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Elucidating the functional role of *olf413* gene in fertility and primary olfaction in *Drosophila melanogaster*

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Abstract
The olfactory system is an animal's capacity to identify and respond to odours, which might indicate the proximity of food or danger. Therefore, life-sustaining qualities like organismal lifespan and food consumption can be affected by organisms' appropriate behavioural reactions to these chemical signals. However, it is still difficult to completely decipher the genetic processes that underlie odour-guided behaviour, the corresponding responses in other characteristics, and the ways in which these either limit or drive their evolution. In order for the organism to engage with their surroundings, they need sensory systems. Sensory detection and subsequent processing of environmental signals govern a wide range of behaviours, including flight from a predator, courtship, and foraging. In the case of olfaction, organisms employ both unpleasant and desirable smell signals to identify and assess potential food sources. Further an animal's overall fitness is heavily dependent on its reproductive success. *Drosophila melanogaster* fertility and fecundity is commonly evaluated experimentally as the number of viable eggs produced as an indicator of reproductive success. The ability of a female to generate eggs is only one factor in reproductive success, but it is crucial since it limits how many offspring the female may have. Genetic basis and network underlying these adaptive traits are complex and very little understood.
In this study, we have investigated the role of gene *olf413*, a paralogue of the *TβH* gene in controlling these two important fitness traits: primary olfaction, and fertility. Our findings indicate that the loss of function of the *olf413* gene, caused by *olf413* knockdown, does not significantly affect primary olfactory response. However, we observed that the loss of function of the *olf413* gene results in sterility in both males and females.

Keywords: *olf413, TβH, Drosophila melanogaster*, sterility, primary olfaction

Introduction

Insects use their keen odour sensing capability to choose safe places to deposit their eggs, obtain food, and distinguish between interspecies mating. These olfactory-driven actions are triggered by mixtures of volatile organic molecules that vary in size, shape, charge, and functional groups. How the brain interprets odours from the environment, even those that are complicated and often conflicting, remains an unanswered question about the olfactory system. *Drosophila melanogaster* has been a popular research model for sorting out the genetics behind olfactory cue perception and discrimination [1, 2]. Olfactory sensory neurons (OSNs) on antennal 3rd segment or the maxillary palp express odorant receptors that allow for the detection of odorants. A unique odorant receptor (OR) type is normally expressed by each OSN, and axons from each OSN go to a separate glomerulus in the antennal lobe [3-7]. These odorant receptors, which make up a family of 60 genes, are thought to generate ligand-gated ion channels [4, 9-12] with the help of the highly conserved coreceptor, Orco. To reach the mushroom body and lateral horn of the brain, axons from first-order OSNs make synaptic connections with second-order projection neurons [13-16]. Consequently, the spatial and temporal representation of glomerular activity enables olfactory discrimination [4, 17, 18].
Fertility and sterility in *Drosophila*, can be influenced by various genetic, environmental, and physiological factors. Fertility refers to the ability of the flies to reproduce and produce viable offspring. Several factors can affect fertility in fruit flies, including 1) Genetic factors, like mutations in genes leading to reduced fertility. 2) Environmental Factors, such as temperature, humidity, and nutrition, which can influence fertility. 3) Reproductive system abnormalities such as structural abnormalities or malfunctions in the reproductive system of fruit flies can lead to fertility issues. 4) Age, where the fertility in *Drosophila* can decline with increase in age. Sterility, on the other hand, refers to the complete inability of an organism to reproduce. In *Drosophila*, sterility can result from a variety of causes, including, 1) Genetic sterility, caused as a result of some genetic mutations causing complete sterility by disrupting crucial reproductive processes. 2) Developmental abnormalities, abnormal development of the reproductive organs can lead to sterility. 3) Environmental factors, where exposure to certain environmental toxins, radiation, or extreme conditions can induce sterility. 4) Incompatibility, explained as sterility as a result from mating between different *Drosophila* species or closely related strains with incompatible genetic backgrounds. Ecdysone and Juvenile Hormone are thought to be the primary hormones responsible for regulating yolk protein production and absorption into the ovary, two key steps in controlling vitellogenesis and egg maturation [19, 20]. Ovulation, egg activation, fertilization, and oviposition are only some of the physiological processes that occur in *Drosophila* throughout the egg laying process. Since it initiates oocyte activation, stimulates oogenesis, and is influenced by feedback mechanisms that regulate the production of mature eggs, ovulation is an especially important reproductive process [19, 21-23].

In our previous screen for genes with a prominent expression pattern in the nervous system [24], we have

identified the P-Gal4 enhancer trap strain SG1.1. This strain is homozygous lethal due to a single P- Gal 4 insertion on chromosome 3. It was determined how this enhancer affects the expression of the LacZ reporter gene in the developing brains of larvae and pupae. Enhancer activity has been seen in neuronal populations of this strain in the adult suboesophageal ganglion (SOG), superior protocerebrum, central brain, and ventral ganglion [25]. SG1.1-Gal4 strain is expressed in a temporally controlled cyclical pattern in the brains of developing larval and pupal stages [25, 26]. SG1.1 P-Gal4 insertion is proposed as a new *olf413* allele by molecular localization and complementation (in press). A protein with copper binding and dopamine decarboxylase domains and a putative molecular function as Tyramine β hydroxylase (*TβH*) is predicted to be encoded by the *olf413* gene, annotated as CG12673 in *Drosophila* genome [27]. In addition, it has been noted as a paralogue of the *TβH* gene [28], a crucial enzyme in the biosynthesis of octopamine [29]. Except for a few genome-wide association screens [30-32], no reports on *olf413* gene's biological role have been published as of yet. The expression in SOG region in SG1.1-Gal4 strain prompted us to investigate the olfactory defects if any in these mutant strains.

Here we demonstrate the role of *olf413* gene in fertility and primary olfaction behavioural paradigms in *olf413* loss of function mutant flies and *olf413* knockdown flies respectively. The present work unravels a novel biological role for gene *olf413* in relation to the fertility and olfactory behaviour.

Materials and methods

1. *Drosophila* stocks

Standard wheat cream agar media was used to culture *Drosophila* stocks in a 12hr /12hr light/dark cycle at 22^0C and 60% relative humidity. Gal4 expression was optimized in experimental crosses by keeping the temperatures at 29^0C. We had used following *Drosophila melanogaster* stocks: Oregon-K from the *Drosophila* Stock Centre at the University of Mysore, SG1.1/*TM3Sb* from our laboratory, Gene disruption strain $olf413^{MI02014}$/*TM3Sb* (#77717, Bloomington *Drosophila* Stock Centre) as described by Lee *et al*. (2018) [33], and *olf413* RNAi line (#29547, BDSC) as described by Perkins *et al*. (2015) [34]. There is no off-target impact in this RNAi line.

2. Fertility assay using *olf413* loss of function mutant strain

To decipher the fertility of the flies the following crosses were set,

1. $olf413^{MI02014}$ homozygous virgin females crossed with $olf413^{MI02014}$ males
2. Oregon-K virgin females crossed with $olf413^{MI02014}$ homozygous males,
3. $olf413^{MI02014}$ homozygous virgin females crossed with Oregon-K males

The crosses were set in an embryo collection container, whose cap is fit with the embryo collection cup containing 2% sucrose agar media with a little yeast paste in the center. This cross was kept in 25^0C incubator for 24 hours. The embryo cup was removed the next day and again incubated at 25^0C for 24 hours to give sufficient time for the embryo to develop and hatch. After a total of 48 hours incubation the embryos were observed for hatchability under a stereo-zoom.

3. Odour sensitivity analysis using *olf413* gene knockdown mutants

UAS *olf413* RNAi virgin females were crossed with $olf413^{SG1.1}$ males to obtain $olf413^{SG1.1}$/*olf413* RNAi F1 flies. *olf413* RNAi knockdown flies were age matched and used for the assay. SG1.1-Gal4/*TM3Sb* and UAS-RNAi stocks were used as controls.

4. T-Maze apparatus

Tully and Quinn (1985) [35] served as basis for the designing of the olfactory experimental device. The equipment consists of 1) a transparent acrylic training tube 2) A sliding compartment supports a T-maze compartment constructed of acrylic sheets with two separate sections, one for instruction and one for testing. 3) A set of tubes for collecting the flies and recording the data. 4) odour tubes are made out of polystyrene cups with eight holes punched in the very tips of the tube. A tiny odour cup is inserted into the odour tube close to the perforations, and the necessary odorant is added to the cup. 5) The vacuum line, which is made of odourless plastic tubing, is connected to the sucking engine through 12V DC current, which provides a steady suction power. The vacuum pump is connected to the testing port while conducting the experiment to ensure uniform distribution of the odour in the testing tube. This equipment was set up in a dark room with a faint red light to conduct the experiment.

5. Odour sensitivity assay

The following odours were employed to investigate spontaneous olfactory response in a T-maze apparatus: ethyl alcohol (an attractant), benzaldehyde (a repulsive). T-maze horizontal testing tube (10cm) was used to introduce a group of 30 flies. Both the control and mutant strains are assayed for primary olfaction towards benzaldehyde and ethyl alcohol. The aversiveness towards benzaldehyde was assayed using 3 day old flies, whereas the attractiveness towards ethyl alcohol was assayed using the same flies at the age of 5 days. The T-maze equipment was inverted from its vertical to horizontal position and tapped three times. Pupal isolation and staging of flies was done to avoid the effect of anaesthetic ether. The flies were allowed 30 seconds to adjust to their new surroundings after being housed inside the central decision point. The bottom port of the sliding center selection point was fit with the suitable vacuum inlet. The middle slide was then lowered into position between the two collecting tubes in a smooth motion. The air was replaced with fresh air from the left T-maze collection tube while the odour was perfumed from the right T-maze collection tube with a full odour cup. The flies were allowed 45 seconds to pick up the odour in the air. The number of flies in each of the choice tubes was determined by collecting them from the collection tube after 45s, anesthetizing them, and counting them. In all of our trial runs, we always positioned the odour-filled cup on the right collecting tube and the odour-free cup on the left. To derive the quantitative odour Response Index (RI), the following formula was used: RI=S-C/S+C. One side has the smell (S), whereas the other doesn't (C) [36].

6. Statistics
SPSS (Version 22) was used for all of the statistical analysis. The mean SEM is displayed for the primary olfaction plots. While comparing more than two groups, we utilized one-way ANOVA, and while comparing the control and mutant groups, we used Tukey's post-hoc honestly significant difference test.

Results
1. *olf413* loss of function mutants are sterile

Table 1: Fertility test of *olf413*^{MI02014} homozygous loss of function mutant

Crosses Parameters	*olf413*^{MI02014} ♀ (Homozygous) X *olf413*^{MI02014} ♂(Homozygous)	Oregon-K ♀ X *olf413*^{MI02014} ♂ (Homozygous)	*olf413*^{MI02014} ♀ (Homozygous) X Oregon-K♂
Number of flies crossed	100♀ X 75♂	100♀ X 75♂	100♀ X 75♂
Number of eggs obtained	5	12	7
% Fertility	0%	0%	0%

Legend
The table shows fertility in percentage of homozygotes *olf413*^{MI02014} strain counted as the number of eggs laid on 2%sucrose agar medium. The fertility/sterility is observed by the viability and hatching of the embryos obtained from the crosses.

The gene disruption strain *olf413*^{MI02014} generated by Lee *et al*., 2018, has a T2A Gal4 insertion in the second intron of the gene *olf413*, along with a 3' polyadenylation site, and hence produce a truncated protein. *olf413*^{MI02014} stock is maintained over TM3 Sb balancer. Initial observation with these *olf413*^{MI02014} homozygous flies were that few flies survive until adulthood (3-4%). We had a question whether these flies can breed and produce fertile offspring. The result of this fertility paradigm is represented in Table 1. To decipher this result we conducted a cross between *olf413*^{MI02014} homozygous virgin females crossed with *olf413*^{MI02014} males. The results showed that these flies when crossed fail to produce progeny (5 eggs for 100 females, fail to hatch into larvae even after 48 hours in 25^{0}C). We conducted two more set of crosses to decipher if the males or females of homozygous *olf413*^{MI02014} survivors were sterile, 1) Oregon-K virgin females were crossed with *olf413*^{MI02014} homozygous males, 2) *olf413*^{MI02014} homozygous virgin females crossed with Oregon-K males. The results of the crosses shows that males as well as females crossed independently to wild type flies failed to produce progeny (7-12 eggs for 100 females, which fail to hatch even after 48 hours at 25^{0}C.) confirming that *olf413*^{MI02014} homozygous males and females are sterile.

2. *olf413* knockdown adult flies show normal primary olfactory response to benzaldehyde and ethyl alcohol

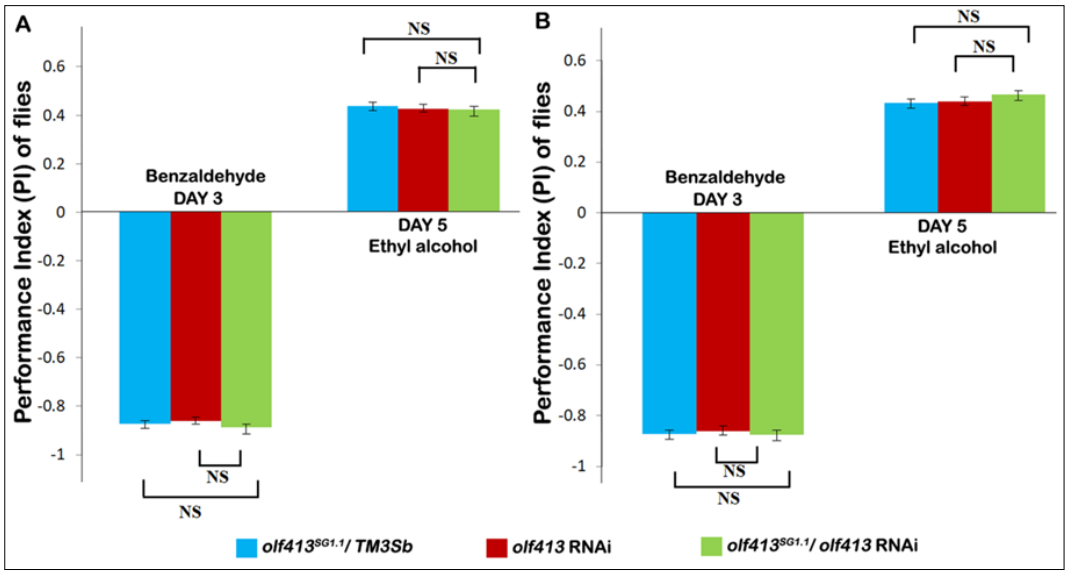

Fig 1: *olf413* knockdown males and female flies do not show primary olfaction defect towards Benzaldehyde and Ethyl alcohol

Legend
Figure 1 represents a bar graph with performance index of male flies (Fig. 1A) and female flies (Fig. 1B) towards Benzaldehyde and Ethyl alcohol odour cues assayed by T-maze apparatus. The bars representing negative value denotes the repulsive response performance index towards Benzaldehyde, whereas the bars representing positive value denotes the attractive response performance index of the flies towards Ethyl alcohol. *olf413*$^{SG1.1}$ and *olf413* RNAi stock served as control for *olf413*$^{SG1.1}$/RNAi mutant strain. The error bars represent the Standard Error of the Mean (SEM). NS indicates No Significance, at level of significance *P < 0.05; **P < 0.01; ***P < 0.001.

The *olf413* RNAi strain carries a transgenic construct for RNA interference specific to *olf413* under UAS regulatory sequence. When crossed to the P-Gal4 strain *olf413*$^{SG1.1}$, the F1 flies express dsRNA in *olf413* specific cell types causing functional knockdown of the gene. In this experiment we check whether these knockdown flies have any primary olfaction deficits using T-maze apparatus. We used a repellent (Benzaldehyde) and an attracted (Ethyl alcohol) as odorant cues. The details of the experimental methodology are explained in materials and methods. From the F1 mutant

flies obtained (Materials and Methods), a total of 300 flies were assayed for the test, 30 flies x 10 trials, for each group. We analysed the results of males and females flies separately. The results of preference towards the odours using male flies are presented in Fig. 1A and the results of preference towards the odours using female flies are presented in Fig. 1B. Comparison between both males and females does not show statistically significant difference between the two sexes. In the graph, the bars representation in negative value denotes the performance index towards the repellent Benzaldehyde, whereas the bars representation in positive value denotes the performance index of the flies towards the attractant, Ethyl alcohol.

olf413$^{SG1.1}$/*olf413* RNAi mutant strain both males and females do not show any statistically significant difference in their performance indices towards both the attractant and the repellent provided, as compared to the respective control group of flies. This confirms that these mutant flies can discriminate between the odours and they show appropriate response to the presented odour. There was no sex specific difference in their response.

Discussion and conclusion

olf413 is a protein-coding gene that has been predicted to have the copper type II ascorbate-dependent monooxygenase domain and the Tyramine/Dopamine beta-hydroxylase signature domains. It is also proposed to be involved in nor-epinephrine biosynthetic process and octopamine biosynthetic process [37]. Tyramine β Hydroxylase is required for conversion of tyramine into octopamine [38], which has been hypothesized as a functional counterpart of mammalian noradrenalin [39]. *TβH* null mutants deficient in octopamine, which serves as a neurotransmitter and neuromodulator [40, 41], have been tested for a number of behavioural abnormalities. Adult *Drosophila* behaviour, including aggressiveness [42], courtship [43], sleep behaviour [44], learning and memory [45], nutritional response [46, 47], and larval movement [48], have been linked to TβH function. *TβH* null flies develop normally and live to up to maturity, but they are unable to reproduce normally [29, 49]. *TβH* mutant females have faulty ovulation, blocking ripe oocytes in the ovaries and this defect could be rescued by supplementing with octopamine [29]. *TβH* expressed produced in the abdominal ganglion cells of the central nervous system that generate octopamine, restores mutant female fertility [29]. In *TβH* mutant, despite mating, the females hold their eggs in the oviduct, resulting in the bloated appearance of their bellies. The inability to lay eggs can recover when the flies are supplemented with octopamine containing food. Post supplementation, the flies initiate the egg laying phenotype. *TβH* gene null mutations have resulted in octopamine-free, female-sterile flies [49].

In our report, the functional role of *olf413* gene using a putative allele of *olf413*, *olf413* $^{SG1.1}$ was investigated. We demonstrate that *olf413* loss of function mutant males as well as females are sterile, implying that *olf413* function is critically required for fertility of male and female flies. The *olf413* knockdown flies do not have any primary olfaction defect. Our study emphasizes on the fertility aspect of a loss of function mutant of *olf413* gene, *olf413*^{MI02014} where we discover that these mutants are sterile and cannot breed. Considering the fact that on an average Oregon-K females lay 34 eggs per day [50], the number of eggs laid by 100 females in our experimental condition is negligibly small in each case (5-12 eggs for 100 females). Further experiments are needed to give a conclusive answer for the reason behind this sterility.

The olfactory sensitivity of the *olf413* knockdown mutants were assayed here using benzaldehyde and ethyl alcohol as odorants with T-Maze apparatus. These knockdown mutants generated using *olf413* RNAi strain are appropriately sensitive towards both the odour cues provided and do not display any preliminary olfaction deficits. Both females and males mutant flies display a similar phenotype in responding appropriately towards the olfactory odour cues.

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Authors' contribution

B.V. Shyamala conceived the idea and designed the experimental study. R. Ramya carried out the experiment and did data analysis. Both the authors prepared the manuscript and are involved in critically reviewing, revising the analysis and the interpretations. Both the authors have read and approved the final manuscript.

Conflict of interest

The authors declare that they do not have any conflicts of interest.

Ethical approval

There are no ethical approval required for the study.

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