylindole (DAPI) (Sigma–Aldrich, St. Louis, MO, USA) as mounting medium. Among all DAPI-stained cells individual sperm was clearly identified based on size and shape. Briefly, dissected tissues (testes and spermathecae) were transferred to a drop of DAPI on a glass slide. Tissues on slide were ruptured using the tips of a pair of fine forceps. After a cover slip was placed on top of the tissues, the slides were examined under a fluorescence microscope and digital images of stained sperm was obtained using microscope-mounted digital camera as described above.

DAPI staining technique was used to examine the spermathecae of female stable flies to determine if successful mating took place, which was recognized by sperm transfer when female flies were put together with males treated with different doses of reserpine through blood feeding. Baseline data on successful mating rates of untreated males and females at different days after adult emergence were generated by setting up a new cage of colony flies to assess the presence of sperm in the spermatheca of female flies on a daily basis for 7 days. A group of ten female flies were randomly taken from the cage each day. The DAPI staining technique was used to detect sperm in the spermatheca of each fly specimen. A female fly was determined as successfully mated when sperms were found in the spermatheca. Mating or sperm transfer success in each sample group was measured as the percentage of females that mated. Three groups of females were sampled each day, and the mean percentage of successful mating or sperm transfer was generated daily between one to 7 days post adult emergence.

2.4. Treatment of male flies with reserpine to determine effects on sperm transfer

Male and female stable flies were separated within 12 h after emergence into two separate cages. Cohorts of 2 or 5 days old male flies were treated by feeding them blood mixed with reserpine. Serial dilutions of technical grade reserpine (Sigma–Aldrich Co., Saint Louis, MO, USA) were made in dimethyl sulfoxide (DMSO) to generate 400 µl each of the

following reserpine concentrations: 82.14 mM (20 mg reserpine), 41.07 mM (10 mg reserpine), 20.53 mM (5 mg reserpine), and 10.27 mM (2.5 mg reserpine). On the day of the feeding experiment, a 50-fold dilution of reserpine was freshly made to feed flies by adding 0.2 ml each of the above reserpine–DMSO solutions to 9.8 ml of cow blood which generated the following reserpine testing concentrations: 1.64, 0.82, 0.41, and 0.21 mM. All reserpine-treated blood contained 2% DMSO.

On the day males were treated, the old blood pad was removed from the fly-holding cage to starve the flies for 4 h prior to feeding with reserpine-treated blood. Three small feeding cages ($5.5 \times 3.0 \times 7.5$ cm), representing three replicates, were prepared for each reserpine concentration. Ten starved male flies were placed into each cage before a piece of cotton pad (6.0×5.0 cm) soaked with reserpine-treated blood was placed on top of the cage to allow male flies to feed for 2 h. Reserpine doses were calculated based on reserpine concentration in blood and the mean blood meal size during 2-h period of feeding for each of the two age groups. Ten fed reserpine-treated males from each feeding cage were then transferred to a new cage, and 10 females of the same age were also added to the same cage. Flies in the new cages were fed by placing cotton pads soaked with untreated blood on cages daily till flies were 7 days old. At that time, mating status of the female was determined using the sperm staining techniques described in Section 2.3. Mortality was recorded daily.

Three untreated control groups of male flies were also prepared, including (1) males from the colony cage that were not separated from females (CT); (2) males that were separated from females at emergence and fed with normal blood on the day of treatment (C1); and (3) males that were separated from females at emergence and fed with blood that contained 2% DMSO on the day of treatment (C2). They were paired with fed but unmated females in the same way as described above to determine sperm transfer/successful mating rates.

2.5. Treatment of female flies with reserpine to determine its effects on oviposition

Female stable flies of the Kerrville laboratory colony start to lay eggs usually on day 6 post-emergence. The effect of reserpine on egg-laying behavior and egg hatch was assessed by feeding blood treated with reserpine to 5 days old females. Female flies were randomly chosen from a cage containing males and females of the same age that were maintained on untreated cow blood. Female flies were starved for 4 h prior to treatment. Dilutions of reserpine in DMSO and blood were prepared, and feeding of reserpine-treated blood to females was done as described above. Reserpine doses were calculated based on reserpine concentration in blood and the mean blood meal size during 2-h feeding period of 5 days old female flies. Following feeding, 10 treated females and 10 untreated males were placed in a test cage. Three cages prepared for each reserpine concentration represented three replicates. Control groups were females fed with untreated blood on the day of treatment (C1), and females fed with cow blood containing 2% DMSO (C2). Three replicates were also prepared for each of the control groups. All female flies in the control groups were fed untreated blood daily. Females laid eggs on the blood pad. Eggs were collected from blood pads that were replaced daily, and the number of eggs on the blood pad of each fly cage was counted daily for 11 days. Mortality was assessed daily. Eggs on blood pads were placed in Petri dishes and incubated at 28 ± 1 °C, 60 ± 1 % RH, and a 12:12 L:D cycle for 72 h before

examination for larval hatching under a dissection scope. The mean egg number/female and mean egg hatch rate in each treatment group were calculated using data obtained during the 11-day collection period.

2.6. Determination of amount of reserpine ingested by male and female flies

Because the blood meal size of both male and female stable flies changed with age, male or female flies may have ingested different amounts of reserpine at different ages when different concentrations of reserpine in blood were fed to the flies. The exact amount of reserpine male or female flies ingested through blood feeding at different ages (2 and 5 days old) was calculated using the following formula:

$$RI (\mu\text{g}) = R (\text{mg/ml})/[B (\text{mg})/BW (\text{g/ml})] \times 1000;$$

where, RI is reserpine (μg) ingested per fly, R is reserpine concentration in blood (mg/ml), B is mean weight (mg) of blood ingested per fly, and BW is blood weight (mg) per ml.

2.7. Serotonin immunohistochemistry

Observations on serotonin immunoreactivity in the stable fly indicated male testes are heavily innervated by serotonergic neuronal processes (Li et al., unpublished). Therefore, this reproductive organ was the target to test the effect of reserpine treatment on serotonin immunoreactivity in male stable flies. Approximately 1000 stable fly pupae were set in a cage to allow adult emergence. Two hundred males that emerged within a period of 24 h were used for this experiment. The male flies were transferred into a new cage and fed daily with untreated cow blood. They were divided into three groups with 50 flies in each group when they were 5 days old. Flies in each group were placed in a separate feeding cage ($5.5 \times 3.0 \times 7.5$ cm) and fed for 2 h one of three types of blood: (1) blood with 2% DMSO, (2) blood with 0.21 mM reserpine, and (3) blood with 0.82 mM reserpine. The estimated reserpine dose in the reserpine-treated group was 0.5 and 2.1 $\mu\text{g}/\text{fly}$, respectively. A subset of flies (7–10 males) was randomly selected from each treatment group at 1, 3, and 5 days posttreatment for immunohistology to examine the profile of serotonin immunoreactivity in testes.

The protocol used to detect serotonin immunoreactivity in testes of males stable flies was modified from Davis et al. (1996) as described previously (Hummel et al., 2007; Siju et al., 2008; Liu et al., 2011). Briefly, paired testes were dissected from each of the male flies and fixed in 4% paraformaldehyde at 4 °C overnight. After multiple washes in stable fly saline, the tissues were blocked in 5% normal donkey serum. The tissues were incubated overnight at room temperature in a 1:1500 dilution of the primary antibody (rabbit anti-serotonin; Immunostar Inc., Hudson, WI, USA). The tissues were blocked in 5% normal donkey serum for a second time, and then washed in stable fly saline before being incubated in a 1:400 dilution of the secondary antibody (donkey anti-rabbit Alexa Fluor 488; Invitrogen, Carlsbad, CA, USA). Donkey anti-rabbit Alexa Fluor 488 was used as the secondary antibody for detecting serotonin, and Fluor 546 Phalloidin (Invitrogen, Carlsbad, CA) was added at the same time for detecting F-actin labeling associated with muscles in testes (Liu et al., 2011). Tissues were dehydrated in a graded ethanol solution and cleared in and mounted in glycerol before examination under an Olympus BX60 fluorescence microscope.

(Olympus America Inc., Center Valley, PA). A testis tissue was designated as positive for serotonin IR if a clear serotonin immunoreactive neuronal process was detected. Positive testis tissues were further imaged using a Zeiss LSM 510 laser scanning confocal microscope (Carl Zeiss Microimaging, Inc., Thornwood, NY) to generate high quality images for publication.

2.8. Statistical analysis

The mean responses of different treatments were analyzed by using the General Linear Models procedure (SAS Institute, 2004). All means were subjected to analysis of variance (ANOVA) followed by a Tukey's Studentized Range test for mean comparisons. In order to stabilize variances, data were transformed by square root of the number plus 0.5 before ANOVA. Non-transformed means and standard errors are reported too. Significant differences among treatments were accepted when $P < 0.05$.

3. Results

3.1. Base line data for adult stable flies from laboratory colony

Changes in the daily mean blood meal size for male and female stable flies are shown in Fig. 1A. Newly emerged adult flies consumed 0.9 ± 0.1 mg of blood per feeding. Blood meal size increased significantly to 4.7 ± 0.2 mg of blood per feeding when flies reached 2 days old. Flies 4 days old ingested the largest amount of blood (5.8 ± 0.2 mg of blood per feeding). Blood meal size decreased significantly thereafter. The mean blood meal size for 2 and 5 days old male flies was 1.5 ± 0.2 and 4.2 ± 0.4 mg of blood per feeding, respectively. In contrast, 5 days old female flies consumed and 5.9 ± 0.1 mg of blood per feeding.

Female stable flies started to lay eggs on day 6 post-emergence (Fig. 1B). At peak, which occurred on the 8th day after emergence, 23 eggs were laid per female (Fig. 1B). Subsequently, the number of eggs laid per female fluctuated through day 13. The last peak of oviposition occurred 20 days post-emergence. Virgin females also laid eggs, but the onset of oviposition was protracted where egg laying started on the 11th day post-emergence. Male flies started to die when they reached 6 days old whereas females began to die 10 days after emergence. Males showed 94.0% mortality on the 14th day, while females of the same age showed 36.0% mortality (Fig. 1C).

3.2. Female mating rates and detection of sperm in spermathecae and testes

Initial experiments to validate staining techniques showed that sperm was detected in testes and spermathecae of males and females, respectively, when older flies (4–5 days old) were sampled. The Live/Dead Sperm Viability staining technique was tested using the dyes SYBR 14 (green) and propidium iodide (red). Live and dead spermatozoa were shown clearly as green and red colors, respectively, in testes (Fig. 2A) and spermathecae (Fig. 2C) according to the dye used to assess viability. The SYBR 14 dye turned the heads of live sperm to green indicating that plasma membranes were intact. Dead sperm had the plasma membrane damaged. The red dye propidium iodide penetrated the damaged membranes and turned the sperm red (Fig. 2C). However, the staining of sperm using the Live/Dead Sperm Viability dyes was temporary. The quality of staining preparations deteriorated after 1–2 h

when most sperm turned red, and cells, including sperm, on slides disintegrated after 4–8 h. Stable fly sperm was also detected in stable fly testes and spermathecae (Fig. 2B) using the DAPI staining technique. Sperm can be identified reliably based on unique shape and size features. The average head length of stable fly sperm was $19.0 \pm 0.4 \mu\text{m}$ ($n = 10$, Fig. 2D), and the average tail length of stable fly sperm was $166.6 \pm 4.7 \mu\text{m}$ ($n = 10$, Fig. 2E). In contrast to the Live/Dead Sperm Viability dyes, DAPI staining was stable and sperm structure and color were preserved on slides for days, even weeks.

The percentages of females that mated successfully at different days post-emergence are shown in Fig. 3. A female was determined as mated when sperm was detected in its spermatheca. No sperm was detected in females that were 3 days old or younger, which suggests that no successful mating took place in younger flies. Predisposition to mate was first observed in flies that were 4 days old, when sperm was detected on average in 42.9% of the females. The mean percentage of mated females increased to 69.5, 85.6, and 96.7% when flies reached 5, 6 and 7 days old, respectively (Fig. 3).

3.3. Treatment with reserpine affects sperm transfer rate

Two days old males fed blood containing reserpine at 0.21, 0.41, 0.82, or 1.64 mM resulted in the ingestion of 0.2, 0.4, 0.8 and 1.5 μg reserpine/fly, respectively (Fig. 4A). The amount of reserpine ingested by 5 days old males fed the same reserpine concentrations in blood was 0.5, 1.0, 2.1 and 4.1 μg reserpine/fly, respectively (Fig. 4B).

A significant decrease in mating success was observed in 2 days old males, as indicated by the percentage of females having sperm in their spermathecae, when each male ingested 1.5 μg of reserpine ($F_{6, 14} = 8.12$, $P = 0.0006$; Fig. 4A). Lower ingestion rates (0.8 and 0.4 μg /fly) reduced sperm transfer or successful mating rate, but the changes were not statistically significant. The lowest reserpine dose (0.2 μg /fly) did not have any effect when compared with all three controls. Treatment with DMSO did not have any negative effect on mating success or sperm transfer. In contrast, the ingestion of blood containing reserpine by 5 days old males had a significant effect on sperm transfer rate. In contrast, treatment of males with reserpine significantly reduced mating success or sperm transfer at all reserpine concentrations ($F_{6, 14} = 21.64$, $P < 0.0001$; Fig. 4B). Successful mating was totally abolished at the highest reserpine dose (4.1 μg /fly).

3.4. Biogenic amine depletion with reserpine and its effect on female reproduction

Five days old females fed blood containing reserpine at 0.21, 0.41, 0.82, and 1.64 mM ingested 0.7, 1.4, 2.9 and 5.8 μg of the compound/fly, respectively (Fig. 5). The mean mortality of females in reserpine-treated groups ranged from 40.0% to 40.7%, which are not significantly different from the two control groups (C1 = 33.0%, C2 = 36.7%) during the 11 days experimental period ($F_{5, 12} = 19.12$, $P = 0.7570$).

Reserpine treatment had a significant negative effect on oviposition as indicated by the reduced numbers of egg laid by females in all reserpine-treated groups ($F_{5, 12} = 0.52$, $P < 0.0001$; Fig. 5A). The mean number of eggs per female in the untreated control (C1) and the group treated with 2% DMSO (C2) was 174.6 ± 6.7 and 161.4 ± 22.7 , respectively. The

mean number of eggs per female dropped significantly to 82.2, 45.0, 49.9, and 40.8 when each fly ingested 0.7, 1.4, 2.9, and 5.8 μg of reserpine, respectively. Overall, the reduction of oviposition (eggs/female) due to reserpine treatment ranged from 49.1% to 74.7%. However, reserpine treatment of 5 days old females had no effect on egg-hatching rates in any of the treatment groups ($F_{5, 12} = 1.36$, $P = 0.3072$; Fig. 5B).

3.5. Reserpine causes transient depletion of serotonin from neuronal processes innervating testes

A normal serotonergic neuronal innervation pattern of testes was observed in 100% of the male flies ($n = 19$) examined in the DMSO-treated control group (Figs. 6A and 7). Serotonin immunoreactive signal in flies fed blood containing 2% DMSO was virtually the same as for males fed normal blood. However, the same serotonin immunoreactive neuronal processes were missing from the testes in 98.0% of the males ($n = 51$) treated with reserpine at the low (0.5 $\mu\text{g}/\text{fly}$) and high doses (2.1 $\mu\text{g}/\text{fly}$) at 1, 3, and 5 h postreserpine treatment (Figs. 6B–D and 7). Serotonin immunoreactive neuronal processes were observed again in the testes of 33.3% of reserpine-treated males ($n = 12$) at 1 day post-treatment (Figs. 6E and 7). At 3 days post-treatment, serotonergic neuronal processes were detected in the testes from 94% of the males ($n = 18$) examined (Figs. 6F and 7). Serotonin depletion by reserpine was evident not only for the fine mesh-like serotonergic neuronal processes that innervate the testes, but also for the nerve bundles that innervate the entire male reproductive system. Serotonin immunoreactive signal was not observed in the nerve bundle innervating testes at 1–5 h post-treatment. Serotonin IR signals reappeared in the nerve bundle at 1 day post-treatment with an intensity similar to that observed in flies fed with DMSO-treated blood (data not shown).

4. Discussion

The amine-depletion effects of reserpine on reproduction and serotonin IR of the stable fly are reported here for the first time. Baseline parameters established to conduct the studies with reserpine indicated that the blood meal size of newly emerged flies was relatively low (~ 1.0 mg/fly), but blood meal size increased significantly with age. Blood meal size peaked at ~ 6.0 mg/fly when flies were 4 days old and decreased thereafter to approximately 2.0 mg/fly when they were 10 days old. Similar results were reported by Venkatesh and Morrison (1980), although the overall blood meal sizes they reported were higher possibly due to larger fly size. Female stable flies are known to have multiple peaks of blood meal size and can lay multiple batches of eggs during their lifetime (Chia, 1978; Venkatesh and Morrison, 1980). The multiple peaks of oviposition over a three week period noted in this study may reflect the blood feeding pattern of mated females.

Results from sperm staining experiments showed that approximately 40.0% of the females were mated by the time they were 4 days old. The results from this study concur with previous findings by Chia (1978) in that male and female flies required to feed on blood for 3 days before insemination of females was observed. Foil and Hogsette (1994) also demonstrated that female stable flies started mating when they are 3 days old. Over 90% of the flies were mated at 7 days post-emergence; this observation suggests that a direct

correlation exists between insemination rates and the age of female flies. The two sperm staining dyes used in this study allowed direct visualization of sperms in stable fly testes and spermathecae, which was the basis for determining the insemination status of female flies. To the authors' knowledge, this constitutes the first report of stable fly sperm documented with images.

Reserpine treatment of male flies had significant negative effects on sperm transfer and eventually mating success. This effect appeared to be more age-dependent than dose-dependent. When 2 days old males were treated with reserpine, there was a 9.4–55.3% decrease in sperm transfer when compared to the controls. However, the effect of reserpine was most significant at all reserpine doses (0.5–4.1 µg/fly) when 5 days old males were treated, which resulted in 75.7–100.0% reduction in sperm transfer when compared to the controls. Similarly, reserpine treatment of 5 days old females resulted in 49.0–74.4% reduction in the mean number of eggs laid per female. Oviposition was not totally inhibited even at the highest reserpine dose (5.8 µg/fly). All reserpine doses led to significant reduction in oviposition and there was not a clear dosedependent effect. The data suggest that a significant negative effect on female oviposition can be observed when reserpine dose reaches a certain threshold. In contrast, reserpine treatment of 5 days old females did not have any effect on egg hatch rate. It is possible that reserpine treatment interrupted not only the neuromuscular activity involved in oviposition, but also other physiological processes, such as vitellogenesis and oocyte development in female stable flies, which remain to be investigated. Reserpine has been reported previously to severely disrupt mating and oviposition in female house flies and the Mediterranean fruit fly (Hays and Amerson, 1967; Wicht and Hays, 1967; Cohen, 1983). House fly eggs were found to develop normally in the ovary of the treated females (Hays et al., 1969). By comparison, reserpine inhibition of vitellogenesis and oocyte development resulted in significantly reduced fecundity and fertility in tenebrionid beetles and fruit flies (Masner et al., 1970; Cohen, 1983).

Results from reserpine treatment of 5 days old female stable flies by feeding and subsequent serotonin immunohistochemistry analysis at different times post-treatment indicated that serotonin was depleted from the central nervous system (not shown) and neuronal processes innervating the testes. The serotonin-depleting effect of reserpine was observed within 1 h of treatment and serotonin IR in neuronal processes was undetected in approximately 98% of the samples examined 5 h post-treatment. In contrast, serotonin IR was detected in 33% of males examined 1 day following treatment and full recovery was observed in 94% of the flies at 3 days after treatment. Previous studies on reserpine used electrochemical detection methods to examine the biogenic amine contents in tissues of the central nervous system in insects (Sloley and Owen, 1982; Brookhart et al., 1987). Reserpine at a dose of 5 µg per fly caused 70% depletion of dopamine and >90% depletion of both serotonin and octopamine 24 h after injection in the black blow fly (*Phormia regina* Meigen) (Brookhart et al., 1987). In contract to findings in the black blow fly, the effect of reserpine was much faster in onset and less persistent in the stable fly.

Findings on the depletion and recovery of serotonin IR in neuronal processes that innervate the testes of male stable flies reported here could be used for comparative analysis of reproductive biology in dipteran species. For example, the effect of serotonin exhaustion on

sperm transfer or successful mating provides evidence supporting the hypothesis that the reproductive anomalies in the fruitless-mutant of *D. melanogaster* may be associated with a lack of serotonergic neuronal processes that innervate contractile muscles of the male mutant testes (Lee et al., 2001).

Morphological observations obtained through immunohistochemical analysis could be related to results from the behavioral experiments. Although untreated females may be ready to mate, a successful sperm transfer from treated males was not achieved. It remains to be determined if depletion of serotonin and other biogenic amines by reserpine abolished the sequence of mating behaviors totally or only blocked sperm transfer during mating. A near total recovery of serotonin immunoreactivity in testes of the male flies was observed at 3 days after reserpine treatment, but it is unclear if a full recovery of mating and other behaviors were achieved in the same time period. However, 2 days old males had time to recover from the amine-depleting effect of reserpine. That was probably why relatively increased mating and higher sperm transfer rate were observed in 2 days old males as compared to 5 days old males upon treatment with similar reserpine doses. It is hypothesized that a lack of serotonin in nerves mediating testes function locally caused mating failure and the eventual reduction of sperm transfer by male flies.

Reserpine is known to deplete a number of biogenic amines in insects and other invertebrates (Sloley and Owen, 1982; O’Gara et al., 1991). Octopaminergic neurons and neuronal processes have been detected in the central nervous system (CNS) and the reproductive system of adult stable flies, specifically the testes of males and ovary and oviduct of females (Li et al., unpublished). Serotonergic neurons and neuronal processes have been detected in the CNS (Liu et al., 2011) and testes of adult stable flies (Current study). However, no serotonergic neuronal processes were detected in the ovaries and oviduct (Li et al., unpublished). This study documented the depleting effects of reserpine on serotonin immunoreactivity in testes of males and corresponding behavioral effect on successful mating. The presence of octopamine in the nervous system of testes suggests this biogenic amine may also be involved in stable fly mating behavior by influencing sperm transfer. Similarly, the effect of reserpine on female oviposition was likely achieved by depletion of the neurotransmitter octopamine from the octopaminergic neurons that innervate the ovary and oviduct. The results from this study provide evidence supporting the hypothesis that the mode of action of reserpine in disrupting insect behaviors is most likely associated with depletion of biogenic amine neurotransmitters (Alpers and Shore, 1969; Stitzel, 1977). Further pharmacological experiments are needed to determine conclusively the roles of serotonin, octopamine, and possibly other neurotransmitters in mating and oviposition behaviors of male and female stable flies. Testing the effects of specific agonist and antagonist compounds at anatomical and physiological levels will help determine how these neurotransmitters influence specific behaviors in the stable fly.

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References

- Alpers HS, Shore PA. Specific binding of reserpine – association with norepinephrine depletion. *Biochemical Pharmacology*. 1969; 18:1363–1372. [PubMed: 5799110]
- Bertin S, Scolari F, Guglielmino CR, Bonizzoni M, Bonomi A, Marchini D, Gomulski LM, Gasperi G, Malacrida AR, Matessi C. Sperm storage and use in polyandrous females of the globally invasive fruit fly, *Ceratitis capitata*. *Journal of Insect Physiology*. 2010; 56:1542–1551. [PubMed: 20466005]
- Blenau W, Baumann A. Molecular and pharmacological properties of insect biogenic amine receptors: lessons from *Drosophila melanogaster* and *Apis mellifera*. *Archives of Insect Biochemistry and Physiology*. 2001; 48:13–38. [PubMed: 11519073]
- Booth TF. Effects of biogenic amines and adrenergic drugs on oviposition in the cattle tick *Boophilus*: evidence for octopaminergic innervation of the oviduct. *Experimental and Applied Acarology*. 1989; 7:259–266.
- Bram, RA. Current and future status of research on stable flies. In: Thomas, GD.; Skoda, SR., editors. *The Stable Fly: A Pest of Humans and Domestic Animals*; Proceedings of ESA; Baltimore, MD. Dec. 1992 1992 p. 146-148.
- Brookhart GL, Edgecomb RS, Murdock LL. Amphetamine and reserpine deplete brain biogenic amines and alter blow fly feeding behavior. *Journal of Neurochemistry*. 1987; 48:1307–1315. [PubMed: 3102695]
- Campbell JB, Berry IL, Boxler DJ, Davis RL, Clanton DC, Deutscher GH. Effects of stable flies (Diptera: Muscidae) on weight gain and feed efficiency of feedlot cattle. *Journal of Economic Entomology*. 1987; 80:117–119. [PubMed: 3571695]
- Chia, LS. Studies on female reproductive physiology in the stable fly, *Stomoxys calcitrans* (L.). University of Waterloo; Waterloo, Ontario, Canada: 1978. M.Sc. Thesis
- Cilek JE, Greene GL. Stable fly (Diptera: Muscidae) insecticide resistance in Kansas cattle feedlot. *Journal of Economic Entomology*. 1994; 87:275–279.
- Clark J, Lange AB. The neural control of spermathecal contractions in the locust, *Locusta migratoria*. *Journal of Insect Physiology*. 2000; 46:191–201. [PubMed: 12770251]
- Clark J, Lange AB. The association of serotonin with the spermatheca of the locust, *Locusta migratoria*. *Biogenic Amines*. 2002; 17:43–60.
- Clark J, Lange AB. Octopamine modulates spermathecal muscle contractions in *Locusta migratoria*. *Journal of Comparative Physiology A*. 2003; 189:105–114.
- Cohen E. Effect of reserpine on reproduction of the Mediterranean fruit fly, *Ceratitis capitata* L. *Zeitschrift fur Angewandte Entomologie*. 1983; 96:99–104.
- Cook BJ. The oviduct musculature of the stable fly *Stomoxys calcitrans*: properties of its spontaneous motility and neural regulation. *Archives of Insect Biochemistry and Physiology*. 1992; 19:119–132.
- Cook BJ, Peterson T. Ovarian muscularis of the stable fly *Stomoxys calcitrans*: its structural, motile, and pharmacological properties. *Archives of Insect Biochemistry and Physiology*. 1989; 12:15–30.
- Cook BJ, Wagner RM. Some pharmacological properties of the oviduct muscularis of the stable fly *Stomoxys calcitrans*. *Comparative Biochemistry and Physiology C*. 1992; 102:273–280.
- da Silva R, Lange AB. Tyramine as a possible neurotransmitter/neuromodulator at the spermatheca of the African migratory locust, *Locusta migratoria*. *Journal of Insect Physiology*. 2008; 54:1306–1313. [PubMed: 18675822]
- Davis NT, Hildebrand JG, Homberg U, Agricola H-J, Teal PEA, Altstein M. Euroanatomy and immunocytochemistry of the median neuroendocrine cells of the subesophageal ganglion of the tobacco hawkmoth, *Manduca sexta*: immunoreactivities to PBAN and other neuropeptides. *Microscopy Research and Technique*. 1996; 35:201–229. [PubMed: 8956271]
- Donini A, Lange AB. Evidence for a possible neurotransmitter/neuromodulator role of tyramine on the locust oviducts. *Journal of Insect Physiology*. 2004; 50:351–361. [PubMed: 15081828]

- Evans PD. Biogenic amines in the insect nervous system. *Advances in Insect Physiology*. 1980; 15:317–473.
- Evans PD, Gee JD. Action of formamidine pesticides on octopamine receptors. *Nature*. 1980; 287:60–62. [PubMed: 6251379]
- Foil LD, Hogsette JA. Biology and control of Tabanids, stable flies and horn flies. In *Ectoparasites of animals and control methods*. Scientific and Technical Review International Office of Epizootics. 1994; 13:1125–1158.
- Harrewijn P, Kayser H. Pymetrozine, a fast-acting and selective inhibitor of aphid feeding. In-situ studies with electronic monitoring of feeding behaviour. *Pesticide Science*. 1997; 49:130–140.
- Harrow ID, Gratton KAF, Evans NA. Neurobiology of arthropod parasites. *Parasitology*. 1991; 102:S59–S69. [PubMed: 1676150]
- Hays SB, Amerson GM. Reproductive control in the house fly with reserpine. *Journal of Economic Entomology*. 1967; 60:781–783. [PubMed: 6042081]
- Hays SB, Hays RL, Mims IS. Comparative effects of reserpine and serotonin creatine sulfate on oviposition in the house fly. *Annals of the Entomological Society of America*. 1969; 62:663–664.
- Hummel NA, Li AY, Witt CM. Serotonin-like immunoreactivity in the central nervous system of two ixodid tick species. *Experimental and Applied Acarology*. 2007; 43:265–278. [PubMed: 18040871]
- Ismail SMM, Baines RA, Downer GH. Dihydrooxadiazines: octopamineergic system as a potential site of insecticidal action. *Pesticide Science*. 1996; 46:163–170.
- Kaufmann L, Schürmann F, Yiallourous M, Harrewijn P, Kayser H. The serotonergic system is involved in feeding inhibition by pymetrozine. Comparative studies on a locust (*Locusta migratoria*) and an aphid (*Myzus persicae*). *Comparative Biochemistry and Physiology C Toxicology and Pharmacology*. 2004; 138:469–483.
- Lange AB. A neurohormonal role for serotonin in the control of locust oviducts. *Archives of Insect Biochemistry and Physiology*. 2004; 56:179–190. [PubMed: 15274179]
- Lange AB, Orchard I, Laughton BG. Neural inhibition of egg-laying in the locust *Locusta migratoria*. *Journal of Insect Physiology*. 1984; 30:271–278.
- Lee G, Villella A, Taylor BJ, Hall JC. New reproductive anomalies in *fruitless*-mutant *Drosophila* males: extreme lengthening of mating durations and infertility correlated with defective serotonergic innervation of reproductive organs. *Journal of Neurobiology*. 2001; 47:121–149. [PubMed: 11291102]
- Lee HG, Seong CS, Kim YC, Davis RL, Han KA. Octopamine receptor OAMB is required for ovulation in *Drosophila melanogaster*. *Developmental Biology*. 2003; 264:179–190. [PubMed: 14623240]
- Liu SS, Li AY, Witt CM, Pérez de León AA. Immunohistological localization of serotonin in the CNS and feeding system of the stable fly *Stomoxys calcitrans* L. (Diptera: Muscidae). *Archives of Insect Biochemistry and Physiology*. 2011; 77:199–219. [PubMed: 21678485]
- Marcon PCRG, Thomas GD, Siegfried BD, Campbell JB. Susceptibility of stable flies (Diptera: Muscidae) from southern Nebraska beef cattle feedlots to selected insecticides and comparison of 3 bioassay techniques. *Journal of Economic Entomology*. 1997; 90:293–298. [PubMed: 9145029]
- Masner P, Hout L, Corriveau GW, Prudhomme JC. Effect of reserpine on the function of the gonads and its neuro-endocrine regulation in tenebrionid beetles. *Journal of Insect Physiology*. 1970; 16:2327–2344.
- Menzel R, Heyne A, Kinzel C, Gerber B, Fiala A. Pharmacological dissociation between the reinforcing, sensitizing and response releasing functions of reward in honeybee classical conditioning. *Behavioral Neuroscience*. 1999; 113:744–754. [PubMed: 10495082]
- Meola SM. Neurosecretory innervation of the accessory gland region of the male stable flies, *Stomoxys calcitrans*. *International Journal of Invertebrate Reproduction and Development*. 1988; 13:105.
- Monastirioti M. Distinct octopamine cell populations residing in the CNS abdominal ganglion controls ovulation in *Drosophila melanogaster*. *Developmental Biology*. 2003; 264:38–49. [PubMed: 14623230]
- O’Gara BA, Chae H, Latham LB, Friesen WO. Modification of leech behavior patterns by reserpine-induced amine depletion. *Journal of Neuroscience*. 1991; 11:96–110. [PubMed: 1986071]

- Orchard I, Lange AB. Pharmacological profile of octopamine receptors on the lateral oviducts of the locust *Locusta migratoria*. *Journal of Insect Physiology*. 1986; 32:741–745.
- Pitzer JB, Kaufman PE, Tenbroeck SH. Assessing permethrin resistance in the stable fly (Diptera: Muscidae) in Florida by using laboratory selections and field evaluations. *Journal of Economic Entomology*. 2010; 103:2258–2263. [PubMed: 21309252]
- SAS Institute. The SAS for Windows, Version 9.1.3. SAS Institute; Cary, NC, USA: 2004.
- Siju KP, Hansson BS, Ignell R. Immunocytochemical localization of serotonin in the central and peripheral chemosensory system of mosquitoes. *Arthropod Structure & Development*. 2008; 37:248–259. [PubMed: 18424232]
- Sloley BD, Orikasa S. Selective depletion of dopamine, octopamine, and 5-Hydroxytryptamine in the nervous tissues of the cockroach (*Periplaneta americana*). *Journal of Neurochemistry*. 1988; 51:535–541. [PubMed: 3134509]
- Sloley BD, Owen MD. The effects of reserpine on amine concentrations in the nervous system of the cockroach (*Periplaneta americana*). *Insect Biochemistry*. 1982; 12:469–476.
- Stevenson PA, Hofmann H, Schoch K, Schildberger K. The fight and flight responses of crickets depleted of biogenic amines. *Journal of Neurobiology*. 2000; 43:107–120. [PubMed: 10770840]
- Stitzel RE. The biological fate of reserpine. *Pharmacological Reviews*. 1977; 28:179–205. [PubMed: 16280]
- Stork MG. The epidemiological and economic importance of fly infestation of meat and milk producing animals in Europe. *Veterinary Record*. 1979; 105:341–343. [PubMed: 552724]
- Taylor DB, Moon RD, Mark DR. Economic impact of stable flies (Diptera: Muscidae) on dairy and beef cattle production. *Journal of Medical Entomology*. 2012; 49:198–209. [PubMed: 22308789]
- Tennakoon PK, Srikrishnaraj KA, Ramasamy MS. Reserpine induced effects on growth and reproduction of *Culex quinquefasciatus* mosquitoes (Diptera: Culicidae). *Journal of the National Science Council of Sri Lanka*. 1994; 22:213–218.
- Twig E, Yuval B. Function of multiple sperm storage organs in female Mediterranean fruit flies (*Ceratitis capitata*, Diptera: Tephritidae). *Journal of Insect Physiology*. 2005; 51:67–74. [PubMed: 15686648]
- Venkatesh K, Morrison PE. Some aspects of oogenesis in the stable fly *Stomoxys calcitrans* (Diptera: Muscidae). *Journal of Insect Physiology*. 1980; 26:711–715.
- Vieira R, Mancebo MJ, Aldegunde M. Effects of reserpine and *p*-chloroamphetamine on 5-HT metabolism and release in the cerebral ganglia of *Inachis io* (Lepidoptera). *Journal of Physiology and Biochemistry*. 2007; 63:129–142. [PubMed: 17933387]
- Wicht MC Jr, Hays SB. Effect of reserpine on reproduction of the house fly. *Journal of Economic Entomology*. 1967; 60:36–38.
- Yamane T, Miyatake T. Reduced female mating receptivity and activation of oviposition in two *Callosobruchus* species due to injection of biogenic amines. *Journal of Insect Physiology*. 2010; 56:271–276. [PubMed: 19895815]

Fig. 1.

Baseline data for blood feeding, oviposition, and mortality in colonized stable flies. (A) Mean blood meal size (mg) during 2 h of feeding measured in groups of 10 males and 10 females ($n = 3$). (B) Mean number of eggs laid per female during the 22-day test period ($n = 5$). (C) Mean cumulative mortality of male and female flies ($n = 5$). Standard error is shown for each mean.

Fig. 2.

Visualization of stable fly sperm using two tissue staining techniques. (A) sperms from a testis stained using Live/Dead™ Sperm Viability dyes. (B) sperms in spermatheca of a female prepared in 4',6-diamidino-2-phenylindole (DAPI) mounting medium. (C) Sperms in spermatheca stained with Live/Dead™ Sperm Viability dyes. (D) heads and tails of stable fly sperms stained with Live/Dead™ Sperm Viability dyes. (E) picture showing autofluorescence of stable fly sperms.

Fig. 3.

Mean percentage of females with sperms detected in the spermathecae at different days post adult emergence. Means with different letters are significantly different (Tukey test, $P < 0.05$). $F_{6, 14} = 318.98$, $P < 0.0001$. Standard error is shown for each of the means.

Fig. 4.

Dose-dependent effect of reserpine treatment of 2 days old (A) and 5 days old (B) male flies on sperm transfer rate, which was the percentage of females containing sperms. CT: Control – untreated males from colony cohort. C1: Control 1 – males experimentally handled but otherwise untreated. C2: Control 2 – males treated with 2% DMSO. R: reserpine treatment ($\mu\text{g}/\text{fly}$). Means with different letters are significantly different (Tukey test, $P < 0.05$). Standard error is shown for each mean.

Fig. 5.

Effects of reserpine treatment of 5 days old females on fecundity (A) and egg hatch rate (B). (A) Mean number of egg ($\pm$ stderr) laid per female during the 11-day test period. (B) Mean egg hatch rate in different treatment groups. C1: Control 1 – untreated females. C2: Control 2 – females treated with 2% DMSO. R: reserpine treatment (μ g/fly). Means with different letters are significantly different (Tukey test, $P < 0.05$). Standard error is shown for each mean.

Fig. 6.

Visualization of serotonergic innervations in stable fly testes and transient serotonin depletion induced by reserpine treatment. Confocal Z-projections (the top half of each testis) showing serotonin/F-actin double labeling in whole mounts of testes in the DMSO (2%)-treated and reserpine-treated 5 days old male flies. A: Serotonin immunoreactive neuronal innervations in fly testis treated with 2% DMSO only (control). (B–D) Complete depletion of serotonin IR in fly testes treated with reserpine at 1, 3 and 5 h post-treatment, respectively. E and F: Recovery of serotonin IR in testes of male flies treated with reserpine at 1 and 3 days post-treatment, respectively.

Fig. 7.

Transient depletion of serotonin in testicular nerves of 5 days old male stable flies fed blood containing reserpine. The percentages of males with positive and negative serotonin immunoreactive staining of the neuronal processes associated with the testes are shown for the DMSO (2%)-treated group (control) and in males treated with reserpine (0.5–2.1 $\mu\text{g}/\text{fly}$) at different times (hour, h, and day, D) posttreatment.