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SVEUČILIŠTE U RIJECI

FAKULTET BIOTEHNOLOGIJE I RAZVOJA LIJEKOVA

Diplomski sveučilišni studij

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Marta Medija

Utjecaj izolacije na koncentraciju neurotransmitera i parametre mreža socijalnih interakcija kod modela *Drosophila melanogaster*

Diplomski rad

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

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Abstract

Social behavior is an essential need for humans and animals, including *Drosophila melanogaster*. Recently, there has been an increasing interest in studying the impact of social isolation, the deprivation of social connections, on behavior and brain chemistry. These studies provide valuable information about changed social interactions that are symptoms of different neuropsychiatric disorders.

To investigate the relationship between social isolation and chemical alterations in the brain, we conducted a study where we assessed the concentration of neurotransmitters in *D. melanogaster* head after a period of one and five days of social isolation. Our LC-MS/MS analysis revealed a significant decrease in concentrations of dopamine, octopamine, glutamate and acetylcholine. Furthermore, to determine if there was a correlation between neurochemical alterations and behavioral modifications, we recorded the social interactions among a group of 12 flies in a circular arena. Python scripts were used to generate and evaluate Social Interaction Networks (SINs) based on five parameters: global efficiency, assortativity, clustering coefficient, betweenness centrality, and closeness centrality. Network analysis revealed that social isolation led to an increase in the clustering coefficient and a decrease in the closeness centrality. This suggests that flies tend to establish smaller but temporary social groups within the network. To reverse the alterations in SINs caused by isolation, we administered dopamine, octopamine and combination of both during five days of isolation. Examination of SINs characteristics of supplemented groups revealed significant variations in clustering coefficients, centrality measures, and assortativity, with noticeable impacts of dopamine and octopamine. The addition of octopamine and the combination of dopamine and octopamine greatly increased closeness centrality, suggesting that flies established more densely linked networks, like what was found in groups without isolation. Additionally, we observed that differences in the age of

grouped flies significantly influence local SINs parameters, with younger flies exhibiting similar behavioral patterns as isolated flies.

Our results show that social isolation has lasting neurochemical and behavioral effects, in particular the behavior in a group setting. Moreover, because supplementation with dopamine and octopamine reversed some negative effect of social isolation, supplementation with these neurotransmitters might contribute in the creation of novel therapies targeted at alleviating the negative effects of social isolation.

Key words: *Drosophila melanogaster*, social isolation, dopamine, octopamine, social interaction networks (SINs)

Sažetak

Društveno ponašanje ključna je potreba ljudi i životinja, uključujući *Drosophila melanogaster*. Nedavno se povećao interes za proučavanje utjecaja socijalne izolacije, deprivacije socijalnih interakcija, na ponašanje i kemiju mozga. Ove studije pružaju vrijedne informacije o promijenjenim socijalnim interakcijama koje su simptomi različitih neuropsihijatrijskih poremećaja.

Kako bismo istražili odnos između socijalne izolacije i kemijskih promjena u mozgu, proveli smo studiju u kojoj smo procijenili koncentraciju neurotransmitera u glavi *D. melanogaster* nakon razdoblja od jednog i pet dana socijalne izolacije. Naša LC-MS/MS analiza otkrila je značajno smanjenje koncentracije dopamina, oktopamina, glutamata i acetilkolina. Nadalje, kako bismo utvrdili postoji li korelacija između neurokemijskih promjena i modifikacija ponašanja, zabilježili smo socijalne interakcije među skupinom od 12 mušica u kružnoj areni. Python skripte korištene su za generiranje i procjenu mreža socijalnih interakcije (SIN) na temelju pet parametara: globalne učinkovitosti, asortativnosti, koeficijenta grupiranja, betweenness centrality i closeness centrality. Analiza socijalnih interakcija je pokazala da je socijalna izolacija dovela do povećanja koeficijenta grupiranja i closeness centrality parametra. Ovo sugerira da mušice imaju tendenciju uspostavljanja manjih, ali privremenih društvenih grupa unutar mreže. Kako bismo poništili promjene u mrežama socijalnih interakcija uzrokovane izolacijom, suplementirali smo hranu dopaminom, oktopaminom i kombinacijom dopamina i oktopamina tijekom pet dana izolacije. Ispitivanje karakteristika SIN-a suplementiranih skupina otkrilo je značajne varijacije u koeficijentima grupiranja, mjerama centralnosti i asortativnosti, sa značajnim utjecajem dopamina i oktopamina. Dodatak oktopamina i kombinacija dopamina i oktopamina uvelike je povećala closeness centrality parametar, sugerirajući da su mušice uspostavile gušće povezane mreže, poput onoga što je pronađeno u skupinama bez izolacije. Osim toga, uočili smo da razlike u dobi grupiranih mušica značajno utječu

na lokalne parametre SIN-a, pri čemu mlađe mušice pokazuju slične obrasce ponašanja kao izolirane mušice.

Naši rezultati pokazuju da socijalna izolacija ima trajne neurokemijske učinke i učinke na ponašanje, posebice ponašanje u grupnom okruženju. Štoviše, budući da je suplementacija dopaminom i oktopaminom poništila neke negativne učinke socijalne izolacije, suplementacija ovim neurotransmiterima mogla bi pridonijeti stvaranju novih terapija usmjerenih na ublažavanje negativnih učinaka socijalne izolacije.

Ključne riječi: *Drosophila melanogaster*, socijalna izolacija, dopamin, oktopamin, mreže socijalnih interakcija

Table of Contents

1. Introduction

Humans naturally seek social interactions which are necessary for the maintenance of mental and physical health (1). Social interactions shape our thoughts, emotions and understanding of the environment around us. In contrast, voluntary or forced social isolation, or deprivation of social interactions, can have adverse impact on individual's health (2).

Social isolation is defined as deprivation of social interactions among individuals. Various studies have reported correlation between states of loneliness, increase in social anxiety, depression and risks for early mortality with social isolation (1,2). In humans, unvoluntary social isolation during early life shows symptoms of neurodevelopmental disorders, such as attention deficit and hyperactivity (3), while social isolation during adulthood through traumatic event, or solitary confinement increases aggression and induces depressive disorders (4). Recently, after COVID-19 pandemic, more research has been conducted about the effects of social isolation on mental health (2,5). Prolonged periods of social isolation during COVID-19 pandemic were associated with enlargement of putamen, bilateral amygdala and anterior temporal cortices which are important in different cognitive and emotional functions (6). It is still uncertain whether these changes are due to social isolation or other stresses induced during quarantine. Therefore, deeper understanding of how social isolation affects behavior and brain chemistry is necessary.

1.2. Influence of social isolation on reward center and monoamines

Social interactions are considered positive social experiences, due to the activation of the reward center in the brain which releases neurotransmitters associated with pleasure, primary monoamine dopamine (DA) (7). DA is crucial for mediating stress response and reward processing, and its dysregulation is associated with neuropsychiatric and substance disorders (8,9). When the rewarding center is stimulated, DA is released

from neurons of the ventral tegmental area (VTA) in the midbrain, that project DA to various regions, including nucleus accumbens (NAc), hippocampus, amygdala and prefrontal cortex (PFC). There, DA binds to dopaminergic receptors and exerts its stimulating effects, after which is recycled back into neurons by dopamine transporters (8).

Negative social experiences, like social isolation can influence reward center through complex interplay of neurotransmitters. The direct mechanism of how social isolation affects reward system and behavior is still unknown. It was shown that social isolation is a risk for depression and anxiety, that are the basis of many psychiatric diseases and behaviors associated with the development of addiction in which mechanism of reward are well investigated. For example, people with Parkinson disease and schizophrenia, are more susceptible to experiencing social isolation and loneliness due to their lowered levels of dopamine (9). In Parkinson's disease lower levels of dopamine are released due to the neurodegeneration of dopamine neurons, which impair cognitive functions and reward center and makes them susceptible to social isolation (5). Studies investigating behavioral effect of social isolation reported that in periods of adolescence, in which our reward system is more susceptible to stress, social isolation can induce sensitivity to social reward leading to depression, anxiety and addictive disorders (10).

Rodents studies showed that social isolation in adulthood increases reward-related behaviors in males, causing changed responsiveness to addictive substances (11). This alterations are associated with changes in the transcription of genes linked with DA and the expression of proteins, particularly those involved with DA synthesis, release, and receptors (11). Furthermore, alterations in DA projecting regions can result in different levels of basal and extracellular DA. For example, social isolation did not have effect on extracellular DA in Nac in isolated male rats, but basal levels of DA were increased (12), while in amygdala isolation decreased basal DA and increased DA transporter levels (13), indicating not all regions of the

brain are equally affected. Additionality, research in male mice demonstrated that social isolation rearing increases protein levels of DA receptor 2 in the NAc but not in hippocampus or PFC (14). This indicates reward center has an important role in mediating isolation induced conditions influenced by the dopaminergic neurotransmission.

1.2.1. Monoamines in insects

Among the biogenic amines found in insects, such as DA, serotonine, tyramine, and octopamine (OA); OA has been mostly associated with reward responses compared to DA. OA is synthesized from tyramine and is structurally similar to noradrenaline in humans. OA functions as a neurotransmitter and a neurohormone in invertebrates (15). It is found in significant amounts in both neuronal and non-neuronal cells of most invertebrate species, where it binds to OA receptor. Except the role in reward, OA exert effects in learning and memory and mood regulation. OA, along with tyramine, is the only monoamine that has a limited physiological function solely in invertebrates (15). Although DA and OA are involved in the reward center of insects, little is known about their interplay in isolation-induced behaviors.

1.2.2. Interplay of dopamine and octopamine

In *C. elegans*, feeding behavior has been found to increase DA signaling and decrease OA signaling, resulting in *C. elegans* longevity, while starvation causes the opposite, increasing OA and decreasing DA signaling (16), indicating an excluding effect between DA and OA. DA activated the dopamine receptor DOP-3 in the octopaminergic neuron, and this activation potentially reduced OA production. Furthermore, DA suppressed octopamine-mediated signaling in cholinergic SIA neurons by interacting with dopamine receptors DOP-2 and DOP-3. When there was no food, DA signaling was reduced which led to the activation of CREB (cAMP response

element binding protein) via the octopamine receptor SER-3 (16). CREB is a transcription factor that has an impact on learning and memory, cell survival and neuron plasticity, and is associated with various neurological disorders such as schizophrenia (17).

In *Drosophila,* OA and DA are known to mediate a variety of behaviors, including aggression, courtship, sleep and foraging (18). An attempt was made to investigate whether findings in *C. elegans* are similar in *Drosophila* (19). In the study two dopamine receptors, DAR1 and DAR2, in the octopaminergic neurons were knocked down, after which the social behavior and longevity of flies was monitored. Unfortunately, the results did not lead to longevity, instead the DAR2 knockdown reduced: resistance to starvation, male-to-male courtship, activity and increased aggression (19). Therefore, further investigation of interplay between DA and OA is needed.

1.3. Influence of social isolation on behavior in animal models

Until now, many studies investigating the effects of social isolation on behavior have been studied in animals, mainly rodents such as mice and rats. Several studies have been conducted involving the zebrafish, which is a social vertebrate, and a highly effective translational model for several diseases common to humans. (20). Research has shown that adult zebrafish have changes in behaviors related to anxiety and serotonin levels in their brains, as a result of social isolation in maturity (20). Furthermore, isolation in insects like worker honeybees has shown reduced performance in learning tasks and decreased levels of DA (21). Similar studies were done in fruit fly, which emerged as a highly valuable model organism used in understanding mechanisms of isolation-induced behaviors.

1.4. Fruit fly as a model for social isolation

Although fruit flies are considered non-eusocial insects, meaning that they do not form colonies or divide labor, their life cycle and natural environment are greatly influenced by their social environment. The fruit fly exhibits complex behaviors that play a key role in several vital functions including circadian cycles, reproduction, aggression, perception, searching for food, and the formation of dynamic social networks that rely on group interactions (22,23). Adult flies cluster around food sources in order to copulate and lay eggs. During larval stage, fruit flies search for food in decaying fruit, while being surrounded by other larvae (24). Modifications in social environment can influence courtship, aggression, sleep and social interaction networks (22). Therefore, fruit flies can serve as an effective model for investigating isolation-induced behaviors.

1.5. Influence of social isolation on behavior in fruit fly

In fruit flies, social isolation has shown multiple effects on behavior including increased aggression, disturbed courtship and social interaction networks. Aggression is considered complex social behavior, which enables competition for territory, mates and food (22). Social isolation is shown to enhance aggressiveness in various animals from mammals to insects. Fruit flies exhibit aggressiveness by lunging (one fly slams its opponent), threatening another fly with wing extensions and tussling with other flies (22). Studies identified *Drosulfakinin* (*Dsk*), which is a homolog of cholecystokinin in vertebrate, as regulator of isolation-induced aggressiveness. Socially isolated flies become more aggressive after *Dsk* inactivation (25).

Social isolation has an impact on courtship behavior in male and females (22). During courtship fruit flies engage in multiple behaviors to seduce their mate, like singing by vibrating their wings. In isolated male flies song burst were shorter compared to group housed males, decreasing their

chances to seduce a female. *Fruitless* gene was reported as important in courtship behavior. Isolated males with null allele of the *fruitless* gene did not court females, but if those males were grouped with other flies they learned to court (26).

1.6. Social interaction networks

Past experiences, like social isolation can affect how flies communicate and connect with each other and how they form networks or clusters. Social communication of fruit flies includes many visual, auditory, chemosensory and tactile cues (22). Recently, due to technological advancements, more studies have focused on investigating how social communication and connections change in a group setting, by observing group dynamics and their interactions (23). This is referred to as analysis of social interaction networks (SINs), where studies use video recording and statistical analysis of different network parameters to investigate interaction patterns of the group and individuals. Depending on how the experiment is set up, studying flie's behavior allowed researchers to identify certain requirements for social interactions (27). These observations have highlighted the significance of chemosensory signals in controlling the distance and level of contact between flies from which Schneider et al. in 2012 first established criteria for social interaction in fruit flies (27). They noticed that flies approach other flies at a certain angle and at a certain distance, and that flies stay in that position for a certain time (fixed criteria). Using fixed criteria, they determined that social isolation had no impact on the networks (27). However, Simon et al. reanalyzed their data with automated criteria known as the social space index, which measures the distance between flies that are resting and not engaging in social interaction (28). They found that 7 days of isolation of 3-4 days old flies, which are immature leads to an increase in the social space (28). Therefore, defining interaction characteristic is essential for understanding the effect of isolation on group networks.

After interaction criteria is established, flies are usually recorded in circular arenas that have curved edges to restrict fly’s movement only in two dimensions for easier tracking. Two of the most commonly used tracking softwares are Ctrax and FlyTracker (29). Both were developed using the MATLAB programming language and are used to track each individual fly by assigning an identity and calculating the fly's trajectory at every moment during the video recording sessions. Despite frequent use, Ctrax can lead to tracking inaccuracies, mainly due to lost of tracking or identity switching. Consequently, FlyTracker software was created to solve and eliminate these problems.

Data from the tracking software is then extracted and analyzed using various formulas written in programming languages such as Python (30), which calculate parameters related to social interaction. For example, the scripts calculate the fly's movement, interaction rate and duration, how close each fly is to another in the network, called closeness centrality, or whether there is any fly that is more important in terms of information flow than others, called betweenness centrality. Using these and other parameters, researchers can analyze network properties. For example, are random networks formed or are they structured. Based on this principle, studies have shown that isolated flies form less stable and less structured networks compared to the grouped flies (31). In contrast, another study got opposite results showing that isolated flies have stronger interactions with other flies (29). This disparity of results may be due to differences in methodology and data analysis. Furthermore, it remains unclear how does social isolation in fruit flies influence concentration of neurotransmitters upon short- and long-term isolation, and if it does, whether those changes affect their social interaction networks. Therefore, we wanted to investigate these questions in this research.

2. Aims

The main aim of this thesis is to investigate if and how does the experience of social isolation influence the social behavior of *D. melanogaster.* The main hypothesis is that social isolation changes social behavior leading to distinct interactions among individuals. To test this hypothesis, we had several sub aims.

The first sub aim was to determine the influence of social isolation on the concentration of neurotransmitters. Our hypothesis was that keeping flies socially isolated will affect the concentration of the neurotransmitters in the brain. To test this, we kept individual flies in social isolation for one or five days and then measured the concentration of dopamine, octopamine, tyramine, gamma-aminobutyric acid (GABA), glutamate and acetylcholine in the head homogenates using liquid chromatography with tandem mass spectrometry (LC-MS/MS).

The second sub aim was to investigate the influence of social isolation on parameters of social interaction networks (SINs). Our hypothesis was that if social isolation influences behavior, then SINs will differ between flies kept in isolation versus grouped flies. To test this, we recorded flies in circular arenas for 20 minutes after five days of isolation/grouping and analyzed five parameters: global efficiency, assortativity, clustering coefficient, betweenness centrality and closeness centrality.

The third sub aim was to investigate if pharmacological manipulation of neurotransmitter concentrations will reverse effects of isolation. Our hypothesis was that if isolation leads to a decrease in the concentration of neurotransmitters, their supplementation should reverse the effects of social isolation on parameters of SINs. To test this, we isolated flies for five days on food containing dopamine, octopamine and combination of both, and recorded their group interactions and SINs parameters.

3. Matherials and method

3.1. Chemicals

For oral feeding of the flies, we used 3,4-dihydroxy-L-phenylalanine (L-DA) (≥98%), and octopamine hydrochloride (OA) (≥95%) that were purchased from Sigma Aldrich.

3.2. Fly strain and cultivation

Wild type (*wt*) male flies of Canton S background were used in all experiments. Files were cultivated in bottles in an incubator at 25 °C and 70% humidity, in 12h light-dark cycles on a standard cornmeal food. Cultivation food consisted of sugar, agar, corn flour, yeast, and water, with the addition of propionic acid and nipagin to prevent mold growth in the food.

3.3. Comparison of different methods for social isolation and network analysis

Social isolation is already studied in flies. Different methods apply different durations of social isolation, and age of isolated flies. In our approach we have used interaction criteria and recording protocol based on Schneider et al. (32), while social isolation protocol was based on measurement of monoamines. In Table 1, I have summarized protocol steps and highlighted differences between our and previously published methods.

Table 1 Comparison between our method and three other previously published methods for social isolation and network analysis.

	Our method	Schneider et al. (2012) (27)	Liu et al. (2018) (29)	Bentzur et al. (2021) (31)
Fly strain	Canton S	Canton S	Canton S	Canton S
Age of flies when isolated	3-5 days	after enclosion	10 days	after enclosion
Length of isolation	5 days	3 days	1-6 days	3 days
Age of flies when recorded	10 days	3 days	10-16 days	3-4 days
Number of flies in arena	12	12	16	10
Arena diameter	61 mm	60 mm	70 mm	245 mm
Movement restrictions	2D	2D	2D	2D
Arena height	3 mm	2 mm	3.5 mm	6 mm
Arena celling coated	YES	not specified	YES	thermally controlled
Transfer to arena	aspiration	aspiration	cold anesthesia	live transfer
Habituation in arena	15 min	15min	1 min	1 min
Time of day when recording	10 AM	not specified	10-12 AM	not specified
Recording duration	20 min	30 min	60 min	15 min
Recording conditions	light	dark	dark	dark or light
Tracking software	Flytracker	Ctrax	Flytracker	Ctrax
Data representation	Z-score	Z-score	Measurement values	Z-score

3.4. Isolation protocol

Cultivated male flies were isolated on cornmeal food in glass tubes prepared on the day of the isolation. Glass tubes contained on one side cornmeal food that was secured with parafilm, to prevent food from drying, and on the other side were closed with a foam cap, to allow air flow in the tube. Before food was inserted into glass tubes it was placed on a paper towel to absorb excess liquid and prevent water condensation within the glass tubes.

To test the influence of duration of social isolation on neurotransmitter concentrations, 3-5 days old male flies were collected from cultivation bottles using microscope and CO_2 anesthesia. Individual flies were

transferred, using an aspirator, into glass tubes where they were kept in isolation for one and five days on a cornmeal food (Fig. 3).

To test the influence of five-day social isolation on parameters of social interaction networks (SINs), 240 male flies per treatment, 3-5 days old, were collected. Each fly was transferred individually to glass tubes containing standard cornmeal food: without supplementation (**ISO**), with 5 mg/mL 3,4-dihydroxy-L-phenylalanine (**L-DA**), 10 mg/mL octopamine hydrochloride (**OA**) and combination of 5 mg/mL L-DA and 10 mg/mL OA (**L-DA+OA**) (Fig. 1). Isolated flies were placed in an incubator for 5 days under the same conditions as for cultivation. In total, 20 groups of 12 flies for each treatment were tested.

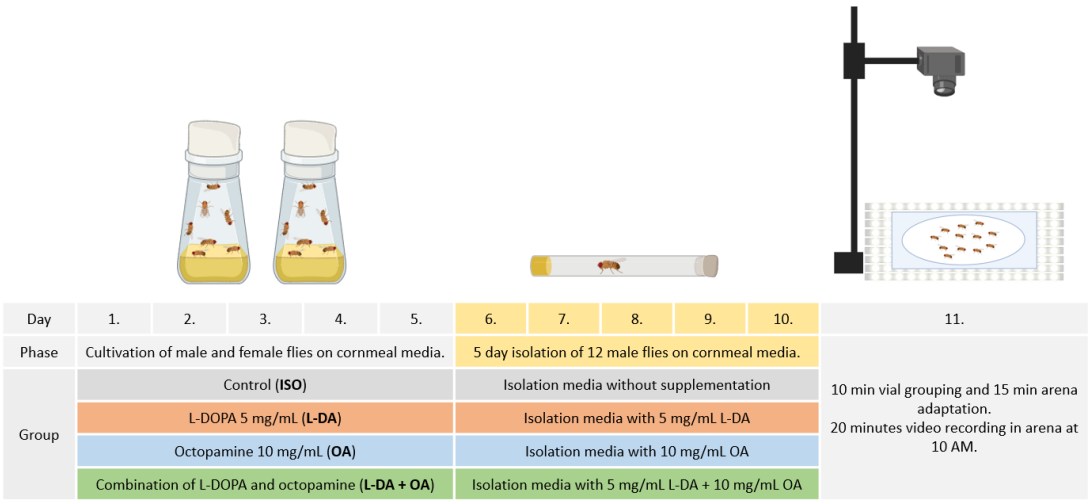


Figure 1. Isolation protocol. The experiment consisted of three phases: 1. fly cultivation, 2. five-day isolation on different media, and 3. video recording.

Control groups consisted of male flies, 3-5 days old that were: grouped in a vial for one day – young control (**YOUNG**) and video recorded the next day, and old control (**CTRL/OLD**) that were flies grouped for 5 days in order to reach the age of isolated flies on the day of video recording (Fig. 2). Flies were housed in an incubator until recording. 20 groups of 12 flies for each control were tested.

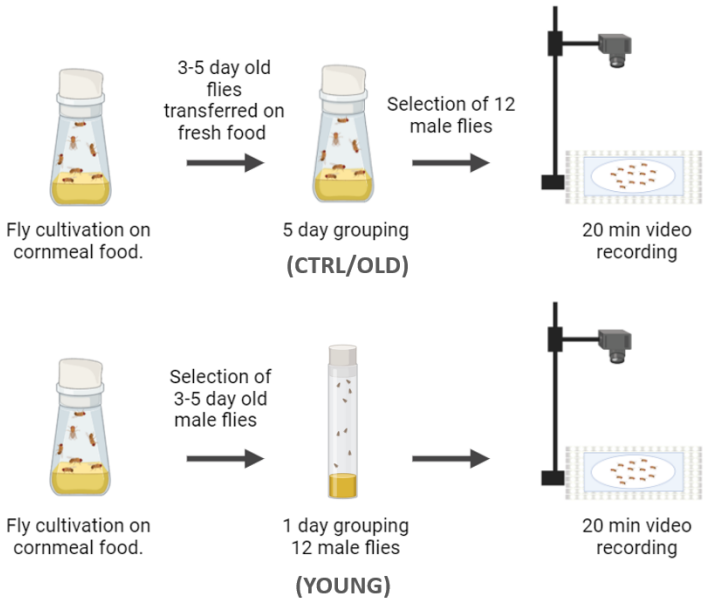


Figure 2. Control groups. Flies were cultivated on cornmeal food. A) 3-5 days old male flies were grouped for five days with females (CTRL/OLD). After five days 12 male flies were recorded in circular arena for 20 minutes. B) 12 male flies, 3-5 days old, were grouped one day (YOUNG), after which they were recorded in circular arena for 20 minutes.

3.5. Sample preparation and LC-MS/MS analysis

After one and five days of isolation, 15 male flies were collected and frozen for 15 minutes. Flies were placed on an ice pad and using dissecting tweezers heads were separated from the body. Heads were homogenized on ice for 10 seconds, using mechanical mixer, in 300 µL of 0.1 M percloric acid (95%, Sigma Aldrich, Buchs, Switzerland). The samples were subsequently centrifuged at 4 °C and 14,000 rpm for 45 minutes. Centrifuged samples were filtered through a cellulose filter with 0.20 µm holes (Ma-cherey-Nagel, Chromafil Xtra RC-20/13, 0,20 µm, 13 mm). Filtered samples were collected in 1 ml vials for analysis using liquid chromatography with tandem mass spectrometry (LC-MS/MS). The levels of dopamine, octopamine, and tyramine were quantitatively analyzed, while the levels of GABA, glutamate, and acetylcholine were semi-quantitatively analyzed. This analysis was conducted using an Agilent 1260 series HPLC chromatograph in combination with an Agilent 6460 triple quadrupole mass

spectrometer (QQQ) connected to an electrospray (ESI) source (Fig. 3) (33). LC-MS/MS analysis was performed by Lara Saftić Martinović.

LC-MS/MS data was statistically analyzed and visualized using GraphPad Prism 10.0.3 version. Differences between neurotransmitters treatments at one and five days of isolation were analyzed using one-way ANOVA with Tukey's multiple comparisons post-hoc test for parametric data, and for nonparametric data; Kruskal-Wallis test with Dunn's multiple comparisons test with statistical significance level $p < 0.05$. Bartlett's test or Brown–Forsythe's test was used to test the normality of data.

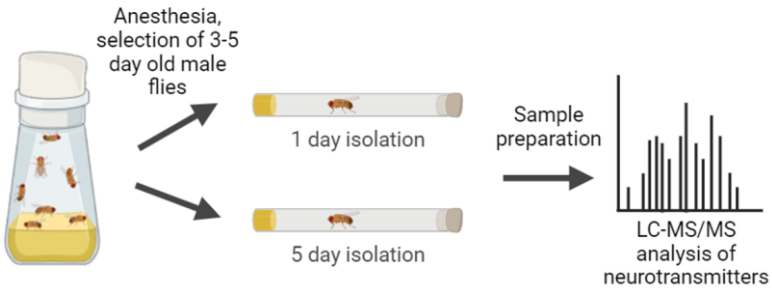


Figure 3. Neurotransmitter analysis. Flies were cultivated on cornmeal food. Male flies, 3-5 days old were anesthetized and isolated in glass tubes for one or five days. After isolation flies heads were homogenized and analyzanaed using liquid chromatography with tandem mass spectrometry.

3.6. Video recording

To acquire data for network construction 12 flies per treatment were recorded in circular arena. First, 12 flies from the same treatment were grouped in a vial for 10 minutes (31), to avoid differences in the aspiration time and for the ease of transfer to arenas. Flies were then transferred into circular arena using an aspirator. The arena measured 61 millimeters in diameter, enabling flies to comfortably move without crowding (23), and 3 millimeters in height to limit mobility to two dimensions. The top of the arena was a clear 2 mm-high Plexiglas cover, covered with sigmacote (Sigma Aldrich) to prevent flies from climbing the arena ceiling. The bottom of the arena consisted of white translucent Plexiglas (34)(30). Four arenas

were placed on a luminous LED surface, above which was a camera (Fig. 4).

Flies were filmed with ACA3800-10GM Basler industrial camera with 3856 x 2764 pixels video resolution (30). The camera was connected to a computer with Basler program for recording flies. Once flies were aspirated in the arena, they were given 15 minutes habituate, after which each arena was filmed for 20 minutes. Video recordings were made at 10:00 AM to avoid the influence of the time of day on behavior. Between each recording arenas were cleaned with water and dried with paper towels, to remove any olfactory cues from previous flies.

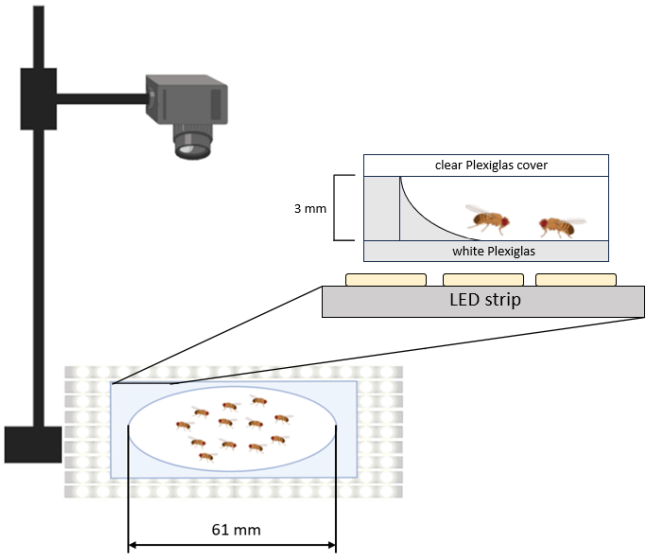


Figure 4. Graphical representation of the video recording setup. 12 flies were recorded for 20 minutes in a circular arena. Arena measured 61 mm in width and 3 mm in hight with a bottom made of white Plexiglas and the top made of clear Plexiglas. Light source was LED strips.

3.7. Fly tracking

Recorded videos were tracked using FlyTracker software (35). FlyTracker, developed using MATLAB, recognizes each fly and assigns it an identity. Knowing the flies identity software calculates flies spatial position (x and y coordinates) and fly orientation (30). Each recorded video was reviewed

manually to eliminate the potential mistakes, like identity swaps. After revision, social interaction networks (SINs) were constructed based on established interaction criteria (Fig. 5) (32,36):

(a) distance between two flies less than 2.5 fly body length (approximately 5 mm)
(b) facing angle (α) of two flies in range of 160 degrees
(c) time spent near each other more than 0.6 seconds

Based on these criteria Python scripts were used for trajectory data processing, network construction and analysis (37).

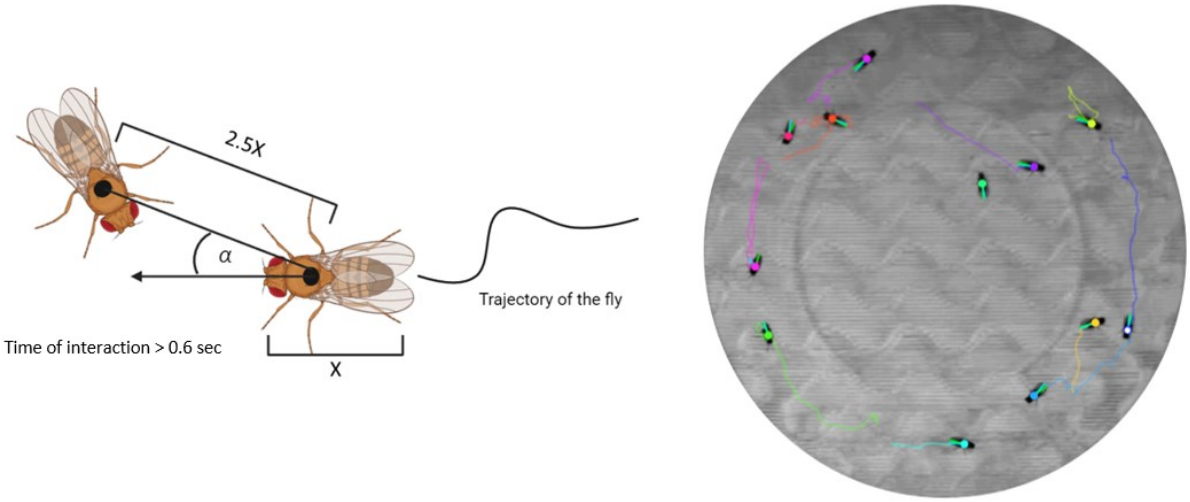


Figure 5. Interaction criteria. Recorded videos were tracked using FlyTracker, which calculated flys position and trajectory as shown in the circle (left). Fly interaction criteria were given as a distance between two flies less than 2.5 body lengths (2.5X) or 5 mm (one fly equals one body length (X) or approximately 2 mm), angle at which one fly approaches another fly in various orientations was under 160 degrees (α) and time spent in this position of a minimum of 0.6 seconds.

3.8. Data analysis and characterization of social interaction networks (SINs)

Constructed SINs were represented as weighted directed graphs containing two sets of data, called nodes and edges. Nodes represent each fly in a group and edges represent a link between two nodes with weights

(Fig. 6). In this research two types of weights were used: 1. Number of interactions (count) – how many times given fly interacted with another and 2. Total duration (duration) – time given fly spent interacting with another fly (30). For every group a network was constructed, and parameters were analyzed on global and local levels. Global level analyzes properties of the entire network and local level analyze connections and behavior of an individual fly within the network. Analyzed parameters were (Fig. 7):

1. **Global Efficiency** – measures how connected are all nodes (flies) in the network based on distance. Higher global efficiency indicates there is a smaller average distance between nodes which increases information flow in the network (38).
2. **Assortativity** - measures the degree of mixing in the network between flies and subgroups based on specific attributes (sex, age, number of connections). High assortativity indicates flies with similar interaction frequencies tend to connect with one another (38).
3. **Clustering Coefficient** – measures how interconnected flies are on a local level, and the probability that flies neighbours will also connect to each other. High clustering coefficient indicates there are tightly knit communities around a particular fly (38).
4. **Betweenness Centrality** – evaluates importance of individuals that are preserving group cohesion, individuals that act as a bridge that connects different individuals. High betweenness centrality indicates that particular individuals are essential for information flow and cohesion (38,39).
5. **Closeness Centrality** – measures the average distance of the shortest paths between each node in the network, which indicates how close a fly is to all other flies in the network. Higher closeness centrality indicates that flies are close to each other and thus well connected (30).

All SINs data were normalized using Z-scores to enable the comparison of networks between isolated and grouped flies. Z-scores were generated using real (observed) and random networks, according to the formula (27):

$$Z - score = \frac{(measurement_{real} - mean(measurement_{random}))}{std(measurement_{random})}$$

For each experimental group we calculated 10 000 random networks in a way that for each experimental group one fly was randomly selected from each arena and 12 such randomized flies were used to calculate the random network.

SINs data was statistically analyzed and visualized using GraphPad Prism 10.0.3 version. Mann-Whitney test was used to analyze differences in behavioral elements between grouped and isolated flies. Unpaired t test or Mann-Whitney test was used to compare differences in SINs parameters between grouped and isolated flies. One-way ANOVA with Tukey's multiple comparisons test or Kruskal-Wallis test with Dunn's multiple comparisons test was used to analyze differences in behavioral elements and SINs parameters between different monoamine supplementation groups. Statistical significance was set to $p < 0.05$. Kolomogrov-Smirnov test was used to test the normality of data.

Visualisation of constructed Social Interaction Networks (SINs) using data from FlyTracker

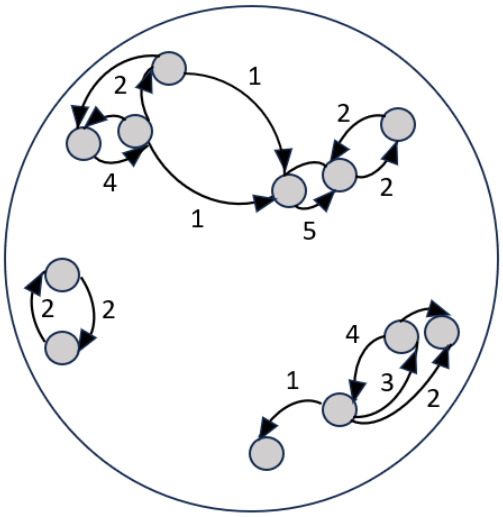


Figure 6. Network representation. Data extracted from FlyTracker was used to construct and visualize social interaction networks (SINs) at a local, and global level. Circles represent flies and arrows represent directed connections between flies. Numbers next to arrows represent weights – the number of times one fly interacted with another fly or duration of interactions.

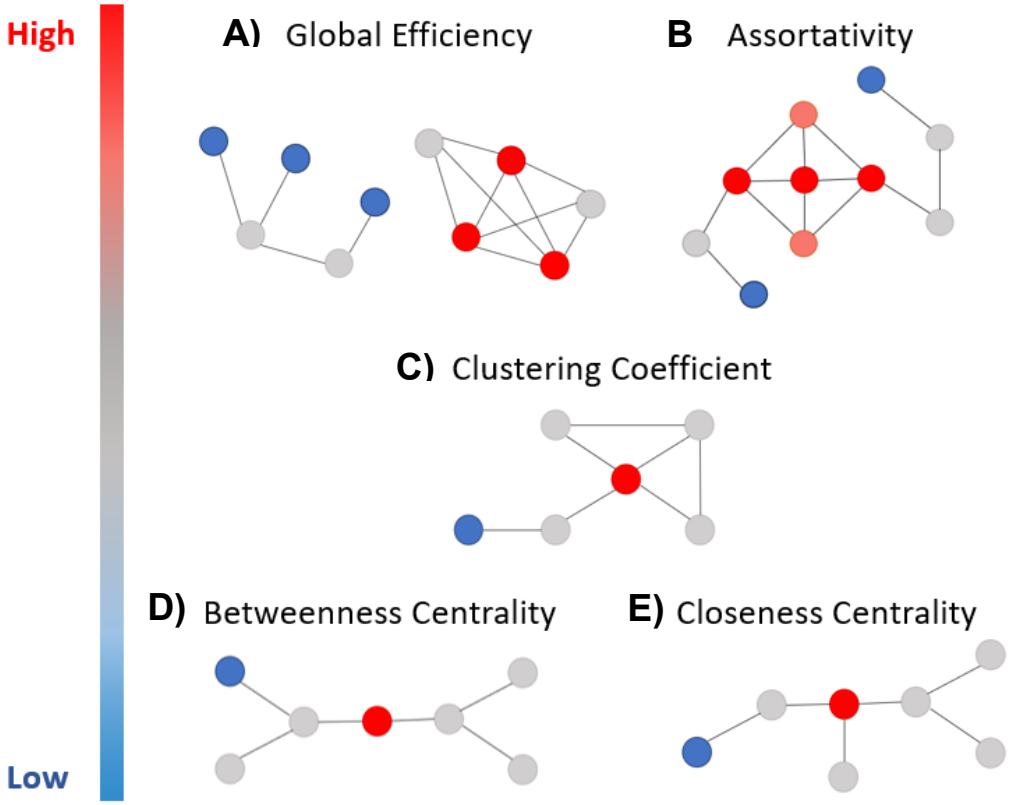


Figure 7. Social interaction networks (SINs) parameters. A) Global Efficiency measures how connected flies are based on distance. **B) Assortativity** measures a degree of mixing based on an attribute on a global level. **C) Clustering Coefficient** measures interconnection between neighboring flies at the local level. **D) Betweenness centrality** identifies flies important for information flow. **E) Closeness Centrality** indicates how close a fly is to all other flies in the network. Dots represent nodes (flies), and lines represent edges (connections). Red dots represent nodes with the highest value of that measure, while blue dots represent nodes with lowest values of that measure.

4. Results

Through the conducted experiments we investigated the effects of social isolation on the concentration of neurotransmitters and on the parameters of social interaction networks (SINs). First, we wanted to determine the influence of social isolation and its duration on concentration of neurotransmitters. For that, we isolated male flies one and five days in glass tubes on cornmeal food. After isolation concentration of dopamine (DA), octopamine (OA), tyramine (TA), gamma-aminobutyric acid (GABA), glutamate (GLU), and acetylcholine (ACh) were measured in head homogenates, using LC-MS/MS analysis. Control group consisted of male flies, 3-5 days old, cultivated on cornmeal food with females (CTRL).

4.1. Prolonged social isolation changes concentration of neurotransmitters in male *D. melanogaster*

One-day social isolation led to a statistically significant decrease in concentration of DA and GLU (Fig. 8A and E), while concentration of OA and GABA were not changed significantly (Fig. 8B and D). Interestingly, concentrations of TA and ACh were increased, but the difference was not statistically significant (Fig. 8C and F). In the five-day isolated flies we observed statistically significant decrease in the concentration of OA, GLU and ACh compared to CTRL and one-day isolated flies (Fig. 8B, E and F), while concentration of DA was statistically decreased compared to CTRL (Fig. 8A). Concentrations of TA were also decreased but the difference was significant only compared to one-day isolated flies (Fig. 8C). Surprisingly, the concentration of GABA remained at very similar levels after one or five days of social isolation (Fig. 8D).

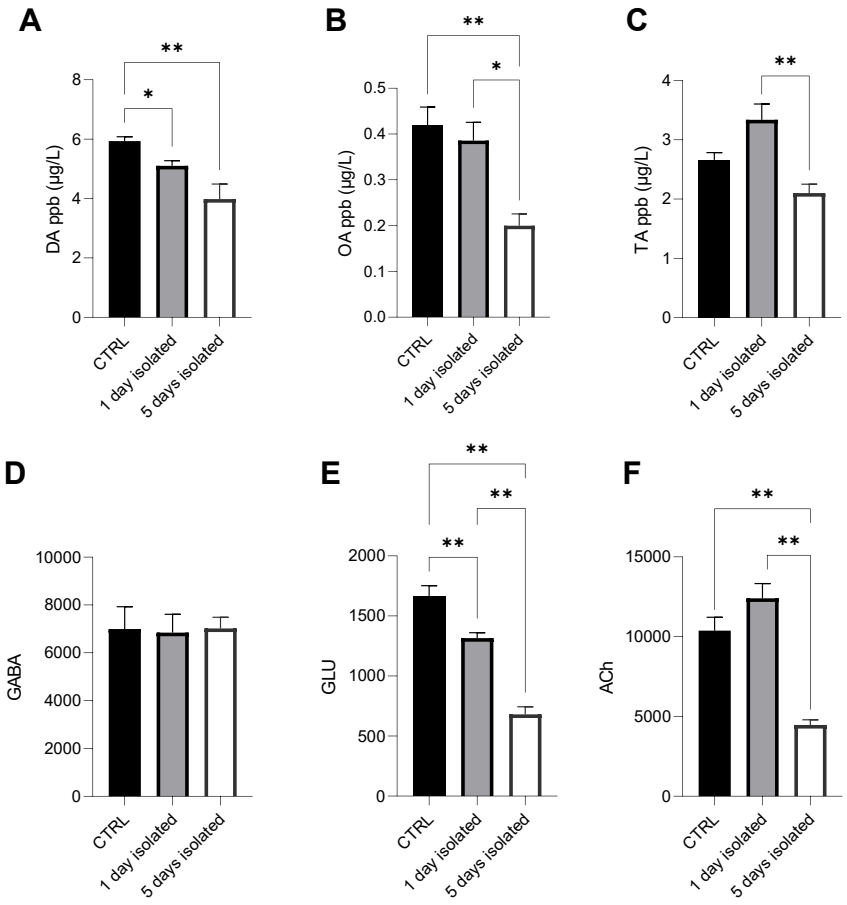


Figure 8. Five-day social isolation decreases concentration of monoamines, glutamate, and acetylcholine, more than one-day social isolation. Male flies were isolated one and five days on the cornmeal food. After isolation 15 male flies were decapitated, and their heads were homogenized for neurotransmitters measurement. Concentration of: **A) dopamine (DA)**, **B) octopamine (OA)**, **C) tyramine (TA)**, **D) gamma-aminobutyric acid (GABA)**, **E) glutamate (GLU)**, and **F) acetylcholine (ACh)** was measured using LC-MS/MS. Control group **(CTRL)** consisted of 3-5 days old male flies cultivated in bottles. Statistical analysis was done using Kruskal-Wallis test with Dunn's multiple comparison post-hoc test that showed multiple significance *: $p < 0.05$, and **: $p < 0.01$. Bartlett's test or Brown–Forsythe's test was used to test the normality of data.

4.2. Five-day social isolation changes behavioral elements and parameters of SINs

The following experiments were focused on the influence of social isolation on parameters of social interaction networks (SINs). Considering that results for measured concentrations of neurotransmitters showed that five-day social isolation decreased concentration of DA, OA, GLU and ACh more than one-day social isolation, we continued to study SINs on five-day isolated flies. First, we tested if SINs would differ between isolated and grouped flies. Therefore, we grouped/isolated male flies five days on cornmeal food. After isolation 12 flies were recorded in circular arena for 20 minutes and SINs were constructed and analyzed with Python scripts through five parameters: global efficiency, assortativity, clustering coefficient, betweenness centrality and closeness centrality. Considering our constructed networks are represented as weighted directed graphs, meaning that we know the direction of interaction, and which fly interacted with which, we have two graphs for each parameter, except global efficiency, because of two weights (numerical value) assigned to each node (fly): count and duration. They provide a more detailed understanding of how many times two flies interacted and for how long. We also calculated three measures related to social interactions as behavioral elements of the network: movement (average distance a fly walks in millimeters per second), interaction rate (number of achieved interactions per minute) and interaction duration (average interaction time in seconds) (40). In this experiment control group (CTRL) consisted of 10 days old male flies, which were cultivated with females on cornmeal food.

Analysis of behavioral elements showed statistically significant differences between grouped (CTRL) and isolated flies (ISO). ISO moved significantly more than CTRL, although all flies were similar age (Fig. 9A), indicating that social isolation increases locomotor activity. ISO group also achieved more interactions (Fig. 9B), while average duration of those interactions was lower than in the CTRL group (Fig. 9C).

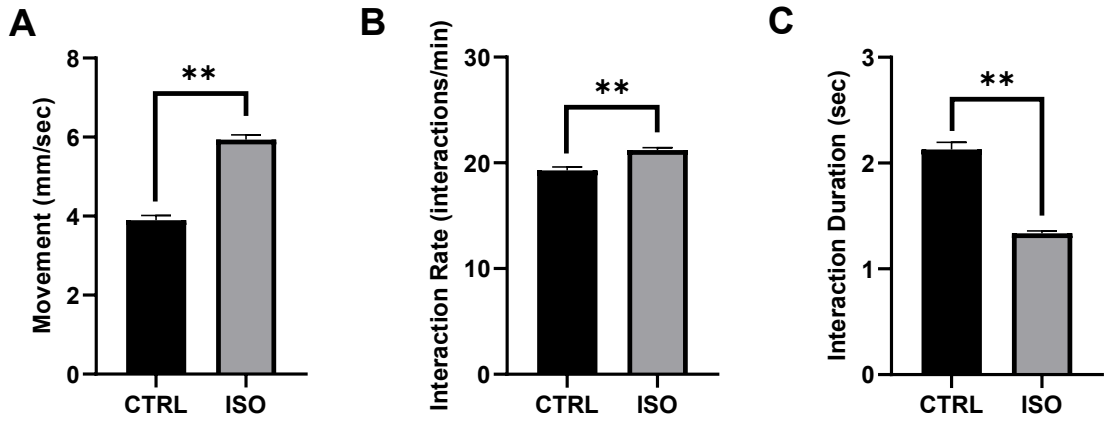


Figure 9. Social isolation increases movement and number of interactions but decreases duration of interactions. 3-5 days old male flies were isolated five days (in total 10 day old) **(ISO)** or they were cultivated with females for 10 days **(CTRL)** in glass tubes on cornmeal food. After the treatment 12 flies were transferred into circular arena and recorded for 20 minutes. Using Python scripts, recorded data was processed, and behavioral elements were calculated: **A) Movement (mm/sec), B) Interaction Rate (interactions/min),** and **C) Interaction Duration (sec).** In total, 240 flies were tested per group. Statistical analysis was done using Mann-Whitney test that showed significance **: $p < 0.01$. Kolomogorov-Smirnov test was used to test the normality of data.

Network analysis at the global level by global efficiency and assortativity did not show statistical differences between CTRL and ISO (Fig. 10A and B). Both groups had high global efficiency (Fig. 10A) indicating networks are globally well connected. However, when looking at the local levels there were statistically significant differences in clustering coefficient and closeness centrality (Fig. 11A and C). ISO showed statistically increased clustering coefficient by number of interactions and their duration compared to CTRL (Fig. 11A). Betweenness centrality did not differ between CTRL and ISO by count and duration (Fig. 11B), indicating no fly acted as a bridge transferring information between subgroups. In contrast to clustering coefficient, social isolation statistically decreased the average number of shortest paths between each fly (Fig. 11C). Their duration was also decreased but the difference was not statistically significant indicating weaker connections between flies (Fig. 11C).

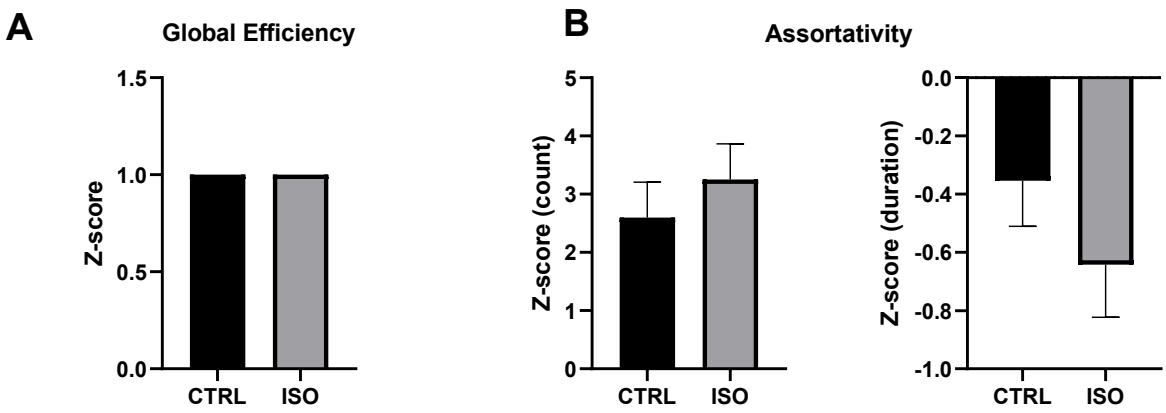


Figure 10. Social isolation does not affect parameters of social interaction networks on a global level. 3-5 days old male flies were isolated five days (in total 10 day old) **(ISO)** or they were cultivated with females for 10 days **(CTRL)** in glass tubes on cornmeal food. After the treatment 12 flies were transferred into circular arena and recorded for 20 minutes. Using Python scripts, recorded data was processed, and global parameters were calculated: **A) Global Efficiency** measures how connected flies are based on distance and **B) Assortativity (count and duration)** measures a degree of mixing based on an attribute. In total, 240 flies were tested per group. Measurements were standardized using Z-scores. Statistical analysis was done using Mann-Whitney test. Significance was set at $p < 0.05$. Kolomogorov-Smirnov test was used to test the normality of data.

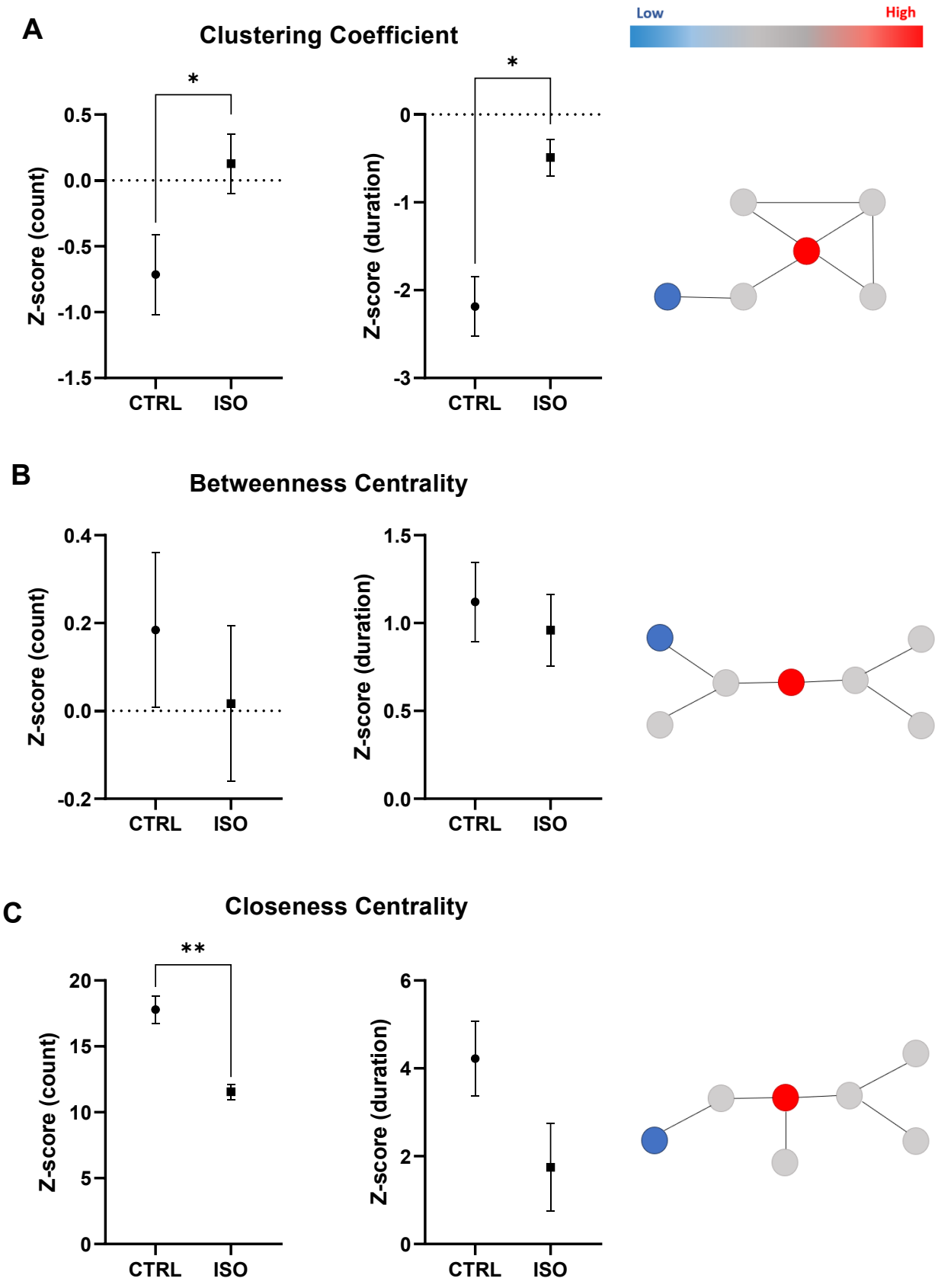


Figure 11. Social isolation increases clustering coefficient but decreases closeness centrality between flies. 3-5 days old male flies were isolated five days (in

total 10 day old) **(ISO)** or they were cultivated with females for 10 days **(CTRL)** in glass tubes on cornmeal food. After the treatment 12 flies were transferred into circular arena and recorded for 20 minutes. Using Python scripts, recorded data was processed, and local parameters were calculated and visually represented next to graphs: **A) Clustering coefficient (count and duration)** measures interconnection between neighboring flies. **B) Betweenness centrality (count and duration)** identifies flies important for information flow, flies that act as a bridge. **C) Closeness centrality (count and duration)** indicates how near a fly is to all other flies in the network. Dots represent nodes (flies), and lines represent edges (connections). Red dots represent nodes with the highest value of that measure, while blue dots represent nodes with lowest values of that measure. In total, 240 flies were tested per group. Measurements were standardized using Z-scores. Statistical analysis was done using unpaired t test or Mann-Whitney test that showed multiple significance *: $p < 0.05$, and **: $p < 0.01$. Kolomogorov-Smirnov test was used to test the normality of data.

4.3. Supplementation with dopamine and octopamine reversed effects of social isolation on local parameters of SINs.

Considering that previous experiments showed decreased concentrations of monoamines and changes in behavioral elements and local parameters in five-day isolated flies we further investigated our third hypothesis, if supplementation with dopamine and octopamine would reverse those effects of social isolation. Therefore, we isolated flies for five days on cornmeal food with different supplements: 5 mg/ml L-DOPA (L-DA), 10 mg/ml octopamine (OA) and combination of 5 mg/ml L-DA and 10 mg/ml OA (L-DA+OA). After isolation 12 flies from each group were recorded in a circular arena for 20 minutes. Using Python scripts, recorded data was processed, and behavioral elements and parameters of SINs were calculated.

Supplementation with L-DA and L-DA+OA led to a statistically significant increase of movement compared to the non-supplemented group (ISO) (Fig. 12A), while OA did not increase movement as we expected (Fig. 12A). Interaction rate showed a gradual statistically significant increase in number of interactions with each supplementation, with L-DA+OA having

the greatest effect (Fig. 12B). In contrast, duration of those interactions did not follow the same increase (Fig. 12C). There was no statistically significant difference in the duration of interactions between ISO, OA, and L-DA+OA, while in the L-DA group duration of interactions was statistically decreased compared to ISO (Fig. 12C), indicating supplementation also affects locomotor activity and number of interactions.

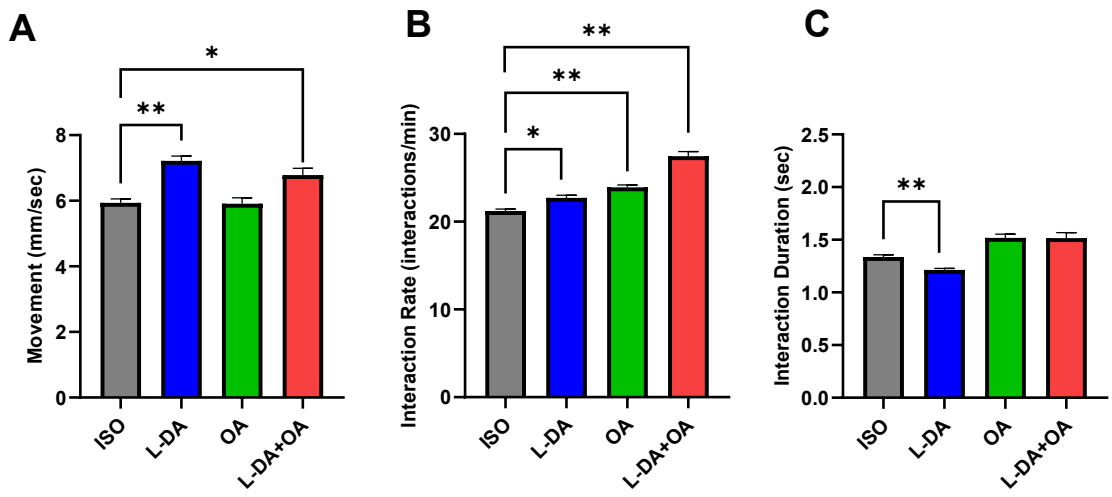


Figure 12. Supplementations containing L-DA increases movement and number of interactions but decrease duration of interactions. 3-5 days old male flies were isolated five days in glass tubes on cornmeal food with different supplements: **isolated flies (ISO)** = food without supplementation, **L-DOPA (L-DA)** = food with 5 mg/ml L-DA, **octopamine (OA)** = food with 10 mg/ml OA, and **L-DA + OA** = food with both 5 mg/ml L-DA and 10 mg/ml OA. After isolation 12 flies from each group were recorded in circular arenas for 20 minutes. Recorded videos were tracked using Flytracker and processed using Python scripts, from which behavioral elements were calculated: **A) Movement (mm/sec), B) Interaction Rate (interactions/min),** and **C) Interaction duration (sec).** In total, 240 flies were tested per group. Statistical analysis was done using Kruskal-Wallis test with Dunn's multiple comparisons test that showed multiple significance *: $p < 0.05$, **: $p < 0.01$ (siginficance was shown only compared to ISO). Kolomogorov-Smirnov test was used to test the normality of data.

Global network analysis by global efficiency did not show differences between supplementation and social isolation (Fig. 13A) but there was a significant gradual decrease in assortativity by the number of flies

connecting with each other based on interaction frequency (Fig.13B left). L-DA+OA group had significantly lower assortativity values compared to ISO and CTRL (Fig. 13B left). Assortativity by duration did not statistically differ between ISO and supplementation groups, but there was statistical difference between CTRL and L-DA+OA group; combination od L-DA+OA decreased duration of interactions (Fig. 13B right).

Considering the occurrence of increased interaction rate induced by social isolation without significant alterations to the global structure of the social network, we analyzed local parameters of SINs. We observed significant differences in clustering coefficient and centrality parameters (Fig. 14). Although clustering coefficient did not differ significantly between supplementation groups and ISO, there were significant differences within supplementation groups and CTRL (Fig 14A left). Supplementation with L-DA significantly increased clustering coefficient by the number of flies that connect to their neighbours compared to OA and CTRL (Fig. 14A left), while duration of those interactions was decreased in all supplementation groups, with OA having the highest effect which resulted in significant decrease compared to ISO and CTRL (Fig. 14A right). Betweenness centrality also did not statistically differ between ISO, CTRL and supplementation groups by count and duration, but there was statistically significant increase in the number of flies that act as connection bridge between other flies in OA group compared to L-DA (Fig. 14B left). Also, betweenness centrality by duration was significantly increased in L-DA+OA compared to L-DA (Fig. 14B right). These differences between supplementation groups indicate different effects that dopamine and octopamine have on SINs parameters.

The highest effect of supplementation during social isolation was observed in closeness centrality parameter (Fig. 14C). There was a statistically significant increase in the number of flies that are closely connected in OA and L-DA+OA group compared to ISO and L-DA, while in L-DA group number of close connected flies was significantly decreased (Fig. 14C left). A similar pattern was reflected on the duration of those

interactions (Fig. 14C right). There was a statistically significant increase in the duration of close connections in OA and L-DA+OA group compared to ISO and L-DA, but here we observed a significant decrease in L-DA group compared to CTRL but not to ISO (Fig. 14C right), indicating on potential effects of supplementation with octopamine in modulating centrality parameters that were changed due to social isolation.

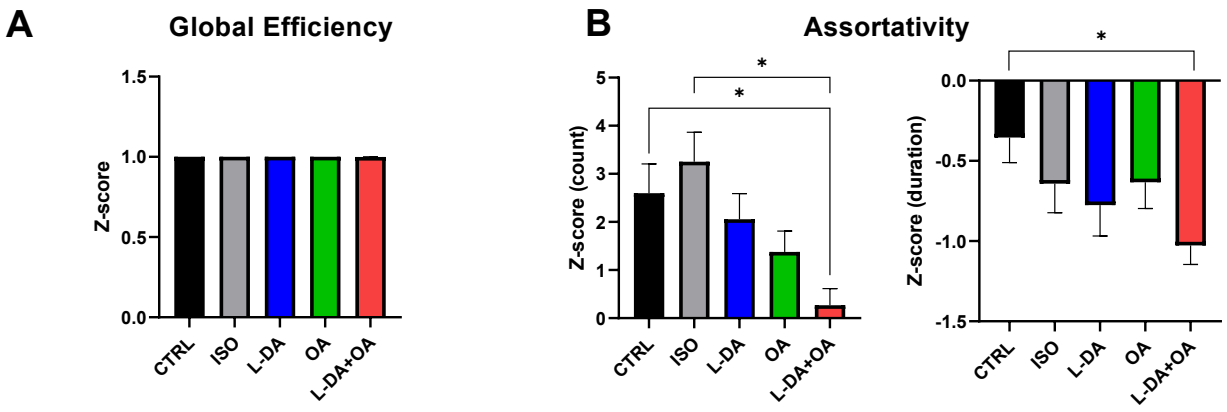


Figure 13. Supplementation with combination of dopamine and octopamine decreases assortativity by the number of interactions. 3-5 days old male flies were isolated five days in glass tubes on cornmeal food with different supplements: **isolated flies (ISO)** = food without supplementation, **L-DOPA (L-DA)** = food with 5 mg/ml L-DA, **octopamine (OA)** = food with 10 mg/ml OA, and **L-DA + OA** = food with both 5 mg/ml L-DA and 10 mg/ml OA. After isolation 12 flies from each group were recorded in circular arenas for 20 minutes. Control group (**CTRL**) consisted of grouped males 10 days old, cultivated od cornmeal food. Recorded videos were tracked using Flytracker and analyzed using python scripts, from which global parameters were calculated: **A) Global Efficiency** measures how connected are flies based on distance and **B) Assortativity (count and duration)** measures a degree of mixing based on an attribute. In total, 240 flies were tested per group. Measurements were standardized using Z-scores. Statistical analysis was done using ordinary one-way ANOVA with Tukey's multiple comparisons test or Kruskal-Wallis test with Dunn's multiple comparisons test and showed multiple significance *: $p < 0.05$. Kolomogorov-Smirnov test was used to test the normality of data.

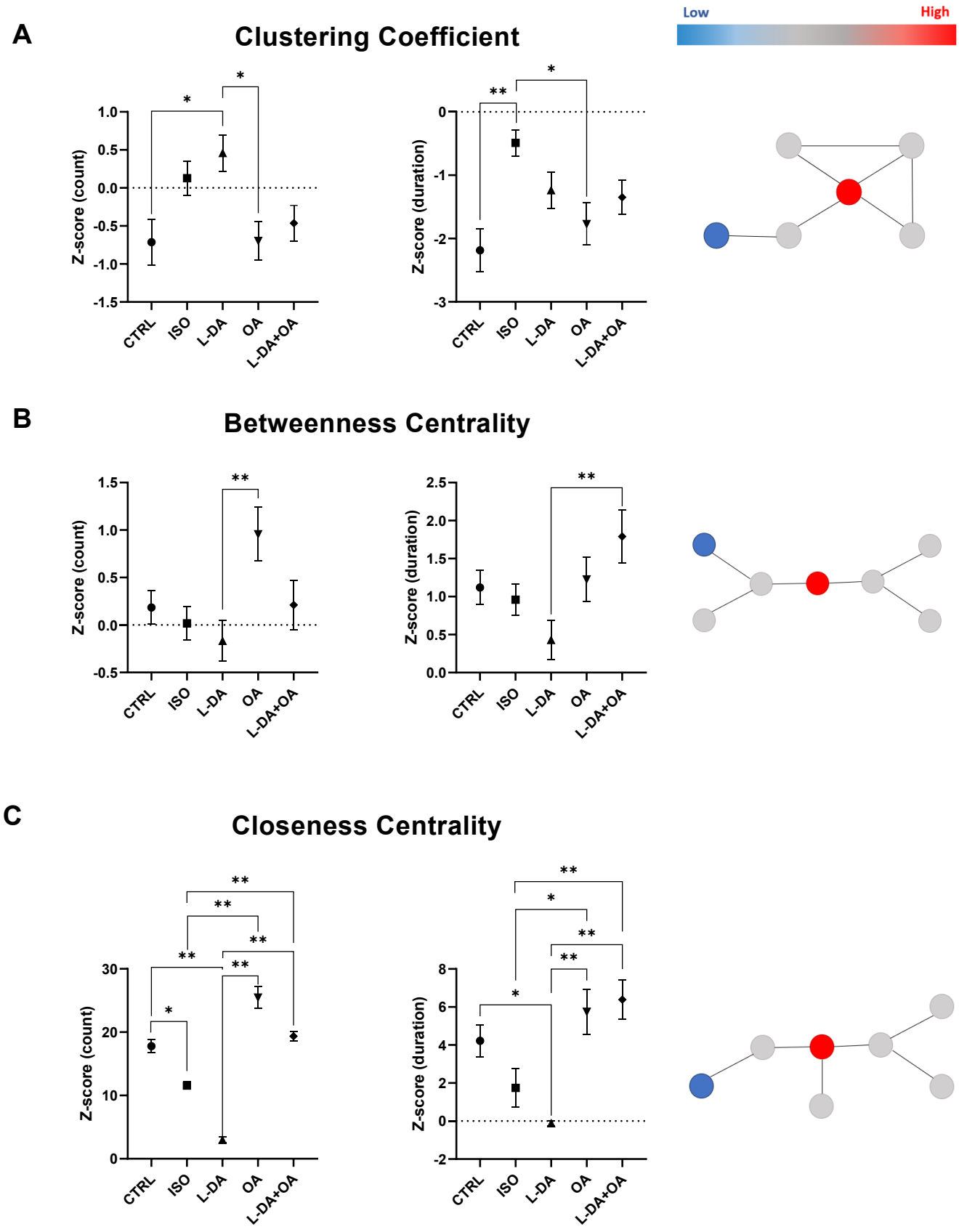


Figure 14. Supplementation with octopamine decreases clustering coefficient by duration and increases closeness centrality. 3-5 days old male flies were isolated five

days in glass tubes on cornmeal food with different supplements: **isolated flies (ISO)** = food without supplementation, **L-DOPA (L-DA)** = food with 5 mg/ml L-DA, **octopamine (OA)** = food with 10 mg/ml OA, and **L-DA + OA** = food with both 5 mg/ml L-DA and 10 mg/ml OA. After isolation 12 flies from each group were recorded in circular arenas for 20 minutes. Control group (**CTRL**) consisted of grouped males 10 days old, cultivated od cornmeal food. Recorded videos were tracked using Flytracker and analyzed using python scripts, from which SINs parameters were calculated and visually represented next to graphs: **A) Clustering coefficient (count and duration)** measures interconnection between neighbouring flies. **B) Betweenness centrality (count and duration)** identifies nodes important for information flow, flies that act as a bridge. **C) Closeness centrality (count and duration)** indicates how near a fly is to all other flies in the network. Dots represent nodes (flies), and lines represent edges (connections). Red dots represent nodes with the highest value of that measure, while blue dots represent nodes with lowest values of that measure. In total, 240 flies were tested per group. Measurements were standardized using Z-scores. Statistical analysis was done using ordinary one-way ANOVA with Tukey's multiple comparisons test or Kruskal-Wallis test with Dunn's multiple comparisons test and showed multiple significance *: $p < 0.05$, **: $p < 0.01$. Kolomogorov-Smirnov test was used to test the normality of data.

4.4. Age alters behavioral elements and local parameters of SINs

In addition to conducted experiments, we observed that age had the highest effects on SINs parameters at the local level. Beside control group (CTRL) we had one more control called YOUNG, for which we also analyzed SINs parameters. YOUNG group consisted of 3-5 days old male flies while in our experiments flies were 10 days old. 3 to 5 days old flied are used predominantly in *Drosophila* experiments as this is considered to be the adult age. YOUNG flies were cultivated in the same matter as CTRL which from here on are referred to as OLD, because they were 10 days of age on the day of the recording. Young flies were grouped for 24h in groups of 12 on cornmeal food before recording. On the day of the recording, flies were transferred in a circular arena and recorded for 20 minutes after which we performed network analysis.

As expected, YOUNG flies moved significantly more than old flies (Fig. 15A), and consequently they had a higher interaction rate (Fig. 15B), but

the duration of those interactions was significantly lower (Fig. 8C), similar to isolated flies.

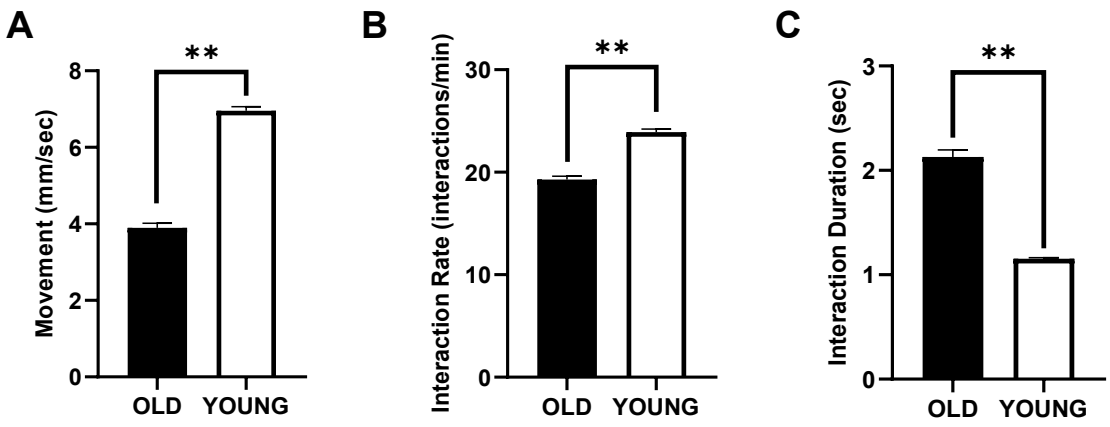


Figure 15. Age decreases movement and number of interactions but increases their duration. 12 male flies, 3-5 days old, were grouped for 24h **(YOUNG)** or cultivated until 10 days of age **(OLD)** and recorded in circular arenas for 20 minutes, after which behavioral elements were calculated: **A) Movement (mm/sec), B) Interaction Rate (interactions/min),** and **C) Interaction duration (sec).** In total, 240 flies were tested per group. Statistical analysis was done using Mann-Whitney test that showed significance **: $p < 0.01$. Kolomogorov-Smirnov test was used to test the normality of data.

Network analysis at the global level did not show significant differences for global efficiency and assortativity (Fig. 16A and B), indicating older flies still maintain globally well-connected networks. At the local levels we observed the highest effect of age on clustering coefficient, betweenness and closeness centrality (Fig. 17). Similarly to the isolated flies YOUNG show statistically significant increase in clustering coefficient by count and duration compared to OLD (Fig. 17A), indicating they are more interconnected to one another. Centrality parameters showed statistically significant differences in both number of interactions and their duration (Fig. 17B and C). Both betweenness and closeness centrality were significantly decreased in YOUNG (Fig. 17B and C) indicating young flies do not have an established structure that enables efficient information flow.

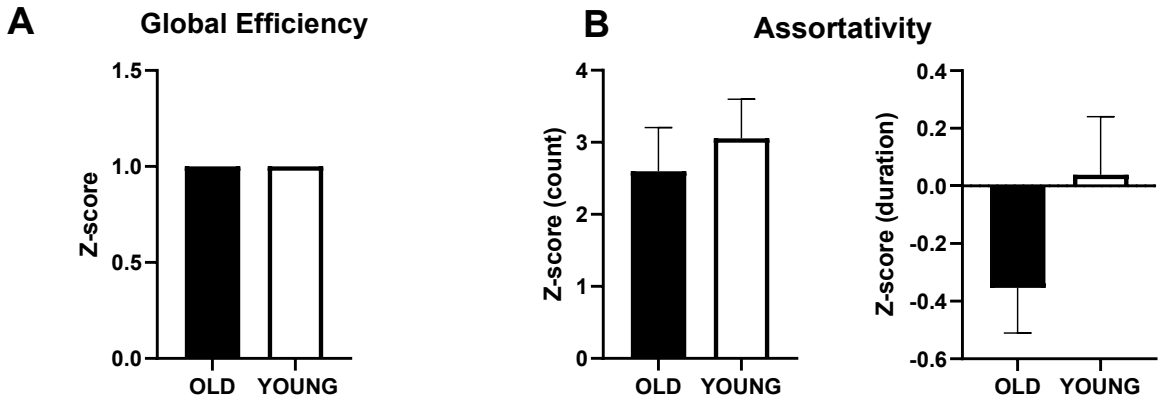


Figure 16. Age does not affect parameters of social interaction networks at the global level. 12 male flies, 3-5 days old, were grouped for 24h **(YOUNG)** or cultivated until 10 days of age **(OLD)** and recorded in circular arenas for 20 minutes, after which global parameters were calculated: **A) Global Efficiency** measures how connected are flies based on distance and **B) Assortativity (count and duration)** measures a degree of mixing based on an attribute. In total, 240 flies were tested per group. Measurements were standardized using Z-scores. Statistical analysis was done using Mann-Whitney test. Significance was set at $p < 0.05$. Kolomogorov-Smirnov test was used to test the normality of data.

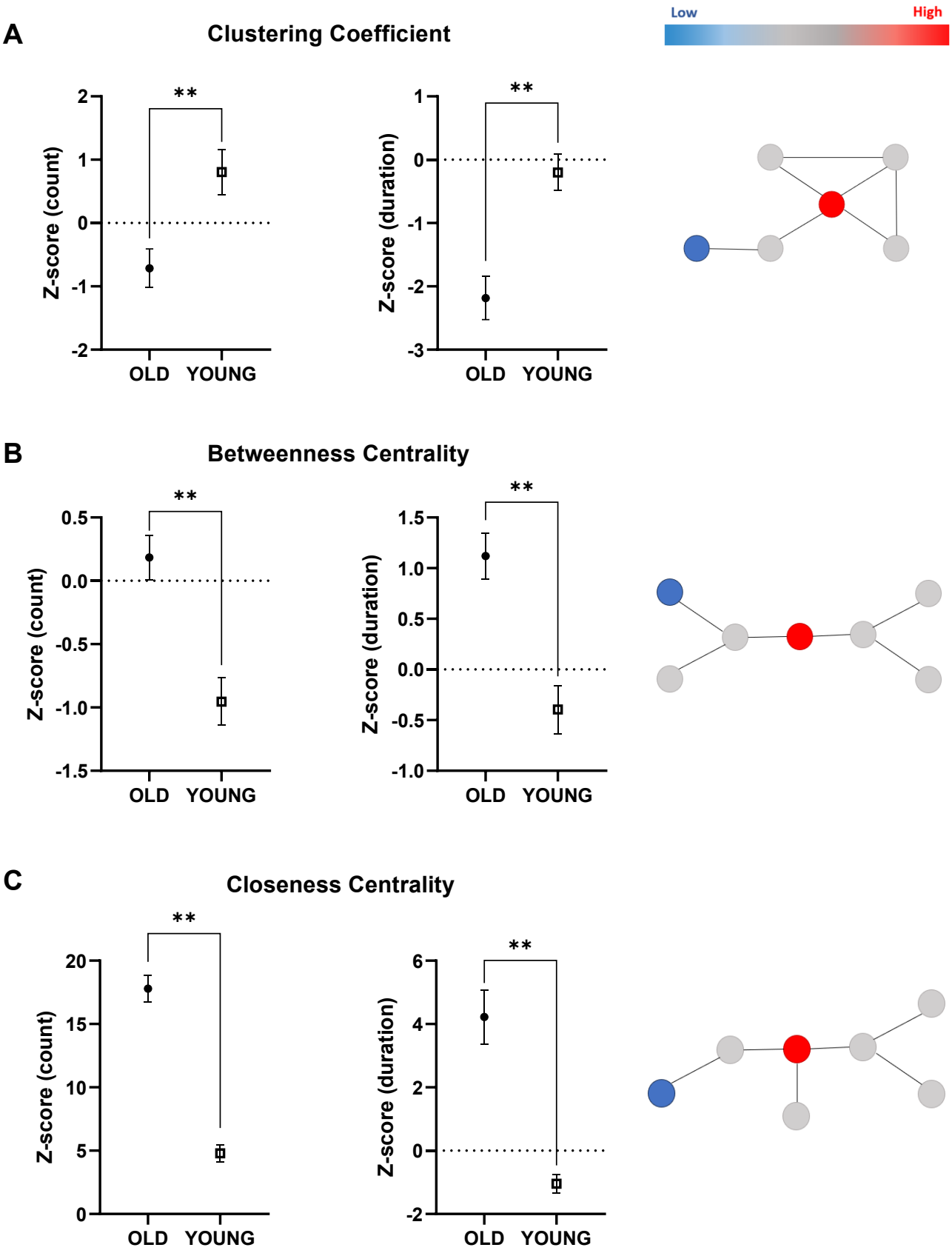


Figure 17. Age decreases clustering coefficient but increases centrality parameters. 12 male flies, 3-5 days old, were grouped for 24h **(YOUNG)** or cultivated

until 10 days of age **(OLD)** and recorded in circular arenas for 20 minutes, after which local parameters were calculated and visualy represented next to graphs: **A) Clustering coefficient (count and duration)** measures interconnection between neighbouring flies. **B) Betweenness centrality (count and duration)** identifies nodes important for information flow, flys that act as a bridge. **C) Closeness centrality (count and duration)** indicates how near a fly is to all other flies in the network. Dots represent nodes (flies), and lines represent edges (connections). Red dots represent nodes with the highest value of that measure, while blue dots represent nodes with lowest values of that measure. In total, 240 flies were tested per group. Measurements were standardized using Z-scores. Statistical analysis was done using unpaired t test or Mann-Whitney test that showed multiple significance: *: $p < 0.05$, and **: $p < 0.01$. Kolomogorov-Smirnov test was used to test the normality of data.

5. Discussion

The behavior of an organism is shaped by its social surroundings. Negative experiences, like social isolation, can have significant and lasting impacts on individual's behavior (41). Therefore, in this thesis we investigated if and how does experience of social isolation influence bahavior of *D. melanogaster.* We measured changes in the concentration of neurotransmitters in one and five day socially isolated flies and behavioral parameters of social interaction networks (SINs) induced by five days of social isolation. We showed that five-day social isolation decreases concentration of dopamine (DA), octopamine (OA), glutamate and acetylcholine more than one-day social isolation. With SINs analysis we showed that five-day social isolation impacts social interactions among isolated flies by enhancing their locomotor activity and social interactions at the local level, while having no influence on the global network structure. Furthermore, due to reduction of concentration of monoamines and changes in local SINs parameters, we investigated if supplementation with DA and OA would reverse effects of social isolation. We showed that supplementation with OA and combination of DA and OA reversed effects of social isolation on SINs parameters at the local level. Finally, we observed similarities between isolated and young flies, as well as a significant effect of age of grouped flies on all local SINs parameters.

Isolation driven behaviors are impacted by neurotransmitters, like biogenic amines; DA and OA which are primary sources of stimulation and reward in insects. Reward center is greatly influenced by DA and OA signalization (42). For instance, in a study on zebrafish when the dopaminergic system was disrupted due to social isolation, it led to changes in the motivation of zebrafish to engage in social behavior, explore the environment, or avoid unpleasant situations. Consequently, this resulted in altered responses to social stimuli, modified locomotor activity, and changes in anxiety (20). Our results show five-day social isolation decreases concentration of DA, OA, glutamte and acetylcholine. Possible

explanations why this happen is that social isolation disturbed reward center signalization. This disturbance possibly affected sensitivity of dopamine receptors. In rats it was found that social isolation reduced expression of dopamine receptor 2, which is an inhibitory receptor that regulates DA signaling (43). Dopamine receptor 2 inhibits DA release when DA levels are high. Dysfunction in these receptors can increase activity and movement, which we see in our isolated flies.

An important similarity between our and other studies of social isolation was the effect on decreasing of DA levels. For example, in a study comparing socially isolated with enriched flies it was reported that DA levels decrease when flies were isolated (44), which also confirms our findings. Similarly, a study that compared group size to several behavioral parameters in the honeybees showed that isolated honeybees exhibited the lowest levels of DA (21). In the same study it was reported that chronic isolation (6 days) of worker honeybees reduces performance of the learning tasks in comparison to grouped honeybees. This study also demonstrated a negative correlation between the size of a group and the level of responsiveness to sucrose, with isolated honeybees exhibiting the highest level of responsiveness. A similar effect of social isolation on learning and memory was observed in *D. melanogaster*. It was shown that isolated flies have fewer mushroom body fibers, which is an important part of the insect brain involved in learning and memory, compared to socially raised flies (45).

Dysregulation of dopamine receptor 1, which is an excitatory receptor that promotes neuronal activity, can lead to impairment in intracellular pathways. In one study researchers examined the behavioral anomalies caused by extended social isolation in adult rats (46). Social isolation caused sensations like anxiety and anhedonia, as well as a reduction in the activity of cAMP response element-binding protein (CREB) in the nucleus accumbens shell, which is a region involved in reward processing, while CREB is a transcription factor that can suppresses the expression of certain

genes. When the expression of CREB was reduced by social isolation, there was an apparent increase in a specific potassium (K+) channel. Ultimately, the expression of seven K+ channels was upregulated as a result of prolonged isolation. These K+ channels have a role in determining the form of action potentials, hyperpolarizing the membrane potential, and influencing neuronal excitability (46). However, additional research is required to determine if CREB functions as a direct inhibitor of transcription for these genes or whether it indirectly controls them via other transcription factors.

Study by Leng et al. reported social isolation did not change levels of acetylcholine in the prefrontal cortex of rats that have been isolated for a year (47), while another study reported increased extracellular concentrations of acetylcholine in prefrontal cortex of isolated flies (48). These findings do not support our results for five-day isolated flies, as we observed decreased levels of acetylcholine. Studies in *Drosophila* show that *Drosophila's* many cholinergic neurons mediate isolation-driven behaviors. Feminizing cholinergic neurons by expressing the *transformer* gene in a screen of 60 GAL4 lines enhanced aggressiveness in male flies (49), suggesting that acetylcholine only indirectly mediates social isolation. It still remains unknown whether this decrease after social isolation is due to increased acetylcholinesterase activity or changes in functions of cholinergic receptors, therefore further investigation is needed.

Glutamate and GABA, are know excitatiory and inhibitory neurotransmitters that were also noticed to have influence in isolation induced behaviors. Our analysis showed social isolation decreased concentration of glutamate but did not affected GABA concentrations. In contast to our findings rodents that were subjected to social isolation after weaning showed that glutamate levels were increased when depolarizing stimulans K+ was given to male rats through microdialysis probe, while basal levels of glutamate were unaffected (50). Interestingly, other studies have reported decreased levels of glutamate and glutamine in dorsal

hipoccampus of rats isolated for 8 weeks (51). The differences among these results could be due to the isolation protocols, and in particular to the age of the animals. Therefore, our findings need further investigation to determine certain effects of social isolation on concentration of glutamate and GABA.

Social isolation in humans is a contributing factor that may lead to decreased cognitive performance and function, as well as enhanced negativity and depression (52). In fruit flies social isolation leads to significant behavioral changes, such as elevated aggression (25) and increased locomotor activity (29,31). We observed similar changes in behaviors. Isolated flies had increased locomotor activity and number of interactions, but on average those interactions were shorter than in the non-isolated flies. These results are in concordance with other two studies where total distance, number of interactions (29) and velocity (mm/sec) of flies (31) was higher in isolated flies. Five-day social isolation compared to one-day isolation, is considered chronic in flies (53), and acts as a stressor. One study reported decreased levels of antioxidant enzymes superoxide dismutase and catalase and elevated levels of hydrogen peroxide, indicating social isolation caused oxidative stress in isolated rats (51). A speculative interpretation could be that isolated flies seek to fulfil their social needs with a higher number of superficial interactions. Although isolation has a profound long-term effect on individuals' behavior, most changes at the level of gene expression occur during the first day and significantly diminish on day 3 and day 6 as Liu et al. reported. Effects of short-term isolation on gene expression were reported in other studies. Piglets isolated for only 4 hours exhibited behavioral abnormalities in open-field tests, neuroendocrine alterations, including elevated levels of cortisol, known as a stress hormone and alterations in the gene expression that control the glucocorticoid response (54).

Our network analysis showed that social isolation does not affect global SINs parameters: global efficiency and assortativity. All tested groups had

the same values of global efficiency because our networks were completely connected, meaning that all 12 flies connected in a period of 20 minutes. In previously mentioned isolation studies, there was also no differences in global efficiency between grouped and isolated flies (29,31), supporting our finding that social isolation does not disturb global structures in flies. One approach which would give us information if there were differences in global parameters would be the analysis of temporal networks. Temporal networks analyze one fly at a specific time frame, for example in the first ten one-minute frames. This kind of analysis would give us more in-depth insights on how the network dynamics change through time for different SINs parameters.

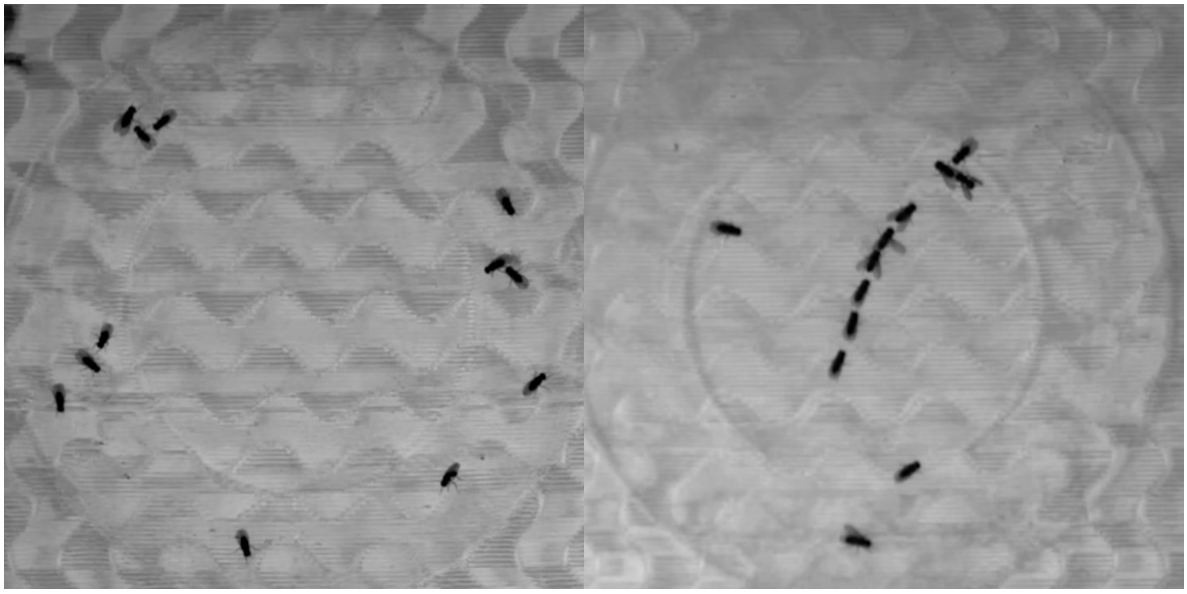
Network analysis at the local level showed high clustering coefficient and low closeness centrality values. These results suggest that isolated flies all connected with each other, but those connections were not close by distance as in grouped flies. This indicates that social isolation impairs flies' ability to maintain potentially more informative interactions with other flies in a group. One explanation is that it is due to stress response and changes in the reward processing resulting in decreased interaction duration and increased movement as well as interaction rate. Similarly, Liu et al. showed that 6 day isolation increased clustering coefficient (29). In contrast, Bentzur et al. reported that 3 day isolation decreased clustering coefficient. These differences could be explained by different methodological steps and different lengths of social isolation.

Although articles investigating social isolation have the same goal of understanding effects of social isolation on behavior and group dynamics through analysis of social interactions, there is not an established protocol (Table 1). Instead, there are a lot of different methods that differ in isolation procedure and network analysis, which result in large discrepancy of the results, as mentioned previously. Differences vary from how many flies are used in an experiment, their age, how long are flies isolated, the recording time and data analysis. For example, Liu et al. (2018) collected 9 days old

flies, grouped them for one day and isolated them from 1 to 6 days. They recorded 16 flies in fly chamber for 60 minutes in complete darkness and tracked recorded videos with Flytracker software. In contrast, Bentzur et al. (2021) isolated flies 3 days after eclosion and recorded 10 flies in a FlyBowl for 15 minutes using Ctrax tracking software. Interestingly, both studies got similar results concerning movement of isolated flies, while their SINs parameter differed. Our results are more in concordance with Liu et al. considering that we had flies similar of age. Although direct comparison is not justified because of differences in the recording time, conditions, and data aquisition (Table 1). For example, Liu et al. considered only head to tail touch as social interaction, while we scored all interactions that satisfied criteria for specific angle at which one fly approaches another. Therefore, it is important that in the future studies of SINs agree on a standardized method.

In all isolated groups in our study, we noticed an interesting behavior that is usually observed among males that have increased sexual arousal due to genetic or environmental manipulations. We observed that males formed “train” like chasing formations containing 5-7 flies, where they aggressively chased each other and tried to couplate with one another. This was the most prevalent in the L-DA+OA supplementation group (Picture 1 right). We tried to quantify that behavior, but our model to detect “train” like formations was not precise enough. Although Benzur et al. reported increase in the proportion of time that isolated flies spent engaging in chase and approach behavior, they did not reported any chasing formations (55). This formations occur with increased sexual arousal, and in flies it can be a consequence of the manipulation of the *fruitless* gene that regulates courtship in males, and the manipulation of DA, especially increased DA (56). Another thing is that in aggressive behavior between two males, there is often a combination of aggressive elements with elements of courtship because of the neural networks that control this partially overlap. One study found that when subjected to social isolation, one mutant known as

sex pistol displayed elevated aggression and exhibited a significant degree of male-male courtship (57). Therefore, isolation in our case may encourage aggressiveness and feeding with DA sexual arousal. Furthermore, it was shown that OA has a role in regulating aggression in fruit flies (18). In one study OA was genetically depleted in mutant flies and that decreased lunge frequency, which is a known aggressive behavior. They also reported that decreased lunge frequency was not influenced by the presence or absence of tyramine (18). Moreover, one study reported that administration of L-DA to cultured ganglia of the migrating locust led to the production of m-OA, which is the physiologically active isomer of OA (15). This could potentially contribue to OA induced aggressive behavior seen in isolated flies fed with comabination of DA and OA.



Picture 1. Social isolation increases chasing and aggressive behavior. The left picture represents flies that were grouped (CTRL) and the right picture represents isolated group – L-DA+OA from which "train like" formation was observed.

Studies conducted on rats indicate that the effect social isolation has on the brain may be reversed. These studies have shown that resocialization may enhance memory and decrease symptoms of anxiety and depression, as well as reverse changes in the structure of neurons in the hippocampus due to stress induced by social isolation (58). Moreover, many therapeutic

strategies are aimed at relieving negative effects of social isolation. Supplementation with different neurotransmitters are usually approaches for mediating effect of age and nuropsychiatric disease like anxiety, depression, Parkinson disease and schizophrenia, on lowered cognitive functions due to lower concentrations of DA (7,59,60). In the study done on elderly people it was shown that supplementation with L-DA, which is a precursor for DA, did not have positive effects on learning and cognitive performance and that administration of L-DA disturbed the balance of dopaminergic system (59). In contrast, in Parkinsons disease clinical studies confirmed efficiency of L-DA therapy (60).

There are not any studies investigating how supplementation affects social structures among fruit flies. In our experiment we tried to reverse the effects that social isolation had on SINs parameters. We showed that supplementation with OA and combination of DA and OA reversed effects social isolation had on social structure in flies, with distinct effects between DA and OA. Those effect are prominent already in movement where OA alone did not increase fly's locomotor activity like DA and combination of DA and OA did. A likely explanation is that DA is a neurotransmitter known to increase locomotor activity. The increase in the amount of the locomotor activity in all supplemented groups caused an increase in the interaction rate, which was the most prominent in the L-DA+OA group. Supplementation with combination of DA and OA potentially led to increased release and/or synthesis of DA and OA causing overstimulation of the postsynaptic DA and OA receptors causing hyperactivity. Furthermore, duration of interaction was decreased only in DA supplementation group due to significant increase in the locomotor activity resulting in the reduced interaction duration, which can be interpreted as decreased time for the information transfer between flies. However, because we did not measure the concentration of DA and OA in the heads after isolation in supplemented groups, we cannot empirically support previous explanations. Therefore, in future concentrations of these

neurotransmitters should be measured before and after the supplementation in order to help in providing a better mechanistic explanation.

Global network analysis showed that grouping due to frequency of interactions and their duration (assortativity) was decreased in flies fed with combination of DA and OA, indicating flies with high interaction frequencies interacted with flies that have low interaction frequencies. This can be contributed to the overstimulation of DA and OA in the flies brain that led to randomized connection between flies.

Local network analysis showed the suplementations with OA and combination of DA and OA reversed effects of social isolation on SINs parameters at the local level. Those flies had lowered clustering coefficient and betweenness centrality, while closeness centrality was increased, meaning those flies were able to achieve closer connections with each other like grouped flies. One of the explanations could be that OA induced strong intracellular response. It is known that OA elevates the levels of cAMP, due to the synchronized activation of Ca2+/Calmodulin triggered adenylate cyclase, which results in a sustained activation of protein kinase A (PKA) and can phosphorylate the nucleus cAMP-responsive element binding protein (CREB). Consequently, this may activate the transcription of genes regulated by CRE-elements, thus generating novel proteins to facilitate the formation of long-term memory and enhance cognitive performance (15). Additionally, one study in *C. elegans* showed that stress caused by starvation, increased OA and decreased DA signaling (16). This could indicate an excluding effect of DA and OA, and therefore explain differences in our parameters of SINs between the two neurotransmitters.

A somewhat surprising finding was that we observed a strong effect of small age difference on SINs parameters of grouped flies. We observed differences in behavioral elements and SINs parameters at the local level between older (10 days old) and younger flies (3-5 days old). Younger flies moved more and had many shorter interactions compared to older flies. In

our SIN analysis older flies had decreased clustering coefficient and elevated centrality values (betweenness and closeness centrality), which indicate older flies, based on previous experience established more close connections that are longer lasting. As well they had more established structure where there were individuals that act as information hubs, controlling information flow. A speculative explanation for these results is that young flies due to better locomotion and shorter exposure to social interaction want to explore and interact with other individuals more than older flies.

Based on longevity study (61) done on aged fruit flies (45-55 days old) our 10 days old flies cannot be classified old. Nevertheless, we suggest that age is a significant factor in observing SINs between 3-5 days old and 10 days old flies. Although, we did not analyzed concentrations of neurotransmitters in 10 days old flies, previous statement can be to some extent supported with neurotransmitter analysis from longevity studies. In that study they showed that DA, OA and serotonin were significantly increased in adult flies (3-5 days old) compared to newly enclosed (8h old) and mature flies (45-55 days old), while concentrations of acetlycholine and tyramine were not changed (61). Similarly, Piccirillo et al. (2014) demonstrated that the DA concentration reaches its peak in flies between 3 and 7 days after eclosion. Once flies become fully sexually mature, their DA levels start to decline (62). Therefore, social isolation during life stages when DA levels are changing as a function of maturation can significantly change DA signaling and their social interactions. The underlying molecular mechanism of DA impairment and social isolation should be further investigated.

Finally, we noticed positive correlation in local SINs parameters between isolated and young flies, both had similar network properties (high clustering coefficient and low centrality values). This indicates previous social experience and social interaction are important for group communication and connectivity. Isolation at young age might prevent flies

from acquiring social experiences necessary for the formation of structured networks. Many studies show that social isolation at an early age can impair individual's social abilities. For example, in zebrafish, long-term isolation at early age led to hyperactivity in adulthood (20).

6. Conclusion

Social isolation can have long-lasting neurochemical and behavioral impacts on individuals affecting their social interaction. Negative consequences of social isolation due to COVID-19 pandemic are still an unexplored area that has been shown to be associated with psychological disorders.

In our research using model organism *Drosophila melanogaster* we show that social isolation indeed changes behavioral patterns. Isolated flies exhibited increased locomotor activity, higher interaction rates, but shorter interaction durations compared to the control group. We have reported increased clustering coefficient and decreased closeness centrality in isolated flies, indicating more local interconnections but weaker overall network integration. Isolation led to reduced concentrations of monoamines, primary dopamine and octopamine, who act as rewarding signal molecules. We showed that supplementation with octopamine and combination of dopamine and octopamine can to some extent reverse and improve behavioral consequences of social isolation with significantly increased movement and interaction rates but decreased interaction durations. We also reported age related effects. Three to five days old flies moved more, had higher interaction rates but shorter durations compared to mature, ten days old flies. Young flies had higher clustering coefficients and lower centrality measures, indicating more interconnected but less efficient information flow networks compared to older flies.

Our results need to be further confirmed and examined on a molecular level, and further research should focus on unraveling the complex interplay of neurotransmitters that were impacted by social isolation. Nevertheless, we showed that supplementation with selected neurotransmitters could potentially help in the development of novel strategies potentially affecting the reward center, which would ultimately reduce and improve the negative effects of social isolation.

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8. Biography

Marta Medija

Rijeka, Croatia, marta.medija8@gmail.com

PROFILE

Highly motivated Master's student in "Biotechnology in Medicine" at the Faculty for biotechnology and drug development. I am an easy learner with a background in biomedicine. I have valuable practical research experience, and I am eager to contribute to innovative medical approaches.

EDUCATION

Oct 2022 — Present

Master student, Faculty for Biotechnology and Drug Development — Rijeka

My Master thesis is focused on the influence of social isolation on the development of addiction, based on social interaction network analysis.

Oct 2019 — Sep 2022

Bachelor of Biotechnology and Drug Development, Department of Biotechnology — Rijeka

Graduated cum laude.

Bachelor thesis: "The effect of quercetin on the developmental consequences of methamphetamine in fruit fly".

INTERNSHIPS

Aug 2022 — Present

Volunteer, Laboratory for Behavioral Genetics — Rijeka

I have acquired skills and independence in performance and analysis of behavioral assays associated with sleep, locomotor activity, and induction and quantification of behaviors associated with addiction. I gained skills in tissue collection, and sample preparation for different biochemical processes.

Aug 2021 — Sep 2021

Institute for Public Health Rijeka

I worked in the "Department for food control, general use items, nutrition improvement and microbiology". My job included processing food samples, determining the proportion of fat, carbohydrates and proteins in food samples and microbiological analysis of food samples. I gained practical skills in food analysis and microbiology.

SKILLS

Teamwork	Problem Solving
Time Management	Data analysis
Adaptability and flexibility	Behavioral assays
Presentation skills	Drosophila research

EXTRA-CURRICULAR ACTIVITIES

Publication

Filošević Vujnović A, Saftić Martinović L, Medija M, Andretić Waldowski R. **Distinct and Dynamic Changes in the Temporal Profiles of Neurotransmitters in *Drosophila melanogaster* Brain following Volatilized Cocaine or Methamphetamine Administrations.** *Pharmaceuticals*. 2023; 16(10):1489. (**Contribution:** Conducted part of the experiment, helped with data curation and visualization)

Student teaching assistant

Presented and guided students through practical exercises in courses: "Basics of biotechnology and drug research" (2023.) and "Analytical chemistry" (2024.).

Popularization of Science

I participated in 2022 and 2023 European Researchers' Night, project "Flies in classroom", STEM picnic, Festival Tobogan where I gave a lecture to young children about fruit flies and science.

LANGUAGES English

AWARDS AND SCOLARSHIPS

Jun 2022	**1st Place, "PosteRi"** scientific student conference Presented my bachelor research.	Rijeka
Oct 2020 — Jul 2021	**STEM Student Scholarship**	Rijeka
Oct 2021 — Jul 2024	**Municipal Student Scholarship**	Dražice,Jelenje

REFERENCES

Associate professor Rozi Andretić Waldowski,
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