The effect of preferential methamphetamine consumption on lifespan and behavioral phenotypes of Drosophila melanogaster

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SVEUČILIŠTE U RIJECI

ODJEL ZA BIOTEHNOLOGIJU

Diplomski sveučilišni studij

“Medicinska kemija”

Laura Fućak

Utjecaj preferencijalne konzumacije metamfetamina na dužinu životnog ciklusa i bihevioralne fenotipove *D. melanogaster*

Diplomski rad

Rijeka, 2023

Mentor: izv. prof. dr. sc. Rozi Andretić Waldowski

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Abstract

Addiction is a relapsing disease caused by substance abuse. Methamphetamine and cocaine are potent psychostimulants whose abuse induces numerous negative consequences for the abuser. Studying substance abuse in humans is challenging, however its effects can be analyzed in model organisms such as *Drosophila melanogaster*. Alongside their genetic traceability, fruit flies are extensively researched making them exemplary models for addiction research. Methamphetamine and cocaine disbalance monoaminergic levels, damage axons and create reactive oxygen species which disrupt normal organism functions. Considering that addiction shows around 72% heritability, we were wondering if the effects of paternal methamphetamine abuse can be seen in filial generations. After artificial selection of the 28th *Canton S* fly generation with high (HP) and low (LP) preference for methamphetamine, we observed life cycle and behavioral phenotypes of flies, their morphology and voluntary self-administration of methamphetamine through FlyCAFE assay. Our aim was to determine if there are any differences between *wt*, HP and LP lines after 28 generations of selection. The obtained data shows impaired activity, sleep, and negative geotaxis of flies, alongside possible changes in flies weight and size compared to control *wt* line. Locomotor activity of HP, LP, and *wt* flies increases after volatilized methamphetamine and cocaine administration with various significances among lines. The HP and LP flies maintain the same preference for methamphetamine, high and low, respectively. Finally, there is no difference in length of life cycle among lines, but HP flies have lower reproduction success rate. Our findings point to a transgenerational effect of paternal methamphetamine abuse, posing further questions about the epigenetic and/or genetic changes induced by substance abuse.

Keywords: *Drosophila melanogaster*, behavioral phenotypes, psychostimulants, methamphetamine preference, transgenerational heritability

Sažetak

Ovisnost je relapsirajuća bolest uzrokovana zlouporabom opojnih supstanci. Metamfetamin i kokain su snažni psihostimulansi čija konzumacija ostavlja brojne negativne posljedice na organizam ovisnika. Izučavanje zlouporabe doga u ljudi vrlo je izazovno, stoga se njihovi učinci analiziraju u modelnim organizmima poput vinske mušice (*Drosophila melanogaster*). Vinske mušice pogodne su za istraživanja ovisnosti jer imaju dobro proučen i sekvenciran genom pa se njihove genetske promjene mogu jednostavnije pratiti i izučavati. Metamfetamin i kokain uzrokuju poremećaje u nivoima monoamina, oštećuju aksone te stvaraju reaktivne kisikove vrste, što otežava razne funkcije organizma. S obzirom na činjenicu da ovisnost pokazuje oko 72% nasljednosti, interesiralo nas je da li se učinci paternalne preferencijalne samo-administracije metamfetamina očituju u filijalnim generacijama mušica. Nakon umjetne selekcije 28. generacije mušica *Canton S* linije sa visokom (HP) i niskom (LP) preferencijom za metamfetamin, proučavali smo životni ciklus i bihevioralne fenotipove *D. melanogaster*. Također smo promatrali morfologiju mušica i larvi te samo-administraciju metamfetamina kroz FlyCAFE metodu. Cilj ove teze bio je vidjeti postoje li razlike između *wt*, HP i LP linija nakon 28 generacija umjetne selekcije. Prikupljeni rezultati ukazuju na povećanu aktivnost i smanjen san mušica, kao i na smanjenu sposobnost negativne geotaksije. Nadalje, postoji mogućnost da visoka i niska paternalna preferencijalna samo-administracija metamfetamina utječe na veličinu i težinu mušica, odnosno larvi u odnosnu na *wt*. Lokomotorna aktivnost mušica HP, LP i *wt* linija raste nakon administracije volatiliziranog metamfetamina i kokaina sa raznim nivoima statističke značajnosti unutar linija. HP linija zadržava visoku, a LP linija nisku preferenciju metamfetamina, što je u skladu s prethodno testiranim generacijama. Konačno, nema promjena u duljini životnog ciklusa mušica, no HP linija ima nižu reprodukcijsku uspješnost.

Navedeni rezultati ukazuju na trans-generacijski efekt paternalne preferencijalne samo-administracije metamfetamina, te postavljaju pitanja o potencijalnim epigenetskim ili genetskim promjenama koje su inducirane zlouporabom supstanci.

Ključne riječi: *Drosophila melanogaster*, bihevioralni fenotipovi, psihostimulansi, preferencija metamfetamina, trans-generacijska nasljednost

Contents

1. Introduction

Drug addiction is a complex mental illness characterized by chronic and relapsing drug abuse with negative consequences for the abuser (1). Ingesting low doses of a drug increases central nervous system activity and causes mood elevation by activating rewarding brain mechanisms (2). Substance abuse is increasing and according to the United Nations there is approximately 22 million abusers of cocaine (COC) and 36 million abusers of amphetamines worldwide, which is causing massive socio-economic problems. Methamphetamine (METH) and cocaine are psychostimulants which cause short term euphoric feeling, often leading to dependance, but their chronic abuse has harmful effects on cognition, memory, and attention while overdoses can lead to circulatory collapse, psychosis, and death (3).

1.1. Psychostimulants and neuroplasticity

Psychostimulants intake causes neuroplasticity, meaning that addiction to drugs is based on pathological changes in brain function and homeostasis (4). In terms of addiction, we can divide neuroplasticity into transient changes in neuronal functions that occur during drug abstinence while becoming addicted (hours-weeks) and into stable changes (possibly permanent) that guide to execution of learned behavior (relapse after abstinence) (5). The exact mechanism by which neurological changes occur is still unknown, but current research indicates the connection with rewarding systems of the brain.

In response to drug uptake, rewarding pathways in the brain are involuntarily engaged and generate drug craving and seeking. Despite having different mechanisms of action as well as different pharmacodynamic and pharmacokinetic properties, METH and COC are both connected to concentration changes of neurotransmitters such as dopamine (DA), serotonin (5-HT) and glutamate in specific brain regions (6). METH and COC are lipophilic, so they easily cross brain blood barrier (BBB) and there they

increase extracellular concentration of DA (7) promoting oxidative stress with negative effects on abusers health.

1.2. Psychostimulants induced oxidative stress

Acute and chronic METH and COC abuse can leave neurotoxic consequences thorough oxidative stress and metabolism changes. Psychostimulants cause neurotransmitter accumulation (mainly DA) by reverse transport into synapse through vesicular monoaminergic transporters alongside with lowering the number of available DA transporters (METH) or by blocking neurotransmitter transporters (COC) (7–9).

The accumulated DA is then auto-oxidized or oxidized through monoamine metabolism, causing reactive oxygen species (ROS) formation including hydrogen peroxide (H_2O_2), superoxide (O_2^-), hydroxyl (OH^-), alkoxyl and peroxyl radicals (10). Moreover, ROS can be further oxidized into quinones, that become nitrogen and O_2^- radicals which cause even more damage to the axon terminals of dopaminergic neurons (7).

The negative effects of oxidative and nitrosative stress on metabolic processes have compelled organisms to develop antioxidative defense systems. These antioxidants can be endogenous, namely enzymes superoxide dismutase (SOD) and catalase (CAT) or they can be exogenous, like flavonoids, carotenoids and vitamins C and E which are obtained from food (10). These protective systems are effective in removing damaging ROS molecules in healthy organisms, however they are significantly disrupted by endogenous stressors and toxins, such as psychostimulants. By increasing ROS production and affecting antioxidants enzyme scavenging system, psychostimulants enable protein, nucleic acid, and lipid oxidation, leading to toxicity (7).

1.3. Psychostimulants neurotoxicity

The neurotoxic effects of psychostimulants are mediated through generating a large amount of ROS molecules from accumulated extracellular DA, which overpowers the cells antioxidant defense mechanisms and leads to neuroinflammation. Neuroinflammation is a result of activated microglia whose gene expression is likely changed by quinones and ROS. Microglial activation elevates levels of pro-inflammatory cytokines, prostaglandins, ROS, and causes secretion of considerable amounts of excitotoxic glutamate (7,11).

Microglial activation increases glutamate neurotransmission, especially near damaged dopaminergic and serotonergic axons. Excitotoxicity is characterized by overproduction of glutamate which is induced by nitrogen reactive species (NOS) formed by METH and COC consummation. Exorbitant levels of glutamate result in accumulation of intracellular calcium, followed by series of cellular processes that can activate phosphatases and protein kinases, creating even more oxidative species, leading to apoptosis (7,12,13).

The lipophilic nature of METH and COC enables them entrance into various organelles including mitochondria. Psychostimulant abuse causes mitochondrial impairment by increasing the production of ROS and proteins which cause mitochondrial fragmentation. Furthermore, they increase the expression of pro-apoptotic proteins while decreasing the expression of anti-apoptotic proteins leading to mitochondrial apoptosis (7,11–13).

In humans, psychostimulant abuse shortly creates mood elevation, followed by agitation, aggression, hypertension, and sleep impairment. Higher doses and chronical abuse causes hyperthermia, liver failure, seizures, heart attack and cerebrovascular hemorrhages and death (3,14). In low doses COC is used as a local anesthetic (15), while METH is used to treat attention deficit hyperactivity disorder (ADHD), narcolepsy and autism spectrum disorder (ASD) (8). However, since studying the effects of drugs on humans is

expensive, complicated and an ever-present ethical question, addiction and its consequences are largely observed on model organisms.

1.4. Animal models for studying psychostimulant addiction

Drug abuse is a chronic and relapsing mental disease (1) which has been observed in animal models to study its mechanisms and improve available treatments. Animal models, such as rodents and invertebrates, enable researchers to ethically study drugs acute, chronic, and addictive effects while saving time and money needed for human research (1). Using animal models allows administration of drugs in specific and controlled conditions, as well as dissecting and observing specific behaviors and molecular pathways. In laboratory conditions discerning substance addiction usually entails drug inducing behavioral phenotypes (endophenotypes) in animal models (16). Drug induced endophenotypes can vary from simpler behaviors which are useful in high-through genetic screenings, to more complex behaviors (e. g., relapse, self-administration) which have a closer relation with human studies (6).

Rodent models (dominantly rats and mice) have provided an insight into molecular and genetic mechanisms of addiction and related behaviors. However, rodents are not ideal models for identification of new genes and mechanisms. This promoted the use of invertebrates (such as *Drosophila melanogaster*) as models to study addiction because of numerous advantages compared to rodents.

1.4.1. *Drosophila melanogaster* as a model organism

Drosophila melanogaster, or commonly referred to as fruit fly, is a genetically tractable model organism for studying addiction. Even though there is a significant difference in the number of genes between mammals and *Drosophila*, they have a similar number of gene families (6). *Drosophila* is a valuable model to study genes connected to human diseases due to its

considerable number of conserved genes (about 75% of human disease genes have their orthologs in *Drosophila*) (6). Moreover, fruit fly's genome has been sequenced, so any mutations can be easily traced and analyzed. Fruit flies are cost effective; they do not require much space or food and have high fecundity with a rapid life cycle (1,6). Lastly, fruit flies are invertebrates which can replace higher models in research, following the 3Rs principle (Reduce, Refine, Replace).

1.5. Behavioral sensitization

Experiments of drug self-administration are contingent, while experimenter-administered drug experiments are noncontingent studies (2). Although noncontingent studies are somewhat simpler and of lower validity compared to contingent ones, they have provided insight on how repeated drug administration alters neuronal pathways (2), enabling discovery of involved genes and molecular mechanisms of addiction.

Behavioral sensitization is an in-depth studied noncontingent behavior. The development of behavioral sensitization can be divided into two phases: initiation (immediate neural effects) and expression (long-term consequences of these initial events). In terms of addiction, it is considered as a simple behavioral change induced by repeated drug administration (17). The robustness of the sensitization response, among other factors, depends on intervals between treatment, the dose, sex, age, and genetics (2). Motor activation is one of the most observed and highly conserved effects of COC and METH abuse, making it a source for many behavioral sensitization studies.

1.5.1. Motor activating effects of METH and COC

Drosophila has been used as a model to study substance abuse, such as ethanol and volatilized COC (vCOC) and METH (vMETH). Model organisms show sensitivity (SENS) after first drug exposure (initiation). In *Drosophila* SENS is an increase in locomotor activity after exposure to vCOC and vMETH

in a dose dependent manner. At low doses vCOC increases locomotor activity and promotes stereotypical behaviors of flies behavior (e.g., grooming, circling, fast uncontrolled movements, buzzing) while at large doses it induces akinesia and death (18). Oral METH administration decreases sleep and promotes activity, affects male courtship, and possibly impairs vision (19). LS is an increase in the amount of locomotor activity to repeated doses of the same amount of the administered drug (expression) (18). In *Drosophila* it can also be defined as behavioral sensitization of increasing intensity of stereotypical drug-induced behavior (16). Research shows that flies develop LS after exposure to both vCOC and vMETH (16–18).

Sleep and activity are also affected by COC and METH administration. Flies show gradual increase in activity after oral METH administration, however without hyperactivity and coordination loss (unlike in vCOC) (19). Furthermore, Andretić et all. showed that in addition to promoting active wakefulness METH also decreases the need for sleep (19). The same results were obtained after oral amphetamine consummation (20). Flies ability to negatively geotax (climb the vertical surfaces) is also impaired after vCOC exposure in a dose dependent manner (21,22). These results are consistent with results obtained from rodent models and demonstrate that *Drosophila* is an excellent model for studying psychostimulants addiction.

1.6. *Drosophila* in psychostimulants self-administration experiments

As mentioned above, psychostimulants engage rewarding pathways in the brain, which causes drug craving and dependency. The accumulated amount of DA in synaptic cleft caused by drug consumption leads to neuroplastic changes in rewarding pathways which motivates drug taking. Considering that abusers actively seek and voluntarily administer the drug, this behavior was studied on animal models. Self-administration is a contingent study relying on operant learning and higher validity due to its

relation to human drug consumption (16).

Self-administration of drugs in *Drosophila* is mostly studied with the CApillary FEeder Assay (CAFE), which is similar to the two bottle choice assays in rodent models. The CAFE assay shows that flies preferentially consume ethanol laced food, persisting despite the absence of gustatory and olfactory input, and the quantity consumed is dose dependent (3,6). The problem with ethanol studies in *Drosophila* is that flies are evolutionally conditioned to relate ethanol with food source, since they consume fermented fruit. However, the demonstration that *D. melanogaster* preferentially consumes food laced with METH or COC indicates that rewarding aspects of psychostimulant are independent of evolutionary biased food consumption. (6).

Based on ethanol studies, the preferential self-administration of COC and METH in flies has recently been studied using CAFÉ method. The findings show that flies voluntarily prefer amphetamine and METH food in a dose dependent manner (flies prefer 1mM laced METH food but avoid 10 mM METH laced food) (16,23). Published reports about COC self-administration are contradictory. Flies have shown an aversion to COC laced sucrose food in a dose dependent manner (24). A possible explanation is that cocaine is a plant alkaloid which naturally repels herbivore insects, so the flies aversion is an evolutional consequence. Other COC self-administration research shows that flies preferentially consume sucrose food laced with COC (25). Another study where flies consumed either METH or METH and glucose food, showed flies prefer the METH and glucose food as opposed to the METH food, indicating that glucose might mask the bitterness of the drug (26). The same could be said for the differences in COC self-administration in flies, sucrose might mask the taste of COC, but it could also depend on the concentration differences in studies.

1.7. Life cycle of *Drosophila melanogaster*

Life cycle of *Drosophila* is relatively short and results in a large progeny (female fly can lay around 100 embryos a day) (27). The length of the cycle greatly depends on the temperature, in so called "standard" conditions (25°C, 70% humidity and agar or cornmeal-based food) the cycle lasts approximately ten days, while at 18°C it lasts up to 18 days. The understanding of flies life cycle is necessary to anticipate the possible changes in survival and developmental effects caused by drug abuse.

Flies are holometabolous insects meaning they undergo metamorphosis divided into four stages: embryo, larval, pupal, and adult stage (28). The cycle starts after the female fly lays embryos onto food media, which develop into first-instar larvae after roughly 24 hours. The 1st-instar stadium larvae crawl on the food media surface to feed, and then molt into second-instar larvae which dig through the food for the next 3-4 days. After, they molt into third-instar larvae and start crawling up the container wall to find a suitable place for pupariation and metamorphosis. Pupariation is characterized by release of ecdysteroid hormone secreted from the ring gland, after which the larval cuticle forms a pupal case that lasts until eclosion. Metamorphosis is separated into two stages: a 12-hour prepupal period (the onset of the larval-pupal transition), and a subsequent pupal period lasting around 84 hours. When the process is finished and flies are formed, naïve virgin flies hatch driven by an eclosion hormone (28). Virgin flies can be discerned from adult flies due to their lighter color and puffiness, along with a meconium (a dark spot on the abdomen). The virgin flies are not sexually active, females become active after 1-4 days, while males after 2-3 days (27).

1.7.1. Monoamines in fly's development stages

Drosophila has DA and serotonin transporters homologues to human ones, along with tyramine and octopamine whose functions are homologous to epinephrine and norepinephrine in mammals (29). In the larval stage there

are traces of octopamine, tyramine, DA, and 5-HT in the brain tissue, and in pupal stage octopamine is not present but concentrations of DA, 5-HT and tyramine are higher. In adult stage tyramine, octopamine, DA, and 5-HT are all present. The tyramine probably inhibits octopamine related behaviors (aggression, feeding) and locomotion, explaining its higher levels in pupal stage where these behaviors do not happen. The DA levels are higher in female flies (also observed in rats), likely because females use it for pheromone production (29). DA and 5-HT play a role in cognition, appetite, attention, and locomotion regulation (30). Accordingly, accumulation and degradation of monoamines (predominantly DA and 5-HT) due to METH consumption could affect the development of flies.

1.8. The effect of METH on glucose metabolism

Current research shows that METH consumption disrupts energy metabolism by decreasing glucose levels in flies, but not how it occurs. This led to formulation of two hypotheses; the "metabolic hypothesis" stating that METH disrupts metabolism by altering gene expression and "behavior hypothesis" claiming metabolism disruption by METH induces behavior changes. Over 48 hours of METH exposure, fly's energy storage molecules triglycerides and glycogen show steady decrease, suggesting a negative caloric balance (26). Also, the levels of trehalose (the blood sugar in insects) are depleted in flies consuming METH, as well as levels of intracellular amino acids. Alongside causing anorexia by decreasing food intake, METH increases the locomotion of flies, additionally exhausting carbohydrate reserves (26). This signifies that anorexic METH effects could impact physiology of flies, predominantly by causing weight loss, which could lead to death. This also leads to question if effects of preferential METH consumption could be seen in the physiology of the progeny.

1.9. Artificial selection of *Drosophila*

During the development and validation of the FlyCAFE platform, among the flies that showed preferential consumption of METH it was observed that there are two subpopulations, one with low, other with high METH preference. This was the basis for the artificial selection with the aim to create two divergent lines, the High Preferring (HP) and the Low Preferring (LP) flies. Three male flies with the highest and lowest preference for METH were selected and mated with *wt* virgins to create the HP and LP lines. This process continued through generations, and the preference of each generation (and their filial generations) was evaluated using FlyCAFE.

Numerous addiction studies indicate that COC and METH abuse activates molecular mechanisms leading to neurological changes and lasting epigenetic remodeling (16,31). Drug induced epigenetic changes can make an individual more vulnerable and susceptible to relapse and drug-seeking behavior. This thesis will try to ascertain if preferential METH consumption between HP and LP lines leads to changes in behavioral phenotypes. As the experiment is conducted on 28th generation of HP and LP flies (and their filial generations), the results will provide an insight into behavioral and physiological changes that occur as consequence of high or low preference for METH.

2. Aims

The main aim of this thesis was to determine if there are any behavioral or morphological differences between HP and LP lines after 28 generations of artificial selection for preferential METH administration compared to starting strain (control).

The sub-aims were:

1. Test if there are differences between HP and LP lines in the sensitivity to vMETH and vCOC and the development of locomotor sensitization.

2. Test the stability of the HP and LP phenotypes by testing preferential consumption of METH in HP and LP lines in 6^{th} filial progeny (F6) of the 28^{th} generation relative to the starting strain and the 28^{th} generation.

3. Since preferential METH self-administration affects tyramine levels that is essential for the enclosion during pupation stage, the third aim was to measure duration of the life cycle and reproduction success in HP and LP lines.

4. METH consumption affects the glucose metabolism, so the fourth aim was to measure size and weight of the third-instar larvae and adult males and females in HP and LP lines relative to control flies.

5. METH administration changes regulation of monoaminergic signaling, so the fifth aim was to measure phenotypes that are dependent on monoaminergic concentration: negative geotaxis, sleep, and activity in HP and LP relative to control flies.

3. Materials and methods

3.1. Fly strains

Flies used were *wild type Canton S* strain (donation from C. Helfrich Forster) that underwent 28th and 30th generation genetic selection for high (HP) and low (LP) METH consumption preference and control *wild type* flies of the same strain. In this thesis control flies are referred to as *wt* flies, and they had not undergone artificial selection as is the case with HP and LP lines. The behavioral assays were performed using 3-5 days old male flies raised in an incubator with 12-hour day: 12-hour night intervals, at 25℃ and 70% humidity. The fly food media was cornmeal based and consisted of cornmeal, agar type II, tap water, sugar, dry yeast, nipagin and propionic acid.

3.2. Chemicals

Methamphetamine-hydrochloride, cocaine- hydrochloride (≥ 97,5%) and mineral oil were purchased from Sigma-Aldrich, ethanol from VWR, and food supplies and sucrose from a local store.

3.3. Experimental design and protocol

Behavioral tests were segregated in non-drug and drug induced. Non-drug tests were morphology (measurement of L3 larva and adult length and mass), life cycle (time from embryo to adult fly and the number of pupae and adults), negative geotaxis, activity, and sleep analysis. Drug induced were preferential METH consumption using FlyCAFE assay and motor activating effects induced by volatilized cocaine (vCOC) or methamphetamine (vMETH) administration, measured using FlyBong platform.

3.3.1. FlyBong assay

The FlyBong is a high-throughput drug administration platform used to distribute volatilized methamphetamine (vMETH) and cocaine (vCOC) to flies. The platform consists of drug delivery and activity monitoring part. The drug delivery part consists of a volatilization chamber, an air pump, and a Gas

Distribution Manifold, while the activity monitoring part consists of commercially available monitoring system and air delivery manifold (32).

Under CO_2 anesthesia male flies were collected and individually housed in 32 polycarbonate tubes with small holes for airflow. On one end the polycarbonate tube was filled with food media and sealed with parafilm to prevent starvation and dehydration. On the other end the tube was connected to the dispenser which ensured equal drug aerosol distribution. To track the locomotor activity of flies *Drosophila* activity monitoring system (DAMS) was used. DAMS measured locomotor activity via infrared light beam placed in the middle of the polycarbonate tubes. The assembled structure was connected to the drug delivery part via rubber tubes.

75 µL of 10 mg/mL methamphetamine hydrochloride (METH-HCl) ethanol or cocaine hydrochloride (COC-HCl) water solution was pipetted into three neck flasks placed into heating caps that served as volatilization apparatus. The solution was pipetted 4 to 6 hours before drug administration to ensure the ethanol evaporation. The flasks were then corked, and the heating cap turned on for 8 minutes to achieve the temperature of 185-200℃ and allow METH or COC vaporization. After the heat was turned off, pumps were turned on for 1 minute to deliver METH or COC aerosol to the flies. The first dose of vMETH was administered at 9 AM and the second at 7 PM, while vCOC was administered at 9 AM and 3 PM. Control lines underwent the same process but were exposed only to hot air. All assays were done in triplicates for *wt*, HP, and LP lines.

DAMS measures locomotor activity of individual flies as the number of times that it crosses the middle of the tube, and this allows populational and individual data analysis (32). Population data was collected in one minute resolution for 10 minutes before and after drug administration (without heating and drug delivery time) and presented as mean of counts/min (33).

Individual analysis compares activity in counts/minute of individual flies in the first 10 minutes before (baseline) and after METH administration, and for cocaine the interval is 5 minutes. The locomotor activity of baseline is then compared to activity after first drug administration. The number of flies with increased activity after the first dose, relative to baseline, represents sensitivity (SENS) to the drug (METH or COC) (32). Locomotor sensitization (LS) of individual flies is achieved if flies increase their locomotor activity in counts/minute after the first drug exposure relative to baseline, and then further increase the activity after second drug exposure relative to first dose (32).

3.3.2. FlyCAFE assay

FlyCAFE assay consists of modified two-choice CApillary FEeding (CAFE) assay in DAM system. 32 flies were individually housed in glass tubes with 1,5 cm long rubber caps at both ends. The caps were covered with nylon mesh and secured with parafilm to allow air and water vapor passage to prevent dehydration. On each rubber cap was a hole that fitted a 200 µL pipette tip that held a 5 µL glass capillary (Figure 1A) (34).

Capillary was filled via capillary action and contained liquid food that had mineral oil on top to minimize evaporation (34). The food consisted of 0,1M sucrose and 0,25 g/mL yeast solutions. The METH food had an additional 1 mg/mL of METH dissolved in distilled water added to sucrose solution. The height of food in the capillaries was measured with a ruler, and capillaries were then inserted into the pipette tip to be easily accessible to flies. The amount of self-administered liquid food in the capillaries was measured at 9 AM for three consecutive days, and then replaced with fresh capillaries.

To obtain the amount of consumed food, the difference in measured liquid height was calculated. To correct for the evaporation, there were control evaporation tubes that housed no flies but had liquid food capillaries placed

on both sides. After calculating the evaporation difference, the consumed food amount was corrected for average evaporation and the result was multiplied by the cross–sectional area of the capillaries (34). The result was the volume of consumed food in µL. Lastly, preferential consummation was obtained by calculating the difference in consumption of METH food versus sucrose-yeast food.

The DAMS monitor was connected to a computer and placed on a pedestal in a plastic tub filled with 1 L of tap water, which was covered with cling film to prevent evaporation and minimize humidity fluctuations. The structure was then placed in an incubator at 24℃ in constant darkness to prevent side preference due to environmental cues (Figure 1B) (34).

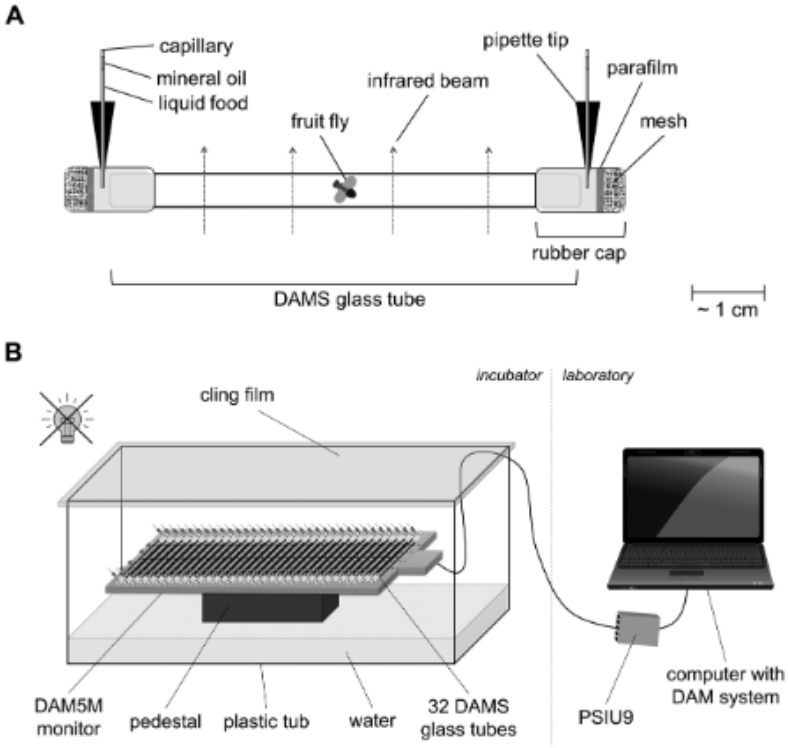

Figure 1. FlyCAFE: an assay for measuring preferential food consummation of individual *Drosophila melanogaster*. Scheme adapted from Rigo et al. (2021) (34). **A)** *Drosophila* activity monitoring system (DAMS) glass tubes with rubber caps and inserted liquid food capillaries. **B)** FlyCAFE apparatus consisting of DAMS monitor

(housing 32 flies) connected to a computer for locomotor activity tracking and placed in a closed water filled container.

3.3.3. Life cycle

The life cycle of flies was tracked by monitoring embryo to fly development. Firstly, an adult male was taken from cultivation vial of the 28th generation and put into a vial containing two *wt* virgin flies. The vials were then left in the incubator for female flies to lay eggs and for 7 days embryos were counted since the first embryo appearance. On the 6th day fist pupae were spotted, and on the 11th day fist flies hatched. Since the first one appeared, both pupae and flies were counted for 10 consecutive days. This was done five times for HP, LP, and *wt* groups.

3.3.4. Morphology of adult flies and L3 larvae

Weight and size of HP and LP flies and their larvae was measured in L3 stage (crawling on the vertical, clean vial wall). Eight to ten larvae were removed from cultivating vials with tweezers and washed in 1 x Phosphate-buffered saline (PBS). The larvae were then placed in a drop of glycerin and heated at 70℃ for 15 seconds in a heat block to immobilize them (35). Using a light microscope, the larvae were visualized and measured using the ImageJ program.

Ten male and female adult flies were separated using CO_2 anesthesia and then frozen at -20℃. Afterwards, they were fixated on a glass slide with a drop of glycerin, visualized under a light microscope and their length was measured using the ImageJ program.

The weight of both larvae and adult flies was measured using an analytical scale. Eight adult male and female flies were removed and separated from the cultivation vials under CO_2 while eight L3 larvae were removed from vials using tweezers and placed in the pre-weighted 2 mL Eppendorf tubes. The tubes

were then put on the scale and weight was measured for each group (HP, LP, and *wt*).

3.3.5. Negative geotaxis assay

Negative geotaxis is a phenotype which refers to the preference of flies to move away from the gravity source (36). It is measured by the elapsed time needed for flies to climb a given height of the plastic vial after mechanical stimulation. Three-day old males were collected under CO_2 anesthesia and transferred into vials. In the experiment there were 10 flies per each of five tubes for three groups (HP, LP and *wt*), and percent of flies that reached given height was measured ten seconds after gently tapping the tube so that all flies fall to the bottom of the tube . This process was repeated five times for each set of five tubes.

3.3.6. Activity and sleep analysis

The locomotor activity of flies was measured by DAM system with one red infrared beam placed in the middle of the glass tubes that measured number of times that an individual fly passed through. The flies were collected under CO_2 anesthesia and individually housed in glass tubes that were sealed with food on one end and sponge at the other which enabled days long data collection. There were six monitors that housed 32 flies: three placed in 12-hour light: dark (LD) conditions, and three in dark: dark (DD) conditions (complete darkness). DAMS recorded the activity and sleep of flies for seven days in one hour range. The raw data of fly activity is characterized by interruption of the light beam in the DAMS monitor (count/min), while sleep is determined as an interval without beam interruption (quiescence). The minimal time frame used for sleep is 5 minutes (37).

3.4. Data analysis

Initial data analysis was done using MS Excel program. Additional statistical analysis and figures were done in GraphPad Prism 9.5.1. statistical analysis program. Statistical significance was determined using Ordinary One-way ANOVA test with Tukey multiple comparisons test.

4. Results

To determine whether artificial selection for high (HP) and low (LP) methamphetamine preference over 28 generations has an effect on the behavioral phenotypes of flies, we tested first filial generation of offspring of distinctly different parental types (F1) and further their progeny (F2).

4.1. Drug exposure and self-administration tests

We have tested the F2 (and their offspring) of the 28th generation for drug induced behavioral phenotypes of acute dose sensitivity, two dose induced behavioral sensitization and the preferential METH consumption of flies using FlyBong and FlyCAFE methods.

4.1.1. Motor-activating effects of vMETH and vCOC are different in selected lines

FlyBong was used to deliver one or two doses of volatilized METH (vMETH) or COC (vCOC) to the flies. Results were analyzed on a population level as average of 5-minute intervals for vCOC and 10-minute intervals for vMETH and on an individual level as percentage of flies responding to the first and to the second exposure. Flies response to one dose was named sensitivity (SENS), and to two doses locomotor sensitization (LS).

Comparison of populational fly lines responses to vMETH can be seen in Figure 2. In baseline (Figure 2A) there are no significant differences in activity, although HP and LP lines are more active than *wt* flies. After the first vMETH dose (Figure 2B) both HP and LP files show significantly higher activity levels than *wt*, with all groups showing an increase in activity when compared to the baseline. After the second dose (Figure 2C) HP and LP flies have lower activity than *wt* flies but with no significant difference, however their activity is increased when compared to baseline. The exposure of the control group to

the hot air also increases the activity of flies from all lines, with HP line showing lowest and LP highest activity levels (Figure 11. of Supplement).

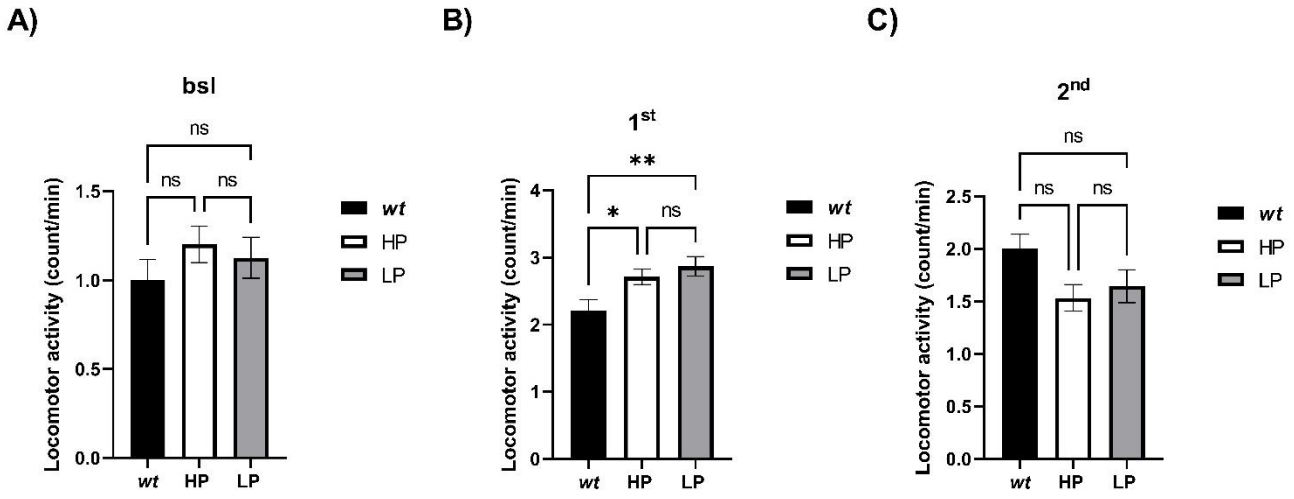

Figure 2. Selected strains show an increase in the locomotor activity after single vMETH administration. Flies used in experiment were 3-5 days old males: wild type *Canton S* (*wt*, *n*=32), F2 progeny of the 28th generation of selection with high (HP, *n*=32) and low (LP, *n*=32) preference for METH. Locomotor activity of the population 10 minutes before (bsl) **A)**, and after exposure to the first (1st) **B)**, and second (2nd) **C)** dose of vMETH. The first dose was administered at 9 AM and the second at 7 PM (10-hour gap). Data are presented as average ± SEM**.** One-way ANOVA with Tukey's multiple comparisons test. **ns**= no significance, ***** p<0,05, ****** p<0,005.

Prior to vCOC exposure flies with low and high preference for METH show significantly higher locomotor activity than *wt* flies (Figure 3A). Following the first dose, the locomotor activity of LP line increases compared to the baseline, similar as in *wt* flies, while the HP line shows a significantly smaller increase relative to the *wt* and LP line (Figure 3B). After the second vCOC dose flies have higher activity than baseline and lower activity than after a single vCOC administration (Figure 3C). HP and LP lines have similar locomotor activity levels, while *wt* shows highest activity with significant difference to LP line. Flies exposed to hot air show increased activity after one and two exposures compared to baseline (Figure 12. of Supplement). HP line shows lowest, while

wt shows highest locomotor activity after one and two exposures of hot air among lines.

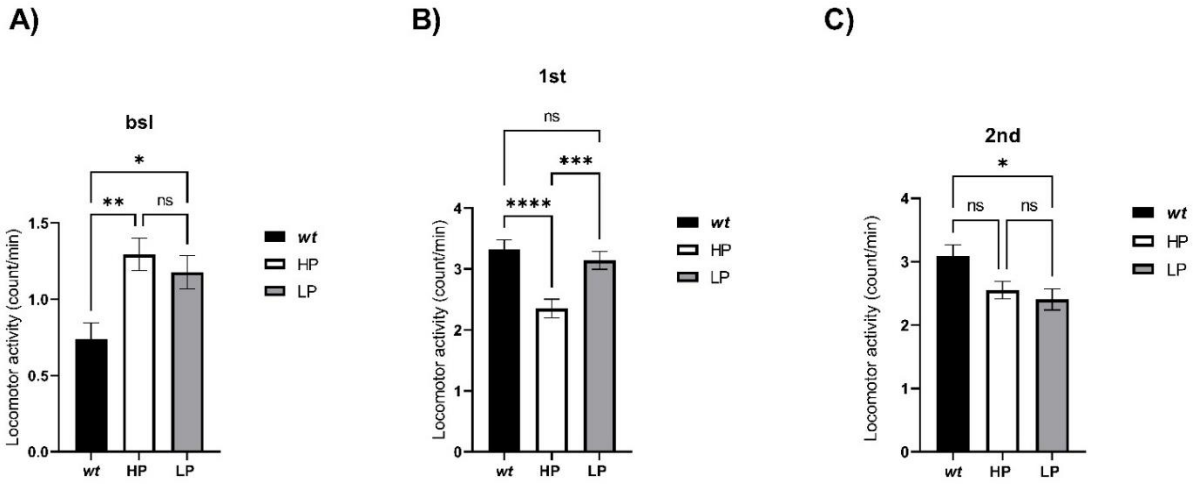

Figure 3. HP and LP lines differ in the locomotor activity after a single vCOC administration. Flies used in experiment were 3-5 days old males: wild type *Canton S* (*wt*, *n*=32), F2 of 28th generation of flies with high (HP, *n*=32) and low (LP, *n*=32) preference for METH. Locomotor activity of the population 5 minutes before (bsl) **A)**, and after exposure to the first (1st) **B)**, and second (2nd) **C)** dose of vCOC. The first dose was administered at 9 AM and the second at 3 PM (6-hour gap). Data was analyzed with One-way ANOVA with Tukey`s multiple comparisons test. **ns**= no significance, ***** p<0,05, ****** p<0,005, ******* p<0,0005, ******** p<0,0001.

On individual level flies show high sensitivity (SENS) to hot air, vMETH and vCOC (Figure 13. of Supplement). Among lines there is no significant difference in SENS, with HP line showing lower SENS to vCOC and hot air than LP and *wt*. Locomotor sensitization (LS) of individual flies from the 28th HP and LP generation does not differ from *wt* after exposure to hot air or psychostimulants vMETH or vCOC (Figure 14. of Supplement). Both HP and LP show lower LS levels than *wt* flies.

4.1.2. HP and LP flies show consistent preference and aversion to METH

The second test was FlyCAFE through which the preferential self-administration of METH over sucrose food was measured. The aim was to determine if F6 progeny of the 28th and F2 progeny of the 30th generation (not shown) retained the HP and LP phenotype as the original selected lines. Food was offered in 5 µL glass capillaries and the intake in µL for individual flies was measured at 9 AM for three consecutive days (each day fresh food was offered).

METH preference of flies diverges within a line during the selection, with high preference (HP) line continuously self-administrates METH food over sucrose, while low preference (LP) line prefers sucrose food (Figure 4A). After the selection was relaxed for six filial generations after the 28th generation of selection, flies maintained the consistent HP and LP phenotype (Figure 4B).

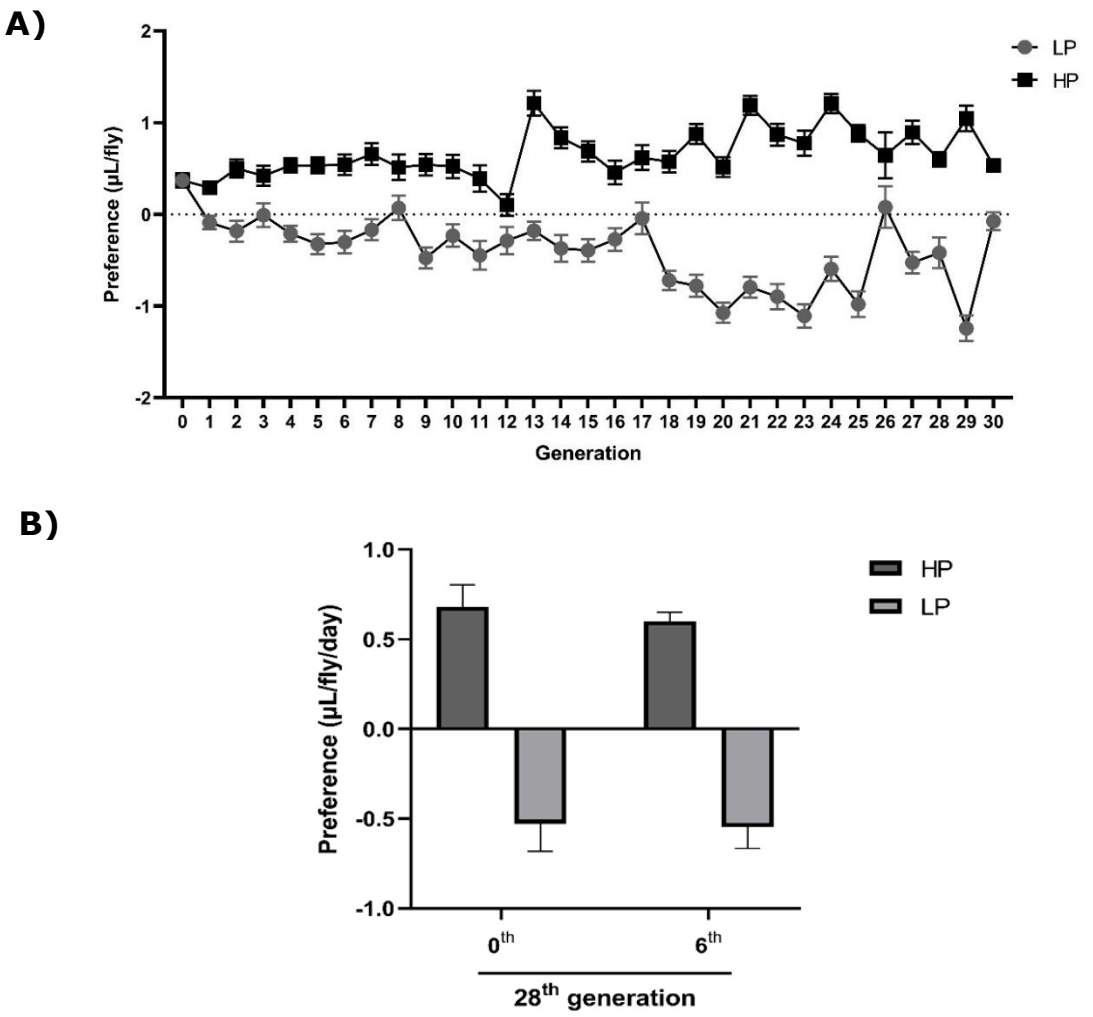

Figure 4. Flies of the 28th generation of selection maintain the HP and LP phenotype after six generations without selection . A) Preferential self-administration of high (HP) and low (LP) preference flies from 0-30th generation. Flies were offered sucrose and METH food in capillaries and for three consecutive days and consumption in µL was measured at 9AM. Points represent mean of preference over three days with SEM. **B)** Food consumption of HP and LP flies from the F0 (*n*=22) and F6 (*n*=22) selection of the 28th generation. Columns represent mean of preference of fly per day in µL with SEM.

4.2. Preferential METH consumption effects on behavioral phenotypes and life cycle

To address other possible genetic effects that were created by artificial selection, by low and high METH preference we have assessed duration of development, morphology, negative geotaxis, sleep, and activity of flies.

4.2.1. HP flies have lower number of adult progeny

To determine if preferential METH consumption transgenerationally affects the life cycle of flies, we quantified the number of embryos, pupae, and flies. The observed HP and LP flies of 3rd generation of the selected 28th line. Vials containing two virgin *wt* females and one male from the experimental and control groups were used as parents. The number of embryos was quantified for seven, and pupae and flies for ten days after the appearance of the first embryo, pupa, and fly.

The results indicate that the number of embryos is the highest in the *wt* group (Figure 5A,B). However, the highest embryo to pupae ratio appears in HP group which had the lowest embryo count, meaning the most of embryos are enclosed to pupae in this group (Figure 5C,D). When observing the daily quantity of pupae (Figure 5C), HP and LP lines have an instant increase in pupae on first day (faster pupariation) which starts varying after the initial growth, while the count of *wt* pupae steadily increases. After the fifth day the number of pupae significantly decreases in all groups. Total cumulative number of eclosed flies shows that *wt* and LP lines have similar cumulative numbers (Figure 5F), even though the daily graph (Figure 5E) shows steady increase of flies for *wt* up until the sixth day after which it gradually decreases. There is similar enclosing pattern for HP and LP lines; their flies enclose earlier then *wt* flies, but they show drastic decrease in number after the sixth day. The results indicate that preferential METH self-administration has a negative effect on fly eclosion, i.e., number of progeny (Figure 5F).

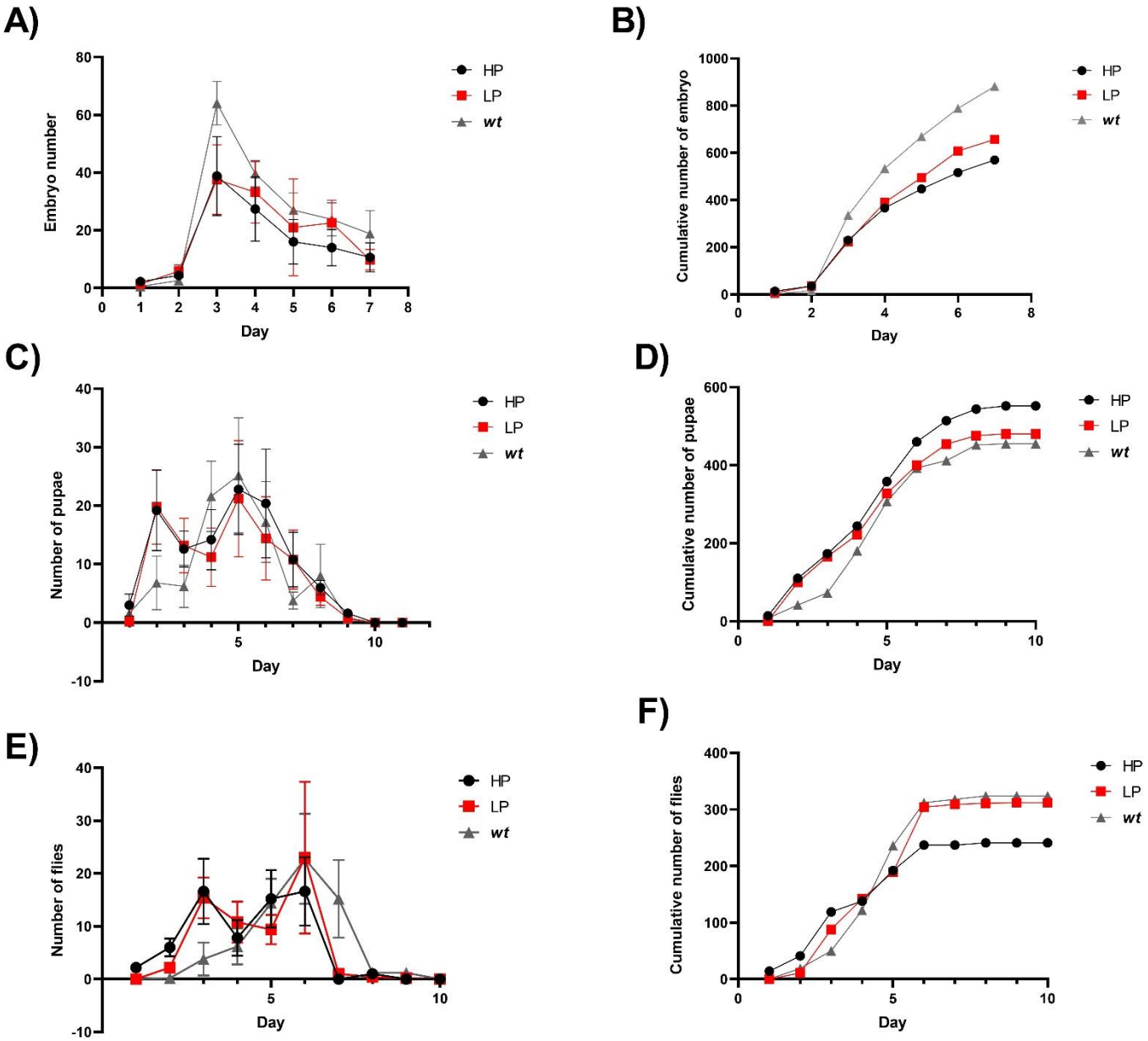

Figure 5. Preferential METH self-administration decreases number of fly progeny. One adult male from a respective group (HP, LP and *wt*) and two *wt* virgins were put into a vial (n=5) and life cycle was tracked since first embryo appearance. Embryos were quantified for seven days and pupae and flies for ten days. Graphs **A)** and **B)** respectively show daily and cumulative number of embryos per line. Number of pupae can be seen in graphs **C)** (daily) and **D)** (cumulative), while number of flies is shown in graphs **E)** (daily) and **F)** (cumulative) for each line (HP, LP and *wt*).

4.2.2. Morphology

The morphology of flies and L3 larvae was determined by measuring their length and weight. The aim was to determine if selection for HP and LP phenotype led to changes in length and weight of adult flies and the L3-instar larvae in the F5 of the 28th generation of HP and LP flies.

The weight of the adult flies and L3-instar larvae was measured using an analytical scale. Eight female and male flies were collected from the cultivation vials using CO2 anesthesia and placed in pre-weighted 2 mL Eppendorf tubes and weighted. L3-instar larvae underwent the same process after they were collected with tweezers. It is noticeable that HP flies weigh the most out of other lines, while LP flies follow closely and show a difference in relation to wt flies (Figure 6A,B). Meanwhile, L3 larvae of HP line seem to be heavier than wt and LP lines (Figure 6C).

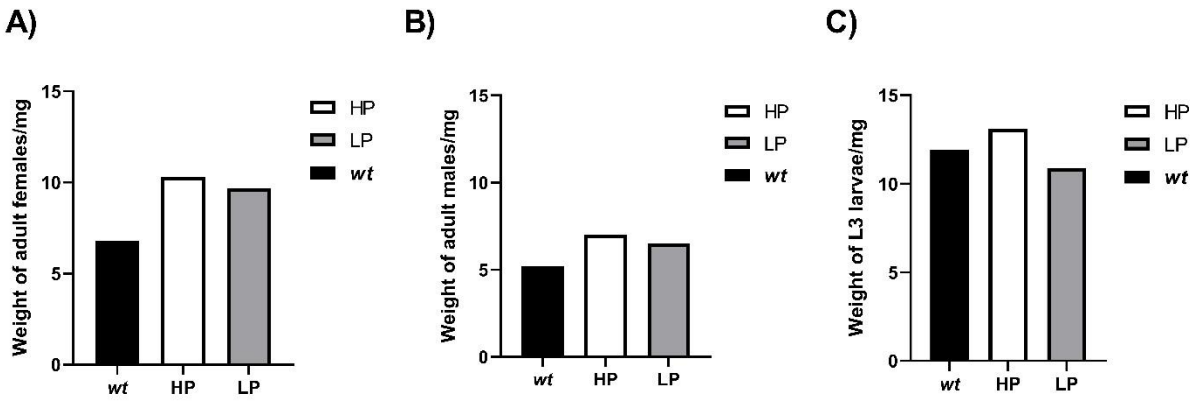

Figure 6. Effect of preferential METH consumption on weight of female flies A), male flies B), and L3 larvae C) of HP, LP, and *wt* groups. Eight samples of flies and L3 larvae were collected from cultivation vials and weighed in Eppendorf tubes. Data is presented as weight of eight specimens and was not analyzed with statistical tests due to small sample size.

The size of flies and L3 larvae was measured using a light microscope and ImageJ program after collecting them from the cultivation vials and immobilizing them in a drop of glycerin. Both female and male LP flies appear to be larger than flies of other lines, in both sexes with statistical significance between HP an LP lines (Figure 7A,B). However, the size of flies does not correlate with their respective larval sizes (Figure 7C) or their larval weights (Figure 6C).

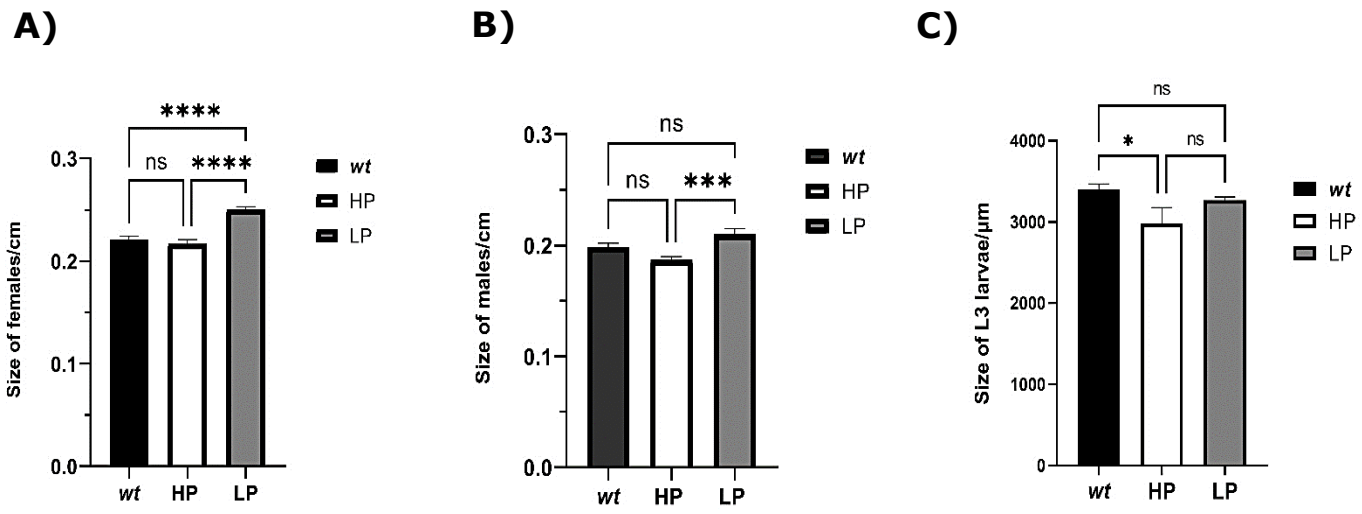

Figure 7. Effect of preferential METH self-administration on the size of *Drosophila* females A), males B), and L3 larvae C). Ten flies were separated under CO_2 anesthesia and immobilized in a drop of glycerin before visualizing them with light microscope and measuring their size using ImageJ program. L3 larvae (*n*=10) were collected using tweezers and washed in 1 x PBS buffer before undergoing the same process described for flies. The data is presented as mean of size ± SEM and was analyzed using One-way ANOVA with Tukey`s multiple comparisons test. **ns**= no significance, ***** p<0,05, ******* p<0,0005, ******** p<0,0001.

4.2.3. Preferential METH self-administration decreases negative geotaxis in *Drosophila melanogaster*

One of experiments that allows the characterization of flies locomotor ability is negative geotaxis. In this experiment it was determined if preferential self-administration of METH influences a fly's ability to vertically climb up the

plastic vial to a given height after mechanical stimulation (startle). We tested the 30th generation HP and LP flies, and the results (Figure 8) show that there is significant decrease in percentage of flies from the LP line to climb the vertical surface, relative to *wt*.

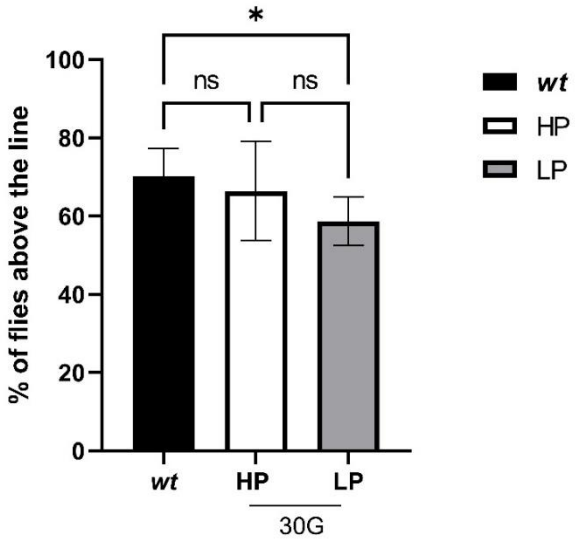

Figure 8. LP flies have decreased climbing ability in the negative geotaxis test. Male flies were collected under CO_2 anesthesia and put into five vials in groups of ten, after which their ability to climb up the plastic vial was timed. The experiment was repeated ten times. The data is presented as percentage of flies that climbed above the determined height ± SEM and was analyzed using One-way ANOVA with Tukey`s multiple comparisons test. **ns**= no significance, ***** p<0,05.

4.2.4. Preferential METH consumption potentially increases locomotor activity and decreases sleep amount

DAM system was used to track the number of midline crossings that flies have made throughout seven days. The aim was to determine if the selection of flies for the preferential METH self-administration affects the activity and sleep of the second generation progeny of the 28th generation of selection. In the experiment there were six monitors housing 32 male flies; three (HP, LP and *wt* line) were kept in 12-hour light: 12-hour dark (LD) conditions and three (HP, LP and *wt*) were kept in 24-hour darkness (DD). The activity of flies measured in counts/minute is higher in DD than in LD conditions (Figure 9).

F2 HP and LP flies of the 28th generation have the higher activity than control *wt* flies in both LD (Figure 9A) and DD conditions (Figure 9B).

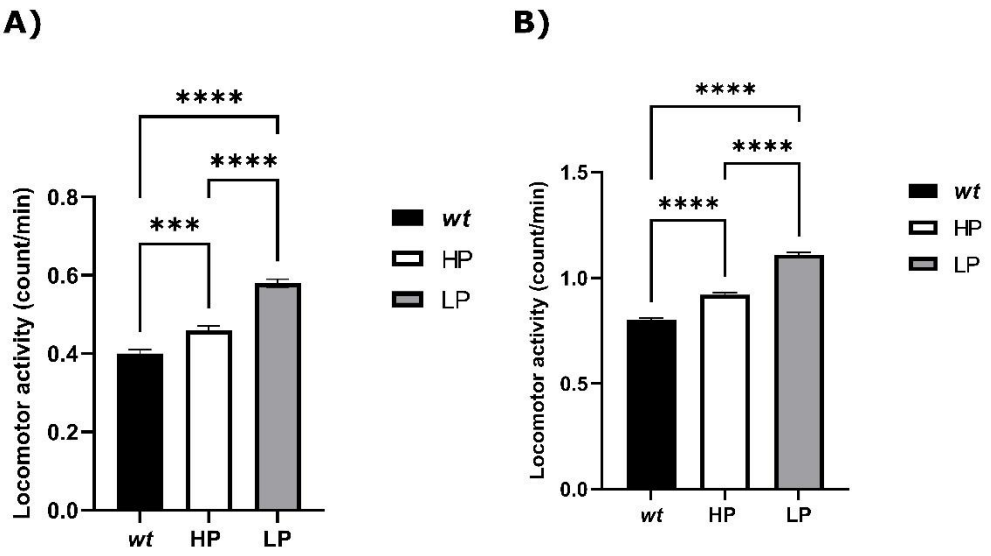

Figure 9. HP and LP lines of the 28th generation of selection have higher activity than *wt* flies in LD A), and DD B) conditions. Adult male flies (n=32) were collected, and activity was tracked by DAM system for seven days in counts per minute. The data is graphed as mean ± SEM and analyzed with One-way ANOVA with Tukey`s multiple comparisons test. *** p<0,0005, **** p<0,0001.

Sleep measured by DAM system was recorded in minutes per hour and was determined as sleep when there was minimally five minutes without light beam interruption. Data obtained for sleep (Figure 10) is consistent with data obtained for activity; flies have a higher sleep amount in LD (at around 40 min/hour) (Figure 10A) than in DD conditions (around 30 min/hour) (Figure 10B). The F2 LP and HP lines of the 28th generation have lower sleep amounts than the control *wt* line, which suggests a transgenerational effect of the METH on sleep. Furthermore, HP line snows significantly higher sleep amount than the LP line.

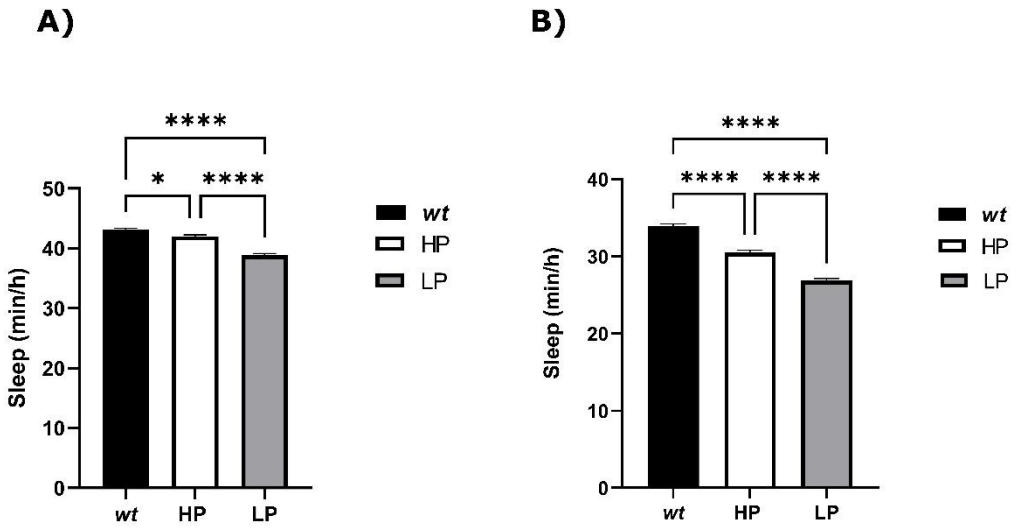

Figure 10. In LD A), and DD B) conditions *wt* flies show a higher amount of sleep than high (HP) and low (LP) preference F2 flies from 28th generation . Adult males (n=32) were collected from cultivation vials and sleep was tracked by DAMS for seven days in minutes per hour. The data is presented as mean of quiescence ± SEM and analyzed with One-way ANOVA with Tukey`s multiple comparisons test. ***** p<0,05, ******** p<0,0001.

5. Discussion

Drug addiction is characterized by voluntary self-administration of the abused substance, leading to neurocircuit changes and pathological conditions. The main aim of this thesis is to determine if preferential METH self-administration affects lifespan and behavioral phenotypes of *Drosophila melanogaster*. The experimental group consisted of 28^{th} and 30^{th} generation male flies progeny with high and low preference for METH, artificially selected based on their voluntary food consumption. We observed the life cycle showing lower progeny in HP line and conducted contingent experiment indicating that HP and LP lines continue to show their phenotypes for at least 6 generations after selection was relaxed. Furthermore, we have done experiments showing increased locomotion of flies after vMETH and vCOC administration, as well as several behavioral phenotypes of *Drosophila* including sleep, activity, and negative geotaxis which showed impairment.

Behavioral sensitization is the one of the most often studies endophenotypes in laboratory animals, primarily in relation to motor-activating effects of psychostimulants COC and METH. The method of drug administration is an important factor affecting the data. The methods like intraperitoneal injection or airbrush required complicated animal handling and was time consuming (18). Therefore, FlyBong was established: a high-throughput method for enabling delivery of precise psychostimulant amount to flies and objective quantification of locomotor activity, while lowering errors due to animal handling (17). The FlyBong was used to deliver vCOC and vMETH to individually housed male flies which show lower sensitivity to hot air than females, and it enabled us populational and individual locomotor activity analysis (16,17).

The exposure to psychostimulants vCOC and vMETH caused an increase in locomotor activity of flies which is consistent with previous studies (16,18,21,26). At population baseline HP flies show highest locomotor activity

possibly indicating that paternal METH consumption led to changes in offsprings DA level since METH increases extracellular DA making the flies more active. Furthermore, after vCOC and vMETH exposure, HP line shows lowest locomotor activity indicating that preferential METH self-administration potentially induces a need for a higher drug dose for it to cause the same rewarding effect as in the *wt* flies. The results obtained for hot air show significant differences among lines after first and second dose administration. The activity measured alongside vMETH (7 PM) shows that LP flies have highest activity, while activity measured with vCOC (3 PM) shows highest activity in *wt* line, with HP having the lowest activity at both times. This could be explained by the fact that hot air stimulates flies with no or with low paternal consumption to METH, while flies with high paternal METH self-administration need a stronger stimulant to express locomotor activity due to DA disbalance caused by the drug. However, we cannot conclude this with confidence because of the unexpected results obtained in the *wt* group, indicating that either the experimental protocol was not properly conducted or that the method itself needs further improvements.

Previous research indicates that individual flies develop SENS and LS to psychostimulants but to much lower percentage to the control hot air (16–18). Our results show that individual flies with prior METH exposure (HP) develop lower SENS and LS than flies with low (LP) or none (*wt*) prior exposure. This is consistent with previous research showing that preferential self-administration of METH food prevents the expression of locomotor sensitization to vMETH (16). All fly lines show high individual SENS but only a subgroup develops LS, indicating that SENS and LS have different regulation mechanisms (17). When comparing both SENS and LS of HP, LP, and *wt* lines, there is no statistical difference in their levels which was unexpected. We expected to see significantly lower SENS and LS of *wt* flies exposed to hot air than *wt* exposed to vMETH and vCOC (17). This could be due to a too high

psychostimulant dose which lead to hyperactivity of flies, so experiment should be repeated with different doses to further examine this result. Also, the FlyBong platform should be further validated since there seem to be issues with hot air delivery, making it difficult to determine if changes in fly locomotion are indeed induced by the drug or by other factors such as hot air.

Assays of voluntary drug intake are methods of high validity that allow quantification of voluntary self-administration of individual flies over time. METH and COC have reinforcing properties acting through mesolimbic DA system. By projecting from the midbrain to striatum and nucleus accumbens, they activate rewarding pathways (38). Alongside causing euphoria, METH abuse lowers serotonin levels, leading to drug-carving and drug-seeking to improve the mood after the effects of METH wear out. Flies will even endure a punishing effect (e.g., electric shock) to obtain the drug (6).

Addiction is a disorder with up to 72% heritability. This prompted us to see if the progeny of selected lines continue to show the preference several generations after the end of selection. We tested the preference of HP and LP flies at F6 of the 28th generation and compared them to null generation *wt.* Even after six generation of no selection the lines showed the consistent high and low preference for METH. The stability of preference is further emphasized within the generation as F6 and F0 HP and LP flies show almost no differences in METH preference. This alludes to heritability (transgenerationally) of METH preference, meaning that the offspring not conditioned to the drug still preferentially consumes it. Corresponding with our results, Le at al. showed that paternal addiction to COC in rats leads to increased COC self-administration in offspring (39). Results suggest that paternal psychostimulants abuse causes epigenetic changes like DNA methylation in mammals and DNA acetylation in *Drosophila melanogaster* sperm, supporting the epigenetic effect on the transgenerational inheritance (39).

Environmental factors such as stress, psychostimulants abuse and malnutrition negatively affect the lifespan and procreation of mammals and flies. METH consumption in pregnant rats increases pup mortality, slows weight gain, impairs cognition, and slows reflexes (40). In humans, maternal METH abuse changes placenta by limiting nutrients intake through vasoconstriction, delays mental development and lowers attention span in offspring by neuroplastic changes (monoamines). METH also negatively affects male reproduction system, although the molecular impact on spermatogenesis is not known (41). We have artificially created HP and LP lines through selective breeding of male flies with *wt* virgins in each generation of selection. By observing lifespan and life cycle of F3 HP and LP lines of the 28th generation we can determine if METH effects on male reproduction can be seen in the filial generations.

Andretić et al. demonstrated METH induced increase of arousal in male flies is characterized by latency in courtship initiation, likely because males cannot interpret female behavior (19). A probable explanation is METH`s effect on DA , which is supported by research where inhibition of DA synthesis caused latency in courtship and mating (19). Our results show that there is no difference in duration of the life cycle among the flies exposed to METH and control, indicating there is no transgenerational effect of METH on copulation. Preliminary research also showed that vMETH exposure has no effect on duration of the life cycle of F1 generation (42).

Preliminary research shows an increase in the numbers of embryos, pupae, and flies after vMETH administration in F1 generation. (27,42). Our results demonstrate that METH has a negative transgenerational effect on the number of flies. LP and *wt* flies follow the same pattern and have similar numbers of progeny, while HP line significantly differs. HP line has the highest embryo to pupa, but the lowest pupa to fly ratio. High pupal and low fly numbers of HP line could be due to METH induced monoamine disbalance.

Tyramine and octopamine are monoamines that potentially control ecdysone production by activating β3-octopamine receptor (Octβ3R) in an autocrine manner (28). Ecdysone is steroid molting hormone responsible for larva-prepupa and prepupa-pupa metamorphosis (28). Tissue analysis showed a high tyramine and low octopamine levels in pupae (29), leading us to assume that METH modulates tyramine levels causing early larval enclosing and arresting fly development in pupal stage. Moreover, if flies even hatch, they will more likely have impaired endophenotypes like sleep, activity, and negative geotaxis than flies with normal monoamine levels.

Drosophila sleep and activity are modulated by environmental (light, social environment) and internal signals (DA, octopamine, circadian clock). DA and octopamine have a role in sleep of flies, where DA plays a more important role (43). METH consumption affects levels of DA which is a strong wake promoting signal in flies and mammals (43). Our results show that flies of all lines (*wt*, LP and HP) sleep less in constant darkness (DD) than light-darkness (LD) conditions, while the opposite is true for activity. This is consistent with the fact that sleep in DD conditions is more sensitive to DA modulation than sleep in LD conditions (43). 28^{th} generation F2 LP and HP lines have lower amounts of sleep than *wt* flies, while having higher activity levels. It should be noted that increased activity and decreased sleep in LP flies ca be explained by their self-administration of METH, even though it is in a small dose. LP flies self-administrate both METH laced and sucrose food, but interestingly their preference index is higher for sucrose food, indicating a change in the fly's phenotype. Therefore, the differences in sleep and activity of LP line should also be considered in terms of phenotype change. Other research also demonstrates that METH promotes wakefulness and raises activity levels in flies and rodents. Flies exposed to METH sleep less often and when they do, they wake up sooner than controls (19). This supports the hypothesis that METH consumption causes neuroplastic changes in large ventral lateral

neurons (responsible for wakefulness) that are retained throughout generations, and that parental drug abuse could affect progeny's sleep and activity transgenerationally.

The effect of paternal METH preference in flies was further evaluated using the geotaxis phenotype, giving us information about their motor activity. Information is obtained by simply observing the fly's ability to climb up after mechanical startle. Studies on COC and ethanol show impairment of locomotion and of negative geotaxis shortly after the administration (6). Offspring of males that self-administrated METH (HP and LP lines) show significant impairment in negative geotaxis compared to *wt*, probably due to DA disbalance that affects locomotion and navigation in flies. Considering that this was observed in the 30^{th} generation of flies, we can assume that METH induced dopamine balance disruption has serious transgenerational consequences. Also, the LP line has the most impaired geotaxis of all lines which can relate to changes in phenotype. The HP line does not exhibit behavioral changes in relation to *wt* i.e., they preferentially consume METH when offered, however the LP line avoids it. Furthermore, while both HP and LP line show impairment of activity, sleep, and geotaxis, LP line does so on a higher level than the HP line. Considering that the HP line consummated more METH than the LP line, we assume that the exhibited behavior is also connected to the phenotype changes of the LP line. Since impaired negative geotaxis is an assay connected to neurodegeneration in flies, our results could also point to the neurodegeneration in the LP line. However, this cannot be decided solely based on the phenotype as these behavioral parameters are regulated through a series of complex mechanisms. Negative geotaxis assay can be further useful in genetic screens for identifying flies with changed phenotype and sensitivity, as it has successfully pointed out candidate genes in the past, which are also conserved in rodents (17).

Acute and chronic abuse of METH disrupts energy balance by affecting glucose metabolism. The mechanisms of disruption are not yet elucidated; however, it is possible that METH inhibits glucose uptake of neurons by affecting glucose transporter protein-3 (GLUT3) (44). There is also a suggestion that METH disrupts BBB integrity by damaging brains endothelial glucose transporter (GLUT-1) causing inadequate supply of glucose rich blood to reach the brain (45). In *Drosophila*, as in mammals, METH diminishes food intake up to 80% leading to anorexia , however the studies of anorexic effect are yet inconclusive (26). Our results indicate that preferential paternal METH intake does not significantly affect the offspring (F5) morphology of the 28th fly generation in either size or weight in relation to *wt*, except for LP females which are larger than *wt*. This is interesting because the artificially selected flies were male, and the effect in seen in female offspring. These results are similar to ones obtained in rats and mice where there was no long-term change in weight of specimens (45). This could indicate that glucose rich food or compensatory decrease in resting metabolism manage to overcome the caloric deficit or that the amount of consumed METH is too low for it to cause anorexia. On the other hand, the difference in size between LP and HP lines, and LP and *wt* females, may indicate that METH consumption does leave a transgenerational mark on glucose metabolism. LP and *wt* flies are larger than HP (even if not significantly), which has been linked to higher fecundity of females (our results show higher reproductive success of LP and *wt* flies as well) (46).

Preliminary results show that HP L3-instar larvae are significantly smaller but heavier than *wt* larvae, which could be explained by activation of certain mechanisms increasing the food intake to increase dietary carbohydrates levels and compensate the diminished endogenous carbohydrates (26). It can also be argued that flies are not susceptible to vasoactive properties of METH like mammals because of the differences in cardiovascular system, so the

nutrients flow is not inhibited. Our data indicates that paternal METH self-administration lowers the size of progeny indicating that even female provision (noticeable in HP larvae) cannot overcome effects of parental drug consumption. The weight difference of HP larvae should be better researched by increasing the number of specimens and conducting it throughout the generations.

6. Conclusion

This thesis shows the heritability of phenotypes, some of which are directly linked to selection agent (methamphetamine), and other that are not obviously related. This was determined by evaluating the offspring's behavioral sensitization and preferential self/administration of METH, as well as observing the life cycle and its duration, morphology, negative geotaxis, sleep, and activity of flies. Artificial selection of flies with the highest and lowest preference for METH enabled creation of high (HP) and low (LP) preference lines through selective breeding of males with *wt* virgins. The stability of low and high METH preference is shown both within the generation (F6 and F0 of the 28th generation) and throughout all tested generations (0-30th). This could point out that there is a transgenerational effect of METH abuse that changes the brain response to addictive drugs, possibly through inherited impairment of the dopamine systems. The progeny of flies which voluntarily self-administrated METH have impaired behavioral phenotypes not directly linked to drug consumption; their ability to negatively geotax is lowered, their activity higher with diminished sleep amount. Furthermore, transgenerational METH effect is seen in reproduction. Offspring of HP line had lower number of surviving progenies, possibly due a disbalance in monoamine levels, primarily tyramine and octopamine which impaired the eclosion of adult flies. It is also possible that METH consumption epigenetically changes male germ cells, impairing reproduction. Sun et al. found seven METH-responsive genes and proteins connected to reproduction in *Drosophila*, with a chance that some are conserved in humans (41). This is consistent with observation in humans METH addicts who show delayed ejaculation and impaired sperm motility (41). However, there was no change in duration of the life cycle, indicating that there is no transgenerational effect of METH on males ability to mate. Lastly, there seems to be a change in morphology of HP and LP lines, potentially implying the glucose metabolism impairment (often connected with

anorexic effect in mammals). Since there seem to be heritable changes in flies by paternal line, it would be interesting to determine if the changes in behavior are due to genetic effect (mutation, allele enrichment) or epigenetic effect (long term change in the level of gene transcription).

7. Literature

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Supplement

4.1.1. Motor-activating effects of vMETH and vCOC

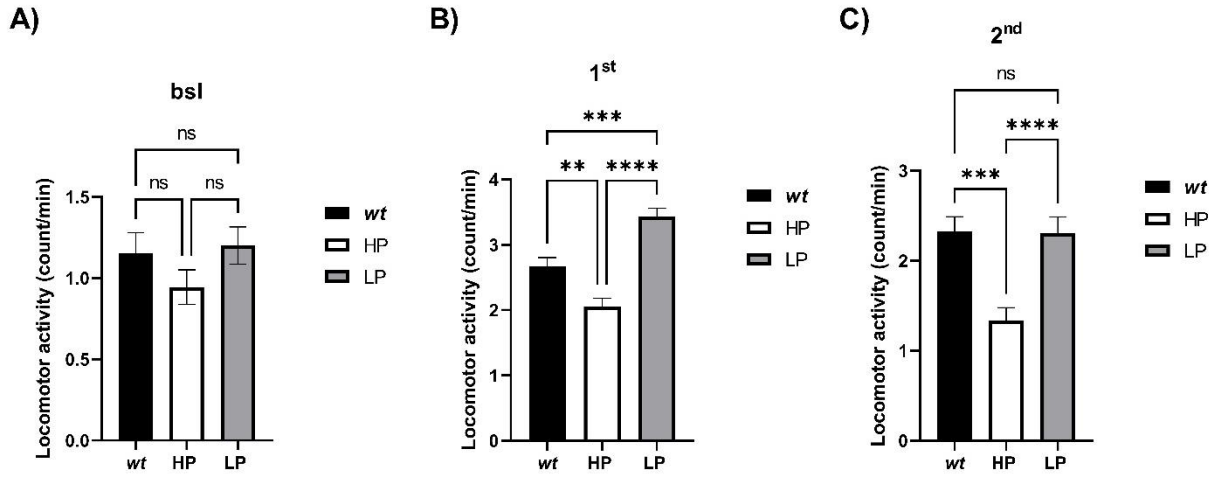

Figure 11. HP line shows lowest increase in locomotor activity after one and two doses of hot air. Flies used in experiment were 3-5 days old males: wild type *Canton S* (*wt*, *n*=32), F2 of 28th generation of flies with high (HP, *n*=32) and low (LP, *n*=32) preference for METH. Locomotor activity of the population 10 minutes before (bsl) **A)**, and after exposure to the first (1st) **B)**, and second (2nd) **C)** dose of hot air. The first dose was administered at 9 AM and the second at 7 PM (10-hour gap). Data was analyzed with One-way ANOVA with Tukey`s multiple comparisons test. **ns**= no significance, ** p<0,005, *** p<0,0005, **** p<0,0001.

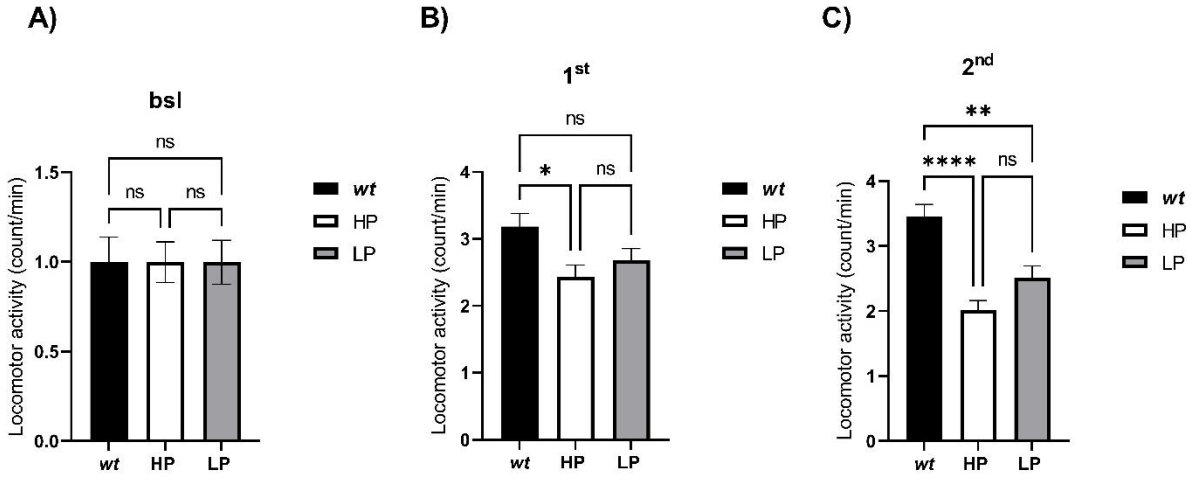

Figure 12. HP, LP and *wt* flies show an increase of locomotor activity after one and two doses of hot air. Flies used in experiment were 3-5 days old males: wild type *Canton S* (*wt*, *n*=32), F2 of 28th generation of flies with high (HP, *n*=32) and low (LP, *n*=32)

preference for METH. . Locomotor activity of the population 5 minutes before (bsl) **A)**, and after exposure to the first (1st) **B)**, and second (2nd) **C)** dose of hot air. The first dose was administered at 9 AM and second at 3 PM (6-hour gap). Data was analyzed with One-way ANOVA with Tukey`s multiple comparisons test. **ns**= no significance, * p<0,05, ** p<0,005, **** p<0,0001.

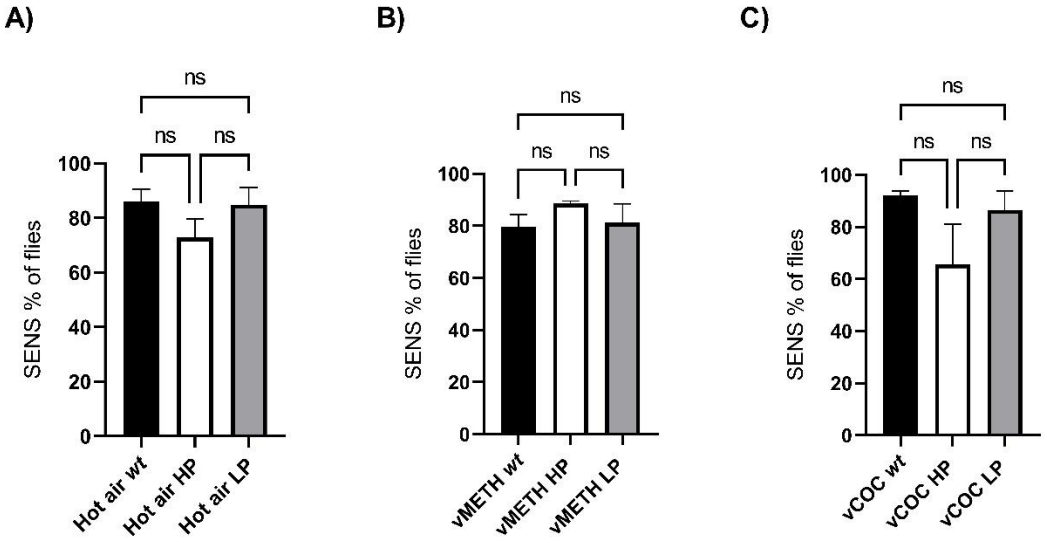

Figure 13. Hot air and psychostimulants vMETH and vCOC induce sensitivity (SENS) in flies with no significant difference among lines. Flies used in experiment were males 3-5 days old: wild type *Canton S* (*wt*, *n*=32), F2 of 28th generation of flies with high (HP, *n*=32) and low (LP, *n*=32) preference for METH. The individual response is shown as % of flies with increased activity after the first dose compared to bsl (SENS) for hot air **A)**, vMETH **B)** and vCOC **C)**. Data was analyzed using One-way ANOVA with Tukey`s multiple comparisons test. **ns**= no significance

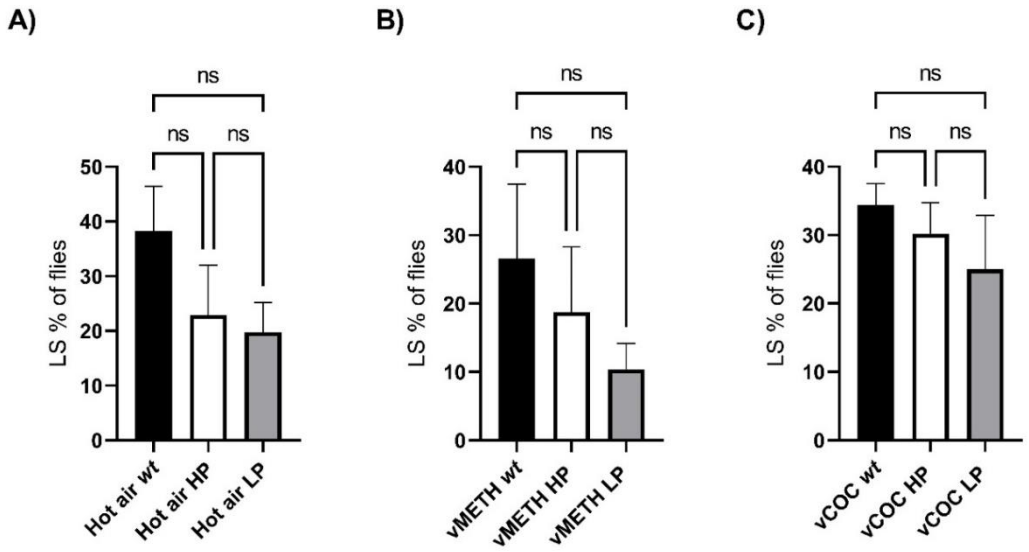

Figure 14. LS in HP and LP differ from *wt* but with no statistical significance. Flies used in experiment were males 3-5 days old: wild type *Canton S* (*wt*, *n*=32), F2 of 28th generation of flies with high (HP, *n*=32) and low (LP, *n*=32) preference for METH. % of flies with a gradual increase in locomotor activity after the first exposure compared to the initial value and an increase after the second exposure, compared to the first exposure (LS) of hot air **A)**, vMETH **B)** and vCOC **C)**. Data was analyzed using One-way ANOVA with Tukey`s multiple comparisons test. **ns**= no significance