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RESEARCH ARTICLE

PRE-FASTING FEEDING RESPONSES OF *DROSOPHILA MELANOGASTER* PRE-ADULT AND POST-ADULT TRAITS TO STIMULANTS

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ABSTRACT

The behaviour paradigms are relatively complicated, it is necessary to understand how the fundamental behaviour is organized at neural level, before a full understanding of the complex behaviour. *Drosophila melanogaster* has shown biased preference when facing sensory stimulation towards varied concentration of stimulants namely nicotine and caffeine. The preference behavioural assays were used to study sensory abilities based on feeding behaviour and climbing ability. The regulation of feeding behaviour in pre- adult and post- adult traits of *Drosophila melanogaster* showed varied responses to the stimulants supplemented in the food regimes with respect to various concentrations of stimulants addicted with organismal stress provided in the form of starvation. The pre-adult (larvae) preferred stimulants (i.e., Caffeine/Nicotine) rather than control and combination of both the stimulants, while the post-adult (flies) preferred the combination of stimulants than the caffeine or nicotine alone.

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INTRODUCTION

Drosophila melanogaster adapt their food consumption to their internal needs and avoid ingesting noxious molecules. Defects in the genes involved in these decisions induce behavioral alterations that are usually screened by monitoring flies feeding in 2-choice or in no-choice situations. Although psychostimulants, opiates and ethanol all have different primary effects and modes of action in the central nervous system (CNS), current theories suggest that their positive reinforcing, or rewarding, properties are mediated in part by an elevation of extracellular dopamine in the nucleus accumbens (Koob *et al.,* 1998).The mechanosensory chordotonal organs and the brain hemispheres are apparently dispensable for these locomotor patterns, arguing they are produced by circuitry in the ventral nerve cord; however, brain and mechanosensory input are required for the integration of these locomotor patterns into adaptive, biologically meaningful behaviour (Ohyama *et al.,* 2013). *Drosophila* larvae being the major feeding stages of the flies' life cycle, have a numerically simple brain, may be 10 million fewer neurons compared to man and possess correspondingly moderate behavioural complexity.

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These features, together with the general potential of the *Drosophila* for transgenic manipulation, (Elliott and Brand, 2008) make them an attractive study case when trained to achieve a circuit-level understanding of the behaviour, in particular with regard to the chemosensory processing and odour-tasting learning. The sense of taste is that component of the contact chemosensory system devoted to organize feeding, allowing animals to prefer edible and avoid toxic substances. In addition, gustatory stimuli can be reinforcers: They can induce memories for stimuli or actions that preceded them, such that the animal can find good and avoid bad food. Gustatory stimuli thus organize both immediate, reflexive behaviour towards food (such as choice and feeding), and, by virtue of their association with predictive stimuli or instrumental actions, the search for food. Trivially, these functions must come about by different sets of neurons on at least some level of processing. While at the level of gustatory interneurons such dissociation can clearly be found (e.g. in terms of the sufficiency of octopaminergic signalling for reinforcement, but not for ingestive behaviour (Menzel *et al*.,1999), it is not resolved in detail whether and how different sets of sensory neurons organize different gustatory reflex behaviours and/ or internal reinforcement signals, respectively. Compared with learned behaviour, an innate behaviour is what an animal can do without practice or training. Animal innate preference behaviour is largely the primitive reaction that an

animal spontaneously demonstrates when choosing between different environmental conditions, such as light, odourant, temperature, or different objects like visual targets and food. Innate preference behaviours are the cornerstones of more complex behaviours. For example, in associative learning behavioural paradigms, the unconditional stimulations, no matter aversive or rewarding, are designed based on innate preferences. In the classical Palvnovian conditioning, food award to the dog is used as unconditioned stimulus (Gong, 2012).

Fruit flies react to taste molecules in a way which is quite similar to humans sometimes more than rodents (Gordesky-Gold *et al.,* 2008) and within the detection range of mammals. They are attracted to sugars, avoid bitter and toxic molecules, and adapt their consumption of acids and salts to their internal needs (Gerber and Stocker, 2007). In *Drosophila* adults, contact chemoreception is mediated through hair-like structures, called sensilla, located on the mouthparts, the legs, the wings margin, and the ovipositor. Nicotine, the major addictive component of tobacco, affects mammalian behavior by activating nicotinic acetylcholine receptors (Nestler, 2005).When exposed to volatilized nicotine, flies exhibit locomotor hyperactivity and spasmodic movements leading to grooming at low doses and hypokinesis and akinesia at higher doses. Similar to cocaine, nicotine exposure dose-dependently impairs negative geotaxis in flies. The locomotor effects of nicotine in flies are similarly dependent on dopamine, as pharmacological depletion of dopamine reduces nicotine sensitivity. Apart from dopamine, little is known about the molecular mechanisms mediating nicotine sensitivity in flies. However, several genes known to mediate cocaine sensitivity in flies have also been shown to regulate nicotine sensitivity: moody mutant flies are sensitive to the effects of both drugs, whereas RhoGAP18B and tao mutants are resistant. These genes suggest that certain shared mechanisms may regulate multiple types of drug addiction in flies (Bainton *et al*., 2000; King *et al*., 2011).

Caffeine is one of the most commonly used psychoactive substances and has been shown to antagonize adenosine receptor signaling, inhibit cAMP phosphodiesterase (PDE) activity, and activate ryanodine receptors. However, the promotion of wakefulness by caffeine is widely thought to be mediated by its antagonism of adenosine receptors, based upon its higher affinity for these molecules. The associated acute locomotor-stimulating effects of these drugs have been proposed to model their rewarding qualities (Wise and Bozarth, 1987). Selective destruction of dopaminergic neurons or pharmacological inhibition of dopaminergic systems prevents the stimulatory effects of most drugs of abuse; these manipulations also curtail drug self-administration (Kuhar *et al*., 1991). One theme that emerges from this large number of pathways is the involvement of molecules affecting molecular or cellular plasticity (e.g. cytoskeletal regulators, ion channels, synaptic molecules), which appear to be recruited to induce behavioural changes. The fly is ideally suited to this task due to the availability of tools to investigate these mechanisms with high spatial and temporal resolution. Thus the aim of the present study is to investigate the effect of stimulants on the behaviour i.e., feeding behaviour and climbing ability in *Drosophila melanogaster* with respect to various concentrations of stimulants addicted with organismal stress provided in the form of starvation.

MATERIALS AND METHODS

The fly stocks were routinely cultured in standard wheat cream agar medium in uncrowded condition at 22± 1ºC with 12:12 h light and dark periods and relative humidity of 70%. The test flies were cultured in wheat cream agar medium along with variable concentrations of stimulants namely nicotine and caffeine i.e 40 mg/100ml, 60 mg/100 ml, and 80 mg/100ml respectively along with control. Further, the present study was emphasizes to reveal the effect of stimulants on the gustatory feeding assay (Andretic *et al*., 2008) and locomotory activity (Gerber *et al*., 2009).

Larval starvation and feeding assay

The larvae were fed for 4 hours in each concentration of nicotine and caffeine along with control prior to starvation. Further the fed larvae were starved for 4 hrs,6 hours and 8 hours. Subsequently, the starved larvae were allowed to feed on the experimental concentrations in the Petri dishes (1mm×100mm) along with control. The food source of molten 1% agarose (control) was poured into the Petri dish demarcated (1 cm wide middle zone) was marked into two equal halves at the centre of the petri dish and was allowed to cool for 10 minutes. Further the combinations of experimental stimulants of variable concentrations were poured into Petri dishes i.e 40 mg/100ml, 60 mg/100ml, 80 mg/100ml caffeine and nicotine on one half of the Petri dish and the other half with control media. The gustatory preference of the larvae was recorded and calculated for the Gustatory Preference index (GPI) for the feeding choice. Thirty larvae were introduced in the centre of each Petri dish and allowed to choice preference of food. The number of larval preference on each half of the petri dish was counted and the gustatory preference index (GPI) was calculated for 20 minutes in an interval of once in every 2 minutes. GPI values range from -1 to +1 with negative values representing preference for control and positive values represent preference for nicotine or caffeine.

$$GPI = \frac{\#\text{Experiment (Nicotine/Caffeine)} - \text{Control}}{\text{Total \# of Larvae-upside (Not preferring either control/ experiment)}}$$

Adult climbing ability

The same set of experimental larvae used for Gustatory index were allowed to eclose into adults and were aged for three days after eclosion to assess the climbing index. The climbing ability was observed and recorded in the measuring cylinders (25cm). The flies were placed at the bottom of the cylinder and the other end of the cylinder was sealed using parafilm. A table lamp was used as a light source and a duster to tap the cylinder. A height of 15cm was considered as standard measure to record the climbing ability. Separate climbing chambers were used for male and female respectively. To perform the assay, the bottom of the tube was tapped on a duster to stimulate flying and the timer was started

simultaneously and the lamp was switched on for every 10 seconds. After 10 seconds the flies that flew successfully above 15cm were counted even the flies that show slight vibration in their wings was also considered. Likewise the climbing ability was recorded in both control as well as treated flies sequentially to allow time for rest and recovery of flies between the 10 trials that was conducted at an interval 10 second (McClung and Hirsh, 1998).

Statistical Analysis

Behavioural assays (Gustatory feeding choice and climbing ability) were subjected to one way ANOVA, Tukey's HSD by using SPSS 20.0.

RESULTS

Larval feeding assay

The mean feeding values in caffeine decreased with starvation hours and increased with increased concentration compared to the control.

Increased significant difference were recorded in lower (40mg/100ml) and higher (80mg/100ml) concentration with that of the control and with mid concentrations (60mg/100ml). As the starvation hours increased, the larvae preferred higher concentration (80mg/100ml) of caffeine than low (40mg/100ml) and mid (60mg/100ml) concentration. Wherein nicotine fed larvae increased values with starvation and increased concentration with that of the control.

The flies showed high significance (P=0.0001) at mid (60mg/100ml) and higher (80mg/100ml) concentration along with that of the control and in between concentrations, larvae fed at 6hours and 8hour duration were insignificant. While, as the starvation is delayed the preference of the larvae towards higher concentration of caffeine is significantly higher. The combination of nicotine-caffeine has also shown that the higher the starvation time the greater preference towards the higher concentration. Interestingly, the larval preference is more towards caffeine neither than nicotine nor in mixed combination of both the stimulants (Table 1).

Table 1. Mean±S. E of Gustatory choice preference of *Drosophila melanogaster* on supplementation of various stimulants

Starvation (Hours) →	Stimulants								
	Caffeine			Nicotine			Caffeine +Nicotine		
	4	6	8	4	6	8	4	6	8
Concentrations ↓ Control	.08±.06	.08±.06	.02±.05	.08±.06	.08±.06	.02±.05	.08±.06	.08±.06	.02±.05
40mg/100ml	*** 5.7±0.5	.06±.06	*** .54±.04	** 0.2±.50	.32±.06	* .24±.04	.25±.04	*** .32±.02	** .06±.02
60mg/100ml	** .32±.04	** .32±.06	* .23±.02	*** .67±.40	*** .37±.06	** .30±.03	.05±.05	*** .53±.04	*** .58±.03
80mg/100ml	*** 7.5±.40	.16±.02	*** .54±.06	*** .46±.40	** .34±.02	*** .45±.06	** .14±.05	** .07±.02	** .23±.04
ANOVA	F=152.41 Df=3,76 P<0.05	F=61.0 Df=3,76 P<0.05	F=31.19 Df=3,76 P<0.05	F=47.69 Df=3,76 P<0.05	F=78.8 Df=3,76 P<0.05	F=11.75 Df=3,76 P<0.05	F=31.5 Df=3,76 P<0.05	F=32.20 Df=3,76 P<0.05	F=43.67 Df=3,76 P<0.05

Note – *P<0.05; **P<0.01;***P<0.001

Table 2. Mean±S.E of Climbing ability of *Drosophila melanogaster* male flies on supplementation of various stimulants

In days →	Stimulants								
	Caffeine			Nicotine			Caffeine +Nicotine		
	3	5	7	3	5	7	3	5	7
Concentrations ↓ Control	1.7±.18	2.2±.39	2. 2±.34	1.7±.18	2.2±.39	2. 2±.34	1.7±.18	2.2±.39	2. 2±.34
40mg/100ml	*** 0.5±.70	* 2.1±0.2	* 2.3±.23	** 2.7±.18	* 3.8 ±..27	* 2.8±.23	*** 4.6±.62	* 2.5±.24	* 1.7 ±.48
60mg/100ml	*** 1.7±.10	*** 4.2±.25	* 3.0±.25	* 2.3±.21	* 2.9±.41	* 3.2±.25	*** 6.7±.35	* 2.9±.22	** 5.6±.92
80mg/100ml	*** 3.1±.56	* 3.0±.40	* 2.4±.26	* 2.1±.41	* 2.9 ±.58	** 3.7±.28	* 3.6±.6.5	*** 4.9±.34	* 3.7±.78
ANOVA	F=55.05 Df=3,76 P<0.05	F=8.94 Df=3,76 P<0.05	F=1.75 Df=3,76 P<0.05	F=2.65 Df=3,76 P<0.05	F=2.45 Df=3,76 P<0.05	F=5.37 Df=3,76 P<0.05	F=18.83 Df=3,76 P<0.05	F=16.59 Df=3,76 P<0.05	F=7.07 Df=3,76 P<0.05

Note – *P<0.05; **P<0.01;***P<0.001

Table 3. Mean±S.E of Climbing ability of *Drosophila melanogaster* female flies on supplementation of various stimulants

In days →	Stimulants								
	Caffeine			Nicotine			Caffeine +Nicotine		
	3	5	7	3	5	7	3	5	7
Concentrations ↓ Control	2.1±.19	2.1±.39	2. 2±.35	2.1±.19	2.1±.35	3.0±.31	2.1±.19	2.1±.35	3.0±.31
40mg/100ml	*** 0.9±.87	** .8±0.14	* 3.0±.29	** 4.2±.80	* 1.9 ±.16	*** 5.3±.33	*** 4.0±.38	*** 3.7±.21	* 2.2 ±.40
60mg/100ml	*** 0.8±.20	* 2.5±.28	* 3.6±.21	* 1.6±.15	* 2.5±.19	** 3.9±.26	* 3.6±.38	* 2.2±.20	* 4.0±.44
80mg/100ml	*** 3.5±.19	* 2.1±.16	*** 1.7±.21	* 2.2±.23	** 3.6 ±.30	* 3.8±.23	* 2.4±.31	* 1.5±.25	* 3.7±.39
ANOVA	F=43.4 Df=3,76 P<0.05	F=9.09 Df=3,76 P<0.05	F=1.16 Df=3,76 P<0.05	F=8.42 Df=3,76 P<0.05	F=7.54 Df=3,76 P<0.05	F=17.04 Df=3,76 P<0.05	F=8.24 Df=3,76 P<0.05	F=13.55 Df=3,76 P<0.05	F=4.37 Df=3,76 P<0.05

Note – *P<0.05; **P<0.01;***P<0.001

Climbing ability

The mean climbing ability of male in Caffeine and as well in nicotine decreased with mid and low concentrations but increased in the higher concentration with that of the control. As the flies were aged with days, the significance of climbing ability increases in control (Table 2). The climbing ability off males adult showed decreased mean with increase in the concentration with that of the control. As the days passed, the climbing ability increased in control in caffeine and as well in nicotine (Table 3). In the stimulant mixture of nicotine and caffeine, the mean climbing ability of male showed increased climbing ability in higher concentration (Table 2). While females have shown decreased mean values as they age (Table 3).

DISCUSSION

In response to contact chemoreception with a phagostimulatory chemical, flies elicit a reflex-like appetitive behaviour wherein they extend the proboscis to attempt feeding (Dethie, 1976). Nicotinic acetylcholine receptors (nAChRs) play an important role as excitatory neurotransmitters in vertebrate and invertebrate species. In insects nAChRs are expressed throughout the nervous system and are the site of action for economically important insecticides such as spinosyns and neonicotinoids (Miller, 2007; Jones, 2007). In the present study higher concentrations was more preferred by the larvae compared to other concentrations with that of the control by the sensory stimulus with increase in starvation hours. Caffeine is naturally produced by plants as an antifeeding and pesticide agent for insects, and in *Drosophila* it was shown to exert its effects through the dopamine receptor, cAMP pathway and protein kinase A activity in the brain. Larvae showed increased feeding response towards the higher concentrations of caffeine compared to that of the control. The activity level monotonically increased with higher concentrations of caffeine and mixed concentrations when compared with nicotine. Caffeine increases the force of contraction of both skeletal muscle and cardiac muscle (Blinks *et al.,* 1970). In skeletal muscle, low concentration of Caffeine have no effect on the resting membrane potential and little effect on action potential (Taylor *et al*.,1969) and the potentiation of contractile force produced by the drug is attributed to an action on sarcoplasmic calcium stress(Weber *et al*.,1968). In atrial muscle, caffeine changes the shape of the action potential (de Gubareff *et al.,*1965) but it is not clear that his the primary cause of the increase in amplitude and duration of the muscle contraction: caffeine may act on sarcoplasmic calcium stores, as well as on the cell membrane in cardiac muscle. Nicotine the primary psychoactive substance in tobacco smoke produces a variety of psychoactive effects and has been believed to be a type of psychostimulants. In humans, NIC produces convulsions, tremors, and excitation of respiration, elevates the arousal level facilitates behaviours and performance, and improves cognition and attention abilities. Repeated exposure to nicotine and other psycho stimulant drugs produces persistent increases in their psychomotor and physiological effects (sensitization), a phenomenon related to the drugs' reinforcing properties and abuse potential. The study shows the climbing ability of the adult flies both male and female showing significance with increased concentrations with that of control. Caffeine and nicotine concentrations fed male flies showed decreased percentage of motor activity as they aged with number of days with that of the control and whereas the mixed concentrations fed male flies showed significantly increased climbing ability as they aged. While the females when fed with different concentrations of stimulants showed lesser climbing ability with that of control when compared to males. The ability of animals to withstand prolonged periods of food deprivation is called 'starvation resistance' (SR), which is a phenotypic trait of great organismal, ecological and evolutionary significance given that starvation is the most ubiquitous environmental stress faced by animals inhabiting environments where food availability fluctuates and is unpredictable (McCue, 2010). Thus in the present study pre-adult (larvae) preferred single stimulant (i.e., Caffeine/Nicotine) rather than combination of both the stimulants, while the post-adult (flies) preferred the combination of stimulants in males. The Pre- adult and the post-adult behaviours elicited were distinct at different doses, there was substantial individual variation among flies of their own preferences towards stimulants.

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