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VREDNOTENJE VEDENJA ZARODKOV RIB ZEBRIC IZPOSTAVLJENIH METILENDIOKSIMETAMFETAMINU

EVALUATION OF LARVAL ZEBRAFISH BEHAVIOR AFTER EXPOSURE TO METHYLENEDIOXYMETHAMPHETAMINE

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Jana Tomc

ABSTRACT

In the last years, following the OECD and FDA regulations, neurotoxicity testing is based on *in vivo* experiments, and zebrafish (*Danio rerio*) as an alternative model is particularly applicable in studies of development and deficits of nervous system and the related behavior. The use of embryonic stages of zebrafish is increasing rapidly due to their good correlations with mammalian systems as well as high genetic and neurodevelopment similarity to humans. Moreover, it complies with the requirements of REACH directive and thus implements its principles of 3R in the use of animals for scientific purposes.

In this thesis, we optimized a method to assess the locomotor activity in early life stages of zebrafish which can provide reliable information about development of nervous system or behavioral selectivity of toxicants and can be simply implemented for the needs of small laboratory. We acknowledged terms and conditions with the use of psychoactive compound *d*-amphetamine, comparing the similarity of our results to the ones from reports. Additionally, a psychoactive substance MDMA in range between 0.005 and 2.59 mM has been tested in order to evaluate developmental and neuronal toxicity, using the ZFET test and behavior test with zebrafish. MDMA is well known as selective neurotoxin for serotonergic system, causing long-term behavior alterations in mammals and assumed to be teratogenic. Our findings indicate that developmental exposure to MDMA between 4 hpf and 52 hpf induced alterations in hatching and a delay in development and the highest 2.59 mM concentration caused neurotoxicity, which was reflected in a decrease of spontaneous movement and an increased occurrence of brain necrosis. However, acute MDMA exposure in this developmental stage did not affect swimming performance at 144 hpf. On the contrary, exposure to MDMA at 144 hours post fertilization (hpf) altered locomotion at much lower concentrations than those used in earlier developmental stage. 0.026 and 0.052 mM MDMA concentrations produced concentration-dependent hypoactivity. Teratogenicity of MDMA was not recorded.

Zebrafish larvae behavior assay allowed us to classify MDMA as a developmental neurotoxicant. Further studies should be done to evaluate in depth if MDMA exposure during interim stages of neuronal development could influence swimming activity of 144 hpf old larvae and therefore the maturation of their nervous systems. Neuropathological studies could be implemented as well to confirm the hypothetical effects of MDMA on neuronal development assessed through locomotor activity.

RAZŠIRJEN POVZETEK

Dandanes smo na različne načine vse bolj izpostavljeni številnim psihoaktivnim snovem. Takšne snovi so tudi amfetamin in njegovi derivati. To so zelo razširjene droge, ki se pogosto zlorabljajo, še zlasti med mladimi, povsod po svetu. Osredotočili smo se na najpogosteje zlorabljen derivat amfetamina, 3,4-metilendioksimetamfetamin (MDMA), ki je dokazano nevrotoksičen za živali in ljudi ter povzroča veliko škodo v delovanju živčnega sistema, kar se dolgoročno kaže predvsem v kognitivnih motnjah in motnjah spomina. Poleg tega naj bi MDMA povzročal tudi razvojno nevrotoksičnost, ki pa je odvisna od razvojne stopnje živčnega sistema v času izpostavljenosti.

Po predpisih organizacij OECD in FDA se nevrotoksičnost kemikalij trenutno ugotavlja s testiranjem *in vivo*, ki temelji predvsem na vrednotenju vedenja, ter nevropatoloških preiskavah pri poskusnih živalih. Takšni testi zahtevajo veliko število živali, so dragi in dolgotrajni. Tako postaja vse večja potreba po cenejših in hitrejših alternativnih metodah z visoko predvidljivostjo, ki pa morajo biti v skladu z načeli 3R direktive REACH o uporabi živali v raziskovalne namene. V zadnjih letih se v raziskavah na mnogih področjih, vključno s toksikologijo, kot alternativni model, vse bolj uporablja vrsta rib zebric (*Danio rerio*), še zlasti njihovi zarodki in larve. Tej vrsti vretenčarjev pripisujejo številne pozitivne lastnosti, še posebno pomembna pa je njihova dobra koleracija s sesalci in visoka genetska podobnost s človekom, kar jim daje veliko prednost kot modelu za določanje številnih vrst toksičnosti. Posebej uporabne so tudi pri raziskovanju motenega razvoja živčnega sistema in s tem povezanega obnašanja.

Glavni namen magistrske naloge je bila optimizacija metode za vrednotenje lokomotorne aktivnosti larv rib zebric, ki bi bila enostavna za uporabo v manjših laboratorijih in bi dajala zanesljive informacije o razvoju živčnega sistema ali o selektivnem delovanju toksičnih snovi na živčni sistem. Na voljo je sicer mnogo literature o presejalnih metodah, s katerimi lahko vrednotimo aktivnost larv rib zebric, vendar še nobena od njih ni bila uradno validirana in standardizirana. Izbrano vedenjsko metodo smo zato optimizirali s pomočjo objavljenih poročil, pri čemer smo se osredotočili na spremljanje že uveljavljenega vzorca aktivnosti larve v izmenjujočih se ciklih svetlobe in teme. Vzpostavljene eksperimentalne pogoje smo nato preverjali z uporabo psihoaktivne snovi *d*-amfetamina ter dobljene rezultate primerjali z rezultati, objavljenimi v študiji, v kateri so uporabili podoben postopek.

Zanesljivost optimizirane metode smo nato preizkusili še s testiranjem druge psihoaktivne substance, MDMA in ovrednotili njen nevrotoksični potencial v različnih stadijih razvoja živčnega sistema rib zebric (od zarodka do larve) ter naše ugotovitve primerjali z rezultati objavljenih študij, opravljenih na ljudeh in poskusnih živalih. Dodatno smo izvedli še test razvojne strupenosti na zarodkih, da bi raziskali vpliv MDMA na celoten razvoj zebrice ter z dodatno metodo vrednotili prvo spontano aktivnost zarodkov v ovojnici.

Ugotovili smo, da je MDMA pri zarodkih, ki so mu bili izpostavljeni v starostnem obdobju od 4 do 52 ur, povzročil spremembe v času valjenja in zaostanek v razvoju. Najvišja uporabljena koncentracija (2.59 mM) je bila nevrotoksična, saj je povzročila znižano frekvenco spontanega gibanja zarodkov in nekrozo možganov. Štiri dni po izpostavitvi MDMA smo vrednotili tudi plavalno aktivnost larv (starost 144 ur), vendar pri tem nismo dokazali pomembnih sprememb. Plavalna aktivnost 144 ur starih larv, ki so bile izpostavljene MDMA 2 uri pred testiranjem, pa je bila znatno zmanjšana, pri čimer so bile uporabljene koncentracije MDMA precej nižje (med 0.005 in 0.052 mM) od tistih, ki smo jih uporabili v zgodnejši fazi njihovega razvoja. Teratogenih učinkov MDMA v razvoju rib zebric nismo opazili.

Na osnovi teh ugotovitev sklepamo, da preučevana metoda daje ustrezne in primerljive rezultate o delovanju MDMA, ki je definiran kot nevrotoksin. Kljub temu so potrebne še nadaljnje raziskave, s katerimi bi lahko ugotavljali ali izpostavljenost MDMA v vmesnem obdobju vpliva na razvoj živčnega sistema in s tem na plavalno aktivnost 144 ur starih larv. Dodatne histopatološke preiskave pa bi lahko dejansko potrdile hipotetičen učinek MDMA na živčni sistem, ki se je pokazal pri vrednotenju aktivnosti poskusnih živali, izpostavljenih delovanju te substance.

ABBREVIATIONS

3 R:	Replacement, Reduction and Refinement
5-HIAA:	5-Hydroxyindoleacetic acid
5-HT:	5-Hydroxytriptamine, serotonin
5-HTR:	Serotonin receptors
5-HT_{2A} receptor:	Receptor of serotonin receptor family
COMT:	Catechol-*O*-methyltransferase
CYP:	Cytochrome P450, large and diverse group of enzymes
DA:	Dopamine
DAT:	Dopamine transporter
DNA:	Deoxyribonucleic acid
dpf:	Days post fertilization
EEC:	European Economic Community
FDA:	Food and Drug Administration
FET:	Fish embryo toxicity test
GABA:	Gamma-aminobutyric acid
GLU:	Glutamate
H_2O_{dd}:	Double distilled water
H_2O_2:	Hydrogen peroxide
$HO^{\bullet}$:	Hydroxyl radical
HHA:	3,4-dihydroxyamphetamine or DHA
HHMA:	4-dihydroxymethamphetamine or DHMA
HMA:	4-hidroxy-3-methoxyamphetamine
HMMA:	4-hydroxy-3-methoxymethamphetamine
hpf:	Hours post fertilization
ISO:	International Organization for Standardization
i.p:	Intraperitoneal
LD_{50}:	Median lethal dose
LSD:	Lysergic acid diethylamide
MAO:	Monoamine oxidase
MDA:	3,4-Methylendioxyamphetamine
MDMA:	3,4-Methylendioxymethamphetamine

NA:	Noradrenalin, norepinephrine
NAT:	Noradrenalin transporter
NMDAR:	N-methyl D-aspartate receptor
OECD:	Organization for Economic Cooperation and Development
PET:	Positron emission tomography
REACH:	Registration, Evaluation, Authorisation and Restriction of Chemical substances
SERT:	Serotonin transporter, 5-HT transporter (5-HTT)
SPECT:	Single photon emission computed tomography
SULT:	Sulfotransferase
THC:	Tetrahydrocannabinol
TPH:	Tryptophan hydroxylase
UBA:	German Federal Environment Agency (Umwelt Bundes Amt)
UDPGT:	Uridine diphosphate glucuronosyl transferase
VMAT:	Vesicular monoamine transporter
ZFET:	Zebrafish embryo toxicity test

1 INTRODUCTION

Nowadays we are more and more exposed to different psychoactive substances in various ways. Especially 3,4-methylendioxymethamphetamine (MDMA) is becoming a very popular drug of abuse among young people all over the world, despite being known for its neurotoxicity in laboratory animals and most likely causing permanent neurotoxic damage in humans (1). Considering that some of MDMA effects can differ widely between different species, it is necessary to include numerous influencing factors when extrapolating the data from animal models to humans. In the recent years the use of zebrafish as an alternative model for drug toxicity screening and other toxicological studies is increasing rapidly due to its good correlation with mammalian systems as well as high genetic and neurodevelopment similarity to humans. It is particularly applicable for studying development and deficits of nervous system and the related behavior. Based on the fact that MDMA neurotoxicity in zebrafish larvae was not known, we decided to explore it by performing locomotor activity analysis through which the influence on neuronal development can be hypothetically inferred. However, it was necessary first to establish and optimize the method in order to assure proper analysis.

1.1 AMPHETAMINES

Amphetamines are well known sympathomimetic and psychotomimetic compounds. They represent a group of psychoactive substances with β-phenylethylamine structure, which is related to catecholaminergic, dopaminergic and serotonergic agonists. Anyway, specific activities of different amphetamine derivatives depend on substituents on the amphetamine molecule (2).

Through the centuries humans have consumed natural amphetamines, like the cathinone (khat) from plant *Catha edulis* and the ephedrine from various plants of *Ephedra* genus (3). The main representative is beta-phenyl-isopropylamine, known as amphetamine, whose activity was discovered in 1929 by the biochemist Gordon Alles, who was searching for decongestant and bronchodilator that would replace ephedrine. Amphetamine rapidly became widespread, being used in various treatments, especially as an ideal therapy for depression and as a slimming agent afterwards (4). Recently, besides the amphetamine

itself, methamphetamine and 3,4-methylendioxymethamphetamine are widely acknowledged and commonly used synthetic drugs of abuse (3).

1.1.1 AMPHETAMINE

Amphetamine is a psychomotor and nervous system stimulant with multiple neuronal effects. It promotes the release of endogenous neurotransmitters, mainly noradrenalin (NA) and dopamine (DA) (3, 5).

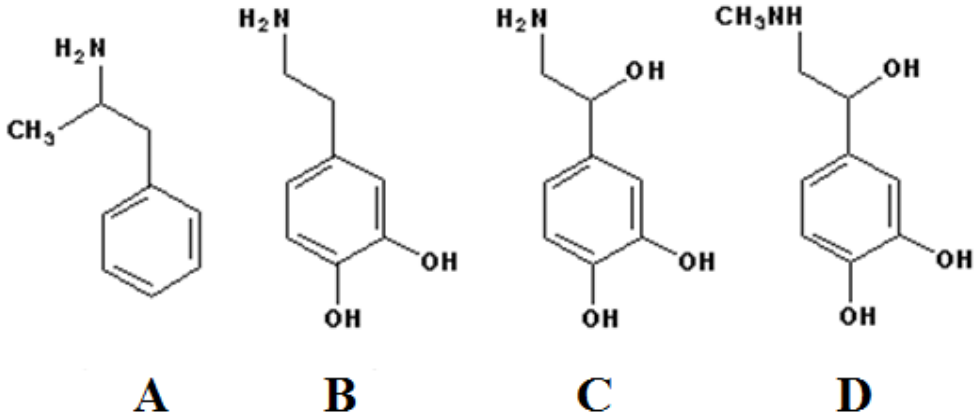

Figure 1: Structural similarity of amphetamine (A) and cateholamines dopamine (B), noradrenalin (C) and adrenalin (D) (5).

Its structural similarity to monoamine neurotransmitters (Figure 1) results in substrate competition for membrane transporters of noradrenalin (NAT) and dopamine (DAT) (3).

PHARMACOKINETICS

Amphetamine is a lipid soluble weak base with a low molecular weight, so it can easily cross membranes and lipid layers including the blood-brain barrier, reaching high concentrations in tissues and fluids with lower pH compared to blood. Equally the presence of α-methyl group in amphetamine structure preserves it from amine oxidation by monoamine oxidase enzyme (MAO), which also facilitates its passage through membranes (3).

Amphetamine has low protein binding capacity and thus high bioavailability as well as volume of distribution. Mainly it is metabolized in liver by cytochrome P450 (CYP)

isoenzymes, mostly CYP2C and CYP2D6 (3). It accumulates in kidney, lungs, cerebrospinal fluid and brain (6). Its elimination half-life is between 6 to 12 hours (3).

MECHANISM OF ACTION

Amphetamine is an indirect sympathomimetic, which increases the cytoplasmic concentration of biogenic amines, particularly DA and NA, as well as serotonin (5-HT) (3, 6). It promotes their release from storage vesicles into the synapse, decreasing reuptake and increasing reverse transport due to the inhibition of their transporters, which are responsible for the reuptake of neurotransmitters into the presynaptic nerve terminals and presynaptic vesicles. Moreover, amphetamine inhibits MAO which is involved in degradation of neurotransmitters (3, 6).
Some animal studies revealed the interaction of amphetamine with NMDA receptors (NMDAR) on serotonergic neurons as well, causing degradation of these neurons (2).

ACUTE EFFECTS

Amphetamine acts mainly on the central nervous system. It stimulates locomotor activity and causes movement disorders, which is related to release of NA from central noradrenergic neurons and DA from dopaminergic neurons (6).
The release of 5-HT and its direct effect on central serotonin receptors (5-HTR) are likely the cause of hallucinogenic amphetamine impacts (2, 6).
Hyperthermia is also one of the consequences of serotonergic and dopaminergic effects.
Regarding the peripheral nervous system, the increased levels of catecholamines can induce tachycardia and arrhythmias, as well as hypertension and cardiovascular collapse. The interaction of amphetamine with NMDAR may lead to seizures (2, 6).

1.1.2 3,4-METHYLENEDIOXYMETHAMPHETAMINE (MDMA)

MDMA, also known as ecstasy, is a psychoactive hallucinogenic compound. It is a ring-substituted amphetamine derivative, structurally related to the hallucinogenic mescaline, amphetamine and endogenous monoamine neurotransmitters (Figure 2) (7).

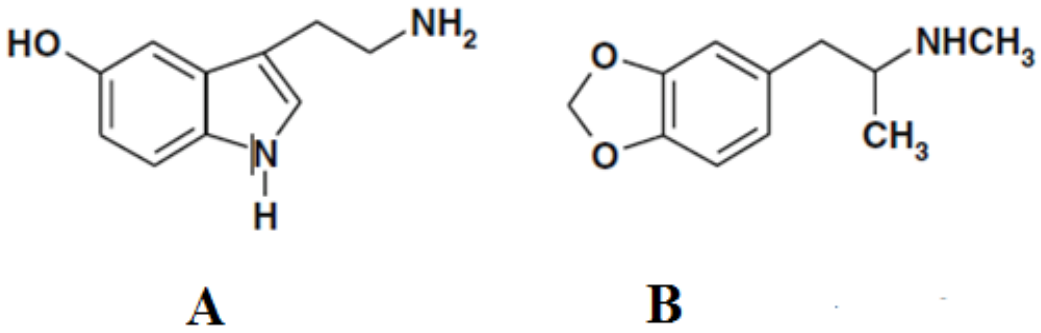

Figure 2: Structural similarity between monoamine neurotransmitter serotonin (A) and MDMA (B) (7).

MDMA is many times presented as selective neurotoxin for serotonergic system, causing long-term behavior alterations.

Numerous studies on laboratory animals proved that MDMA decreases brain levels of 5-HT and its metabolite 5-hydroxyindoleacetic acid (5-HIAA). It also reduces binding to serotonin transporter (SERT) and fibre density in forebrain areas (8).

MDMA is an indirect monoaminergic agonist as it interacts with monoamine transporters and thereby stimulates not only the release of serotonin, but also of DA and NA and also inhibits their reuptake.

Through its action on monoaminergic system MDMA damages the peripheral and central nervous system (7).

PHARMACOKINETICS

Non-linear pharmacokinetics is typical for MDMA. That implies that small increase of its dose causes higher plasma concentrations than assumed (9).

MDMA easily diffuses through cell membranes and lipid layers of tissues and organs, which have more acidic pH compared to blood. This is due to the fact that it is a weak base with a low molecular weight. It also has low protein binding capacity and high volume of distribution (7). Its elimination half-life is approximately 8 to 9 hours (3).

The metabolism of MDMA is mainly conducted in liver and lasts for at least 24 hours (7). The main metabolic route in humans is its *O*-demethylenation to 3,4-dihydroxymethamphetamine (HHMA, DHMA), where several CYP izoenzymes are implicated, followed by *O*-methylation of HHMA to 4-hydroxy-3-

methoxymethamphetamine (HMMA) by catechol-*O*-methyltransferase (COMT). Further on, the enzymes uridine diphosphate glucuronosyl transferase (UDPGT) and sulfotransferase (SULT) catalyse the conjugation of HHMA and HMMA to glucuronides or sulphates (Figure 3) (10).

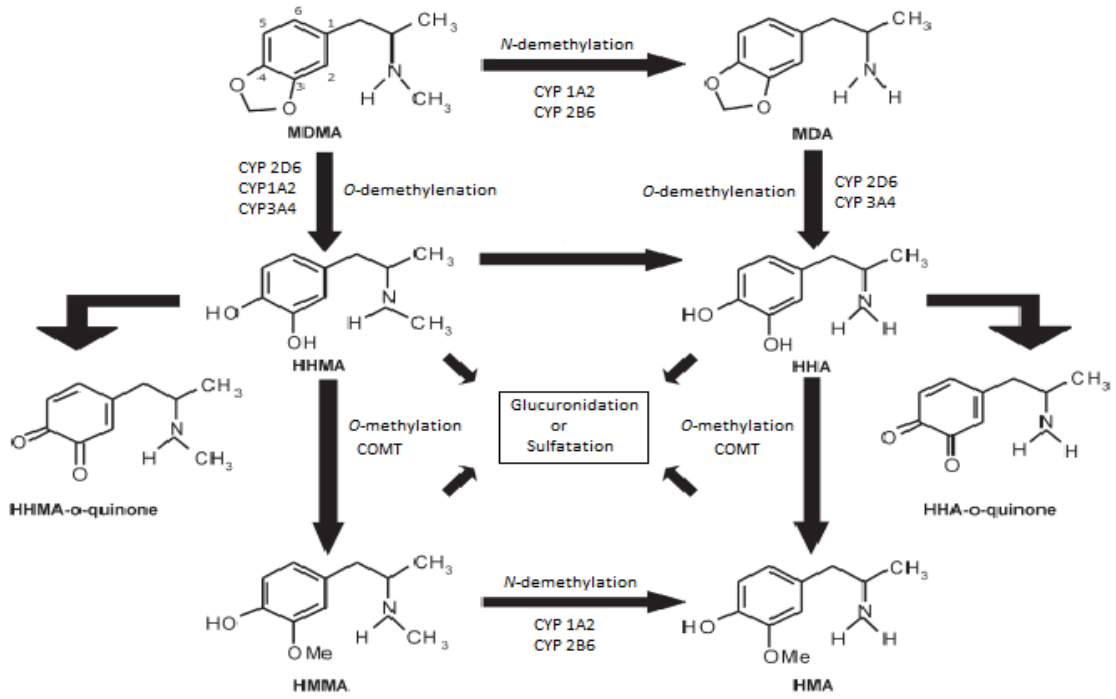

Figure 3: Metabolic pathways of MDMA (11).

The second metabolic pathway of MDMA is its *N*-demethylation to biologically active 3,4-methylendioxyamphetamine (MDA), which is subsequently metabolised to 3,4-dihydroxyamphetamine (HHA, DHA) and *O*-methylated to 4-hydroxy-3-methoxyamphetamine (HMA). Both metabolites can undergo conjugation by UDPGT and SULT. Metabolites HHMA and HHA may also be oxidised to ortho-quinones, which are considered to be highly neurotoxic (Figure 3). Same metabolic pathways were identified in rats, mainly the *N*-demethylation to MDA (7).

O-demethylenation and *N*-demethylation are catalysed by CYP2D6, CYP1A2, CYP2B6 and CYP3A4 in humans and by CYP2D1, CYP1A2 and CYP3A2 in rats (3).

MECHANISM OF ACTION

In all species, MDMA increases synaptic concentrations of 5-HT, DA and NA and has an impact on other neurotransmitters, such as gamma-aminobutyric acid (GABA) and acetylcholine (9).

Methylenedioxy ring in the MDMA molecule causes substantial inhibition of SERT, DAT and NAT (7). MDMA also acts as a substrate-type releaser of 5-HT. It is a substrate for plasma membrane and vesicular membrane transport systems (Figure 4), being responsible for serotonin reuptake and storage in nerve endings (12). MDMA enters the neuron terminal by binding on plasma membrane SERT, which carries it from extracellular fluid into serotonergic neuron or by diffusion, in case its concentration is very high. Inside neuronal terminal, MDMA enters into storage vesicles by vesicular monoamine transporter (VMAT). It increases the release of 5-HT trough serotonin-MDMA exchange thereby depleting vesicular neurotransmitter storages. With increased concentration of 5-HT in cytoplasm, MDMA accelerates the release of intracellular serotonin to the synapse by reversing the activity of serotonin transporter (Figure 4) (7). Long-term exhaustion of serotonin leads to morphological damage of serotonergic nerve terminals. By acting on serotonin transporters, MDMA probably affects behavior (12).

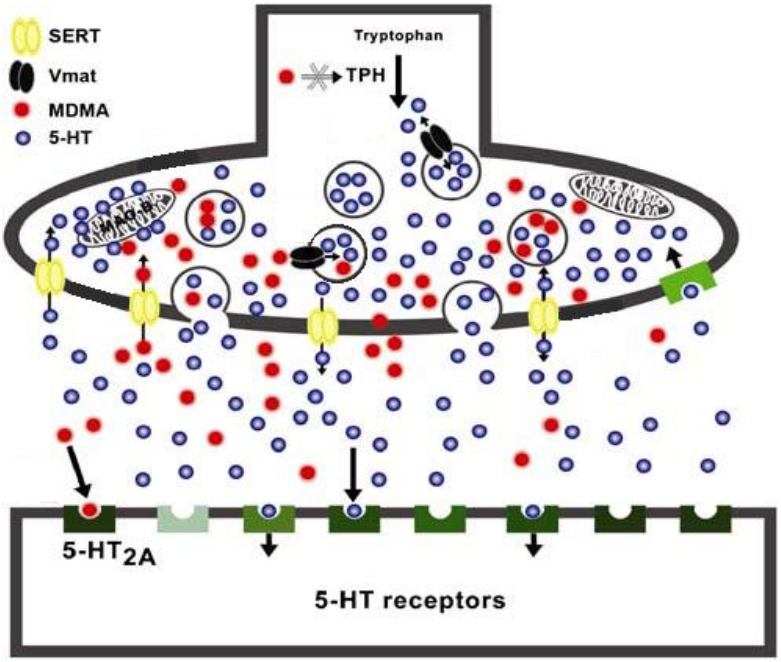

Figure 4: MDMA mechanism of action on neuronal serotonergic terminal and synapse (7).

MDMA is also an agonist of the 5-HT_{2A} receptor (Figure 4), to which it binds with a good binding affinity. The 5-HT_{2A} receptor is expected to have a major role in cognitive functions of the brain. Its stimulation leads to hallucinogenic effects (7).

Neurotoxicity as the major effect of MDMA has many cellular and molecular mechanisms:

- MDMA-induced hyperthermia is important amplifying factor of neurotoxicity, since it is also able to increase the release of serotonin and dopamine, but its direct correlation to long-term neurotoxicity is still not confirmed.
- MDMA inhibits tryptophan hydroxylase (TPH), the enzyme for 5-HT synthesis and MAO, the enzyme for 5-HT degradation (Figure 4). MAO, the enzyme of the outer mitochondrial membrane, participates in MDMA-induced neurotoxicity by catalyzing oxidative deamination of monoamine neurotransmitters along their metabolic pathway. This particular metabolic reaction generates hydrogen peroxide (H_2O_2) and if its removal is deficient, it accumulates. H_2O_2 can be converted to hydroxyl radical ($HO^{\bullet}$), which causes oxidative stress in brain mitochondria and nerve terminals, consequently leading to destruction of the latter and to neurotoxicity.
- Certain reports determined that MDMA neurotoxicity is caused by several successive effects, with DA and MAO metabolism having an important role. First, MDMA induces an acute release of DA and 5-HT which is followed by decreased intraneuronal stock of 5-HT. Postsynaptic 5-HTRs expressed on GABA interneurons are activated due to the initial 5-HT release, resulting in increased DA release and synthesis. The released DA is transported by SERT into depleted 5-HT neuron terminals. Here the metabolism of DA is conducted by MAO. Consequently oxidative stress can occur and serotonergic neurons become damaged.
- Some studies also revealed that MDMA can induce glutamate (GLU) release. This excitatory amino acid induces neuronal damage and it is quite possible that such excitotoxicity contributes to MDMA neurotoxicity as well.
- Regarding the neurotoxicity of metabolites, it is obvious that systemic metabolism of MDMA must occur. After all, highly reactive metabolites can cause oxidative stress which results in serotonergic terminal loss (7).

ACUTE EFFECTS

A variety of studies confirmed that MDMA causes many acute effects. Some of them differ widely between species, while others are more or less similar.

The most frequent "good" psychological effects of MDMA in humans are: mood enhancement, increased energy and thus physical activity, empathy, euphoria, sensory awareness and hallucinations (7, 9). On the other hand, these effects are accompanied by adverse central nervous system effects such as irritability, depression, panic attacks, confusion, insomnia and paranoia (7). Also numerous other physiological side effects, such as: seizures, rhabdomyolysis, hyponatremia, disseminated intravenous coagulation, acute renal failure, thrombocytopenia, delayed leukocytosis, acidosis, hypoglycemia, pulmonary congestion, edema, hepatitis, tremor, nausea, vomiting, jaw clenching, teeth grinding, dry mouth, headache, sweating, hyperflexia and neuroendocrine alterations, may occur (7, 9).

A well known serotonin syndrome in humans is a consequence of intrasynaptic serotonin excess and is reflected in simultaneous occurrence of behavioral hyperactivity, mental confusion, agitation, hyperreflexia, fever, tachycardia, shivering, clonus, myoclonus, ocular oscillations and tremor (13).

In cardiovascular system, cutaneous vasoconstriction, tachycardia and arrhythmia, as well as increased diastolic and systolic pressures are observed in laboratory animals. In humans increased blood pressure, tachycardia and palpitations are present (7).

Also poor immune response and increased susceptibility to infections, both in laboratory rats and humans are common consequences of MDMA use (9).

Hyperthermia is one of the main effects in which serotonergic, dopaminergic and adrenergic functions are involved. This MDMA effect, observed in laboratory animals can be directly compared to hyperthermia induced in humans (7). Some studies report, that MDMA causes excessive cooling of body temperature in cold environment or overheating at high environmental temperature in rats (13). In humans however, MDMA is assumed to cause hyperthermia independently of environmental temperature (9).

An important MDMA effect observed in laboratory rats is also hyperlocomotion, which is related to activation of monoamine receptors, due to increased monoamine release in the brain. Serotonin syndrome is present as well (7).

Although being rare, MDMA lethal effects should not be forgotten. Infamous, the most serious and usually fatal fulminant hyperthermia is followed by disseminated intravascular coagulation, rhabdomyolysis, acute renal and multiple organ failure (7).
Additional severe cause of MDMA lethality is hyponatremia, which leads to cerebral edema and seizures. Also liver and heart failure have been documented, but they are very rare (14).
However, deaths related to "ecstasy", especially to MDMA alone, are very uncommon. Usually their causes are attributed to toxic additives in MDMA tablets or to simultaneous consumption of other drugs (7, 9).

In humans the MDMA toxic dose varies. Fatal or nearly fatal blood concentrations have been reported to be between 0.11 mg/L and 2.1 mg/L (15).

Non-human toxicity values have also been determined:

LD_{50} in mouse i.p 97 mg/kg,
LD_{50} in rat i.p 49 mg/kg,
LD_{50} in guinea pig i.p 98 mg/kg (15).

LONG-TERM EFFECTS

The most important prolonged effect of MDMA is neurotoxicity which affects serotonergic and dopaminergic systems and occurs 24 hours to 2 weeks after its administration in a species-specific way (7, 9). In rats, not only the loss of serotonergic neuron terminals in the higher brain regions occurs but also degradation of other neurons is evident, while the cell bodies are spared. These serious defects cause long-term functional deficits. In mice on the contrary, MDMA impairs dopaminergic nerve terminals and depletes DA, as well as its metabolites (7).
When extrapolating data from animal models to humans, several pharmacological differences in pharmacokinetics and metabolism of the drug, as well as age, sex and concomitant polydrug use must be considered as they all have an influence on a long-term action of MDMA and its neurotoxicity. Some reports suggest that data from animal neurotoxicity studies are comparable with "heavy" MDMA users. However, only a direct study in humans could give the most detailed and reliable results. Current studies of the long-term MDMA effects in humans with a history of its use are based on indirect

methods, mainly on measurements of 5-HT metabolites in cerebrospinal fluid or neuroimaging techniques in the brain. So far, by using positron emission tomography (PET) and single photon emission computed tomography (SPECT), decreased densities of brain serotonin transporter sites, serotonin binding sites, as well as the activity of serotonin transporters were confirmed. Regarding the sex differences in humans, females are expected to be more sensitive to MDMA-induced neurotoxicity than males, since the reduction of 5-HIAA in cerebrospinal fluid is far greater in women. Finally, long-term changes in serotonin neuronal function and serotonin depletion result in cognitive dysfunction and memory deficits. They especially affect working, verbal and visual memory. In addition, MDMA also causes decline in learning and planning abilities, as well as in executive control. Some of the acute effects of MDMA such as hallucinations, depression, paranoia, panic disorders, impulsivity and cognitive impairment can persist for longer times (days, weeks) (7).

Regarding its developmental toxicity, MDMA is expected to be teratogenic in animals and humans, since it causes DNA damage. In rats, *in utero* exposure of fetuses delays their postnatal physical, neurological and sensorimotor development and reduces brain serotonin concentration at birth. In humans, however a higher potential for deformity of foot and heart deficits exists (9).

There are inconsistent reports regarding MDMA-induced developmental neurotoxicity, because in the perinatal and early postnatal developmental stage, metabolism and brain neuronal systems are not fully developed yet. Some animal studies have shown that MDMA presence during pregnancy caused reduced levels of 5-HIAA or decreased brain levels of serotonin in the offsprings, yet the others showed no changes. However, MDMA and its metabolite MDA pass through the placenta and can be measured in the amniotic fluid and fetal brain (7).

INTERACTIONS WITH OTHER DRUGS OF ABUSE

Combinations of MDMA with other drugs can present a high risk in terms of health. The purity of MDMA sold on the “street” is a very variable factor, and also the fact that it is often combined with other psychoactive drugs.

Since MDMA is mostly metabolised by CYP2D6 in humans, competitive inhibition of this enzyme by other concomitantly used drugs (amphetamines, cocaine, some antidepressants and antipsychotics) can increase its plasma concentrations. Moreover, concomitant use of proserotonergic drugs (amphetamines, cocaine) can emphasize serotonin effects of MDMA and thus increase the risk for serotonin syndrome.

Effects of MDMA in combination with:

- ETHANOL: Ethanol increases plasma concentrations of MDMA. In humans this combination causes prolonged euphoric effects and decreases MDMA-induced hyperthermia, while in animals it increases the hyperlocomotion effects, decreases hyperpyretic effects, induces memory deficits and augments rewarding effects caused by MDMA.
- CANNABIS: Tetrahydrocannabinol (THC) in cannabis weakens and delays MDMA-induced hyperthermia and oxidative stress and causes memory deficits in both, humans and animals. In humans a combination of THC and MDMA also leads to various psychological problems, attenuated visual and self motion perception, poor immunity and increased heart rate. On the other hand, THC can prevent some of the noxious effects of MDMA. Namely, in animals it can decrease MDMA-induced hyperlocomotion.
- COCAINE: According to informal theoretic discussions, the effects of MDMA should be reinforced in a presence of cocaine in humans. This combination was shown to increase the hyperlocomotion effect of MDMA in animals.
- AMPHETAMINE: In humans their combination results in serious long-term cognitive, behavioral and neurological alterations, as well as in increased neurotoxicity through oxidative stress and production of free radicals. Serotonin syndrome, increased MDMA-induced hyperlocomotion and anxiety-like behavior with decreased social interaction, all occur in animals.
- NICOTINE: In humans nicotine attenuates the negative effects of MDMA and other way around, while in animals increases the MDMA-induced rewarding effects.

- CAFFEINE: While it decreases drowsiness and fatigue in humans, it increases the MDMA-induced hyperthermia, hyperlocomotion, tahycardia and neurotoxicity in animals.
- OPIATES: The co-use of heroin augments proserotonergic effects of MDMA in humans. A combination of morphine and MDMA alters the rewarding effects caused by morphine in animals.
- LSD (Lysergic acid diethylamide): This combination causes increased animal responses to MDMA. Common effects of LSD and MDMA in humans have still not been formally addressed.
- KETAMINE: It deteriorates the MDMA-induced neurotoxicity in animals while in humans these effects have not been under consideration yet.
- PSYCHOTERAPEUTICS: Antidepressants, as selective serotonin reuptake inhibitors, can decrease the MDMA-induced tachycardia, hypertension or its positive subjective effects. On the other hand, antipsychotics counter the MDMA-induced hyperthermia in humans. In animals some antidepressants inhibit effects and neurotoxicity induced by MDMA (9).

1.2 ZEBRAFISH (*Danio rerio*) AS A TOXICOLOGY RESEARCH MODEL

According to OECD (Organization for Economic Cooperation and Development) and FDA (Food and Drug Administration) regulations, neurotoxicity testing, induced by chemicals, is based on *in vivo* experiments, which mainly involve neurobehavioral assessment of cognitive, sensory and motoric functions, as well as neuropathological examination. The most preferred test species is rat. These kinds of tests are for example: Neurotoxicity testing, Neurotoxicity study in rodents and Developmental neurotoxicity study (16, 17, 18). Due to the fact that such experiments use large numbers of animals, are expensive and time consuming for screening of chemical compounds, there is a continuous need for more rapid, cheaper and more predictive alternative methods. Such new alternative strategies have to comply with the requirements of REACH (Registration, Evaluation, Authorisation and Restriction of Chemical substances) directive and thus fully implement its principles

regarding the replacement, reduction and refinement (3R) in the use of animals for scientific purposes (19).

Due to their numerous favourable characteristics, several OECD guidelines, like Fish early-life stage toxicity test, Fish short-term toxicity test on embryo and sac-fry stages, as well as Fish acute toxicity test, recommend zebrafish, especially their embryos, as an alternative vertebrate model useful in many fields of research, including toxicology and drug discovery studies (20, 21, 22, 23). Zebrafish are suitable for assessing many types of toxicity, like reproductive, developmental, acute, neuro, cardio, ocular, neurobehavioral and vascular, as well as for studying endocrine disruption and carcinogenicity (24).

The benefits of embryonic zebrafish model for toxicology studies are:

- Small size.
- Easy and cheap husbandry.
- High fertility and large number of offspring.
- External fertilization.
- Development *ex utero.*
- Early morphology and fast development.
- Transparency, which enables clear observations and monitoring.
- Low dosage of experimental compounds and thus reduced quantities of waste disposal.
- Small experimental environment (microwell plates) for large-scale chemical screening and a possibility to perform several experimental replicates at one time (24).
- Simple application of tested compound, just by adding it to the water (liquid), i.e. the experimental environment; it is simply absorbed through the skin and gills of zebrafish at embryonic stage and through digestive system at larval stage (25, 26).
- Good correlation with mammalian experimental systems.
- High genetic and neurodevelopment similarity to humans (19).

The use of zebrafish as a model for studying development of nervous system and the related behavior is growing increasingly. Locomotor responses of larvae can already be evaluated since the first day of their development and after 6 days post fertilization (dpf), they are already mature swimmers with completely functional sensory and motor systems (27).

Although they have numerous features similar to humans, caution needs to be taken when correlating the concentration of tested compound in media that causes toxic effects in zebrafish to plasma concentration of this compound causing the same effects in humans. Various substances are not all absorbed equally by zebrafish and that is why the precise determination of quantity of the absorbed compound is highly recommended in order to make appropriate correlations (26).

1.2.1 ZEBRAFISH EMBRYO TOXICITY TEST (ZFET)

All living organisms are very susceptible to chemical exposure during embryogenesis and early development, including fish (28). In addition, zebrafish embryos are perfect for toxicity testing since they are transparent and they develop *ex utero* (outside of the mother) (26). Thus unobstructed assessments of morphological changes in development of the brain, heart, jaw, trunk segmentation and size can be made (24).

Already standardized at international level, the Fish embryo toxicity test (FET) is becoming an increasingly frequent used method in toxicology studies (29). According to the 3R principle imposed by REACH regulations regarding animal welfare, the current draft of the international OECD guideline suggests the implementation of FET by using zebrafish as the finest alternative to the classical Fish acute toxicity test, where mortality is the primary endpoint (30). Moreover, embryonic life stages of zebrafish can perfectly predict later life stages of fish, being used in acute toxicity testing (29).

Basically, the Fish acute toxicity test and the Zebrafish embryo toxicity test are quite similar assays for determination of short-term toxicity.

For Fish acute toxicity testing, different types of adult fish are used and a 96 hours exposure to a tested substance is prescribed and their mortality as a principal endpoint is documented at 24, 48, 72 and 96 hours (22).

In contrast, ZFET is used to determine acute toxicity in embryonic life stages of zebrafish. The exposure to a tested substance lasts for 96 hours and it has to begin as soon as possible after fertilization of fish eggs - up to 3 hours post fertilization (hpf). As an indicator of lethality and thus the acute toxicity, four endpoints of the test are relevant: coagulation of fertilized eggs, non-formation of somites, non-detachment of the tail and non-detection of the heartbeat. They have to be recorded every 24 hours. Furthermore, non-hatching after 108 hpf may be considered as an additional important toxicity endpoint, taking into account that normal zebrafish hatches in the time interval from 48 to 96 hpf (31).

Moreover, ZFET enables determination of developmental toxicity through observation of any other morphological and physiological anomalies, which can be used as test developmental endpoints (Table IV, Table V) (29, 31, 32). In this case it should be noted that the developmental toxicity is defined as any adverse effect which may result from substance exposure prior to conception, during prenatal development, or postnatally up to the time of sexual maturation, interfering with normal development of embryo (33). Ordinarily, such disorders are of transient nature. Within developmental toxicity the term teratogenicity refers to the permanent structural change/damage (malformation) that may adversely affect survival, development or function of embryo and is usually rare (33, 34).
In addition to developmental toxicity screening developmental neurotoxicity can be precisely evaluated as well (18).

The embryonic stage includes period from fertilization to the time when larvae hatch. Thereafter the development of zebrafish continues outside the chorion, which is called the eleutheroembryo stage (free embryo) (29). At first, the ZFET test was based on a 48 hours chemical exposure of newly fertilized eggs, as proposed by the German Federal Environment Agency (UBA) that submitted the draft of FET guidelines (32). The OECD has recently extended the exposure time to 96 hours (after hatching), based on the findings that the chorion hinders the chemical exposure. In validation studies, the toxicity of chemicals with high molecular weight (> 300 000 g/mol) was only noted after hatching while other chemicals with lower molecular weight (< 400 g/mol) were slightly more toxic at 96 than at 48 hours, all due to a possible obstructed passage through the chorion (35).

1.2.2 DEVELOPMENT OF ZEBRAFISH AND THEIR LOCOMOTOR NETWORK FORMATION

The embryogenesis of zebrafish is mainly completed within 96 hpf. At this time most organs are already developed and functioning (36).

Around 6 hpf, when gastrulation begins, the fertilized and unfertilized eggs can easily be discerned (36). Embryos have beating hearts, moving tails, eyes and primitive brain already at 24 hpf. The hatching occurs from 48 hpf on (37).

Regarding the locomotor network organization and the movement modulation, zebrafish spinal cord is the most important. However, a major influence on development and adjustment of spinal network is ascribed to serotonin, dopamine and noradrenaline, the neurotransmitters of the aminergic system, which becomes fully complemented within a period from 96 to 120 hpf (38).

Neurogenesis (Figure 5) occurs at 6 hpf and reaches its peak at 48 hpf. The differentiation of catecholaminergic circuitry starts at 18 hpf while that of dopaminergic and serotonergic begins at 24 hpf (36). Especially serotonin patterns locomotor behavior, as the formation of its innervation temporally correlates with the stages of locomotor development, from spontaneous tail coiling to mature swimming, which occurs within 96 – 120 hpf (39).

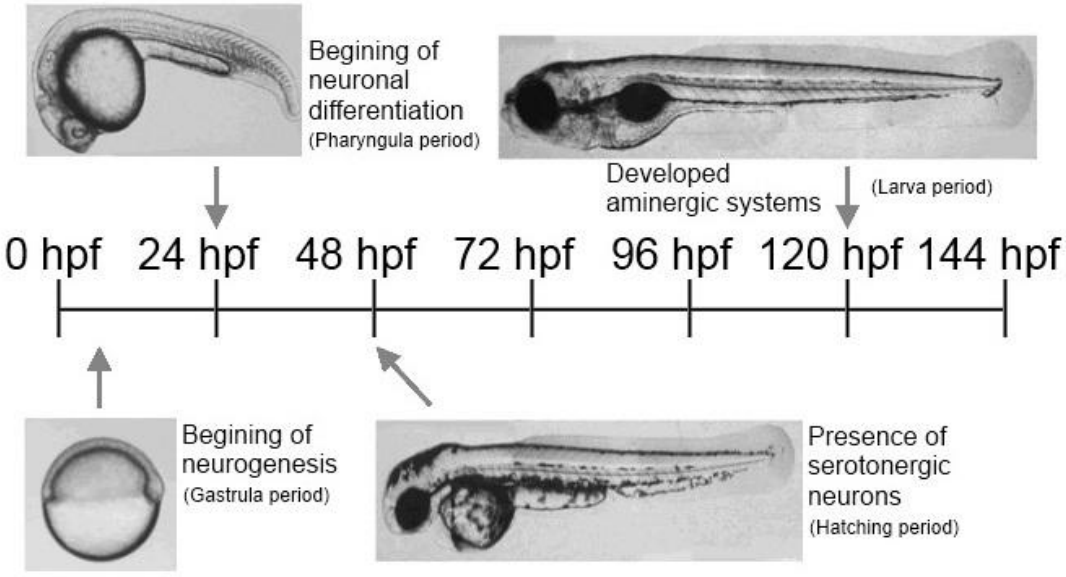

Figure 5: Timeline of serotonergic innervation development in zebrafish embryo (gastrula and pharyngula period)/ eleutheroembryo (hatching period)/ larvae (40).

Despite the fact, that serotonergic neurons are present in the spinal cord and hind brain already around 48 hpf, the serotonin is supposed to influence the swimming from the day 4 (96 hpf) onwards. Several studies have uncovered that in locomotor capacity of developing zebrafish larvae, serotonin increases the duration of swimming by modulating more inactive than active periods, which normally happens in infant or adult experimental specimens of other vertebrates (41).

1.2.3 EVALUATION OF SPONTANEOUS TAIL COILING

The evaluation of spontaneous tail coiling is a method to assess the first locomotor activity, related to developing neuronal network in zebrafish embryo, following its exposure to a neurotoxic substance (19). Normally developing zebrafish embryo starts to move spontaneously after 24 hpf, while still being in chorion (42). Any alteration in spontaneous movement is considered a behavioral test endpoint and can be used to determine developmental neurotoxicity of a tested compound (19).

The method is based on recording the frequency of tail coilings/minute in embryos aged from 24 to 26 hpf which show no malformations (19).

1.2.4 ZEBRAFISH LARVAE BEHAVIOR ASSAY

In mammalian studies, psychoactive drugs are normally used to determine the functioning of nervous system, which is reflected in animal behavior (27). Also in vertebrates, the neuronal development and signalling are mainly reflected in their behaviour. Therefore its evaluation is often used today in determining the alterations in nervous system, especially neurotoxicity (25).

Behavior observation of developing zebrafish larvae is becoming more and more important in determination of healthiness or defectiveness of organisms (43). After hatching, the free-swimming zebrafish larvae express several types of behavior and some of these, like: optokinetic response, optomotor response, swimming activity, social behavior, as well as learning and memory, are very useful in toxicity studies. Automated imaging system for observation and analysis of complex behavior in large numbers of zebrafish larvae enables

the implementation of screening in multiwell plates and thus the simultaneous acquisition of large amount of data and valuable information (37).

The principle of any behavioral response is movement. Therefore, many studies are based on locomotor activity measurement, with its changes as an endpoint. In zebrafish larvae, the impact of neurotoxic compounds on their nervous system and consequently their locomotor activity can be assessed as occurrence of changes in swimming velocity and distances covered (25).

In order to provide an established, reproducible, reliable and quantifiable, i.e. optimized behavioral assay, it is necessary to understand the behavior of developing zebrafish larvae. Many studies have determined influences of various parameters on their activity during characterization of locomotion patterns within a small testing environment used for rapid screening of various chemicals (43). Some researchers have found that the optimal time for diurnal testing is between 13:00 and 15:30 pm, when the locomotor activity is stable and the variability among larvae is the lowest. The locomotion of zebrafish larvae was tested also to evaluate their activity patterns separately in visible light and darkness, as well as during alternating light and dark periods. Duration of each individual period of light and dark was also tested. Based on these tests, the three-phase pattern of activity was determined: moderate acclimatizational activity in darkness, decreased movement in light and promoted activity on return to darkness (43).

Figure 6: The recording box for monitoring of zebrafish larvae movements, equipped with a recording camera and infrared and visible light sources (44).

In principle, the zebrafish larvae behavioral assay is a method in which movements of 6 day old treated larvae are recorded in a special recording box which enables the setting of alternating dark and light periods. The movement is quantified from recorded videos by using special software and presented as the total distance covered. Data are then statistically analyzed (45). As an endpoint, the increased or decreased activity of every treated group of larvae is evaluated, relating to the dose of applied test substance and the time of exposure (25).

Behavior is one of the most variable of all responses in living organisms. Therefore, especially for aquatic species, an extra care needs to be taken, as there are so many intrinsic and extrinsic factors, which, in addition to the neurotoxic compound tested, can also influence the behavior. One of these parameters is water, which can contain several interfering contaminants (25). Furthermore, it has been established that variables, such as the presence of malformations or lower rearing temperature which causes delayed development, can reduce the extent of larval activity. On the contrary, the activity increases in parallel with increasing age of larvae, the size of the circumference of rearing environment and the contrast between the level of light when switched off to create darkness. Although a type of rearing solution does not affect larval behavior, the parameters such as pH, dissolved oxygen content, conductivity and osmolarity of medium still need to be monitored in order to exclude any effects not related to the toxic compound being tested (45, 46).

2 OBJECTIVE

Nowadays, vertebrates are becoming increasingly considered as the best models to determine various mechanisms of neurotoxicity. Especially zebrafish have been proven to be extremely useful models in large-scale drug or chemical screening. There are many reports about behavior tests during zebrafish development in small testing environments, but all of them are still at developmental and experimental level, so that they have not been validated or standardized yet. For this reason the principle objective of this work was to optimize the zebrafish larvae behavior test, which could then be comparable to existing reports. The optimization was focused on assessment of general locomotion of larvae with the use of appropriate psychoactive compound that affects behavior in a well known manner.

Our additional objective was to assess neurotoxic effects of the psychoactive substance 3,4-methylendioxymethamphetamine (MDMA) by performing locomotor activity analysis of exposed zabrafish larvae. *D*-Amphetamine was used as a control psychoactive compound for optimization of the behavior assay. Because many of its derivatives are very popular substances among young people in recent years, we particularly focused on one of them, the MDMA, being commonly used as recreational drug. MDMA is known to cause serotonergic neurotoxicity in laboratory animals and long-term functional disorders in humans, which is consistent with neurological deficits (7, 13). Many times it has been described as a teratogen as well (9). Although being studied for over 15 years, the mechanisms of MDMA neurotoxicity are still not completely understood. Considering the fact that zebrafish have high genetic and neurodevelopmental similarity to humans, we wanted to find out if the use of their embryos and larvae, as a testing model, could give us any useful information regarding MDMA-induced developmental and adult neurotoxicity.

3 MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 EXPERIMENTAL ANIMALS

All experiments were performed at the laboratory of the Toxicology unit, Faculty of Pharmacy, University of Barcelona, Spain.

Adult wild type zebrafish (*Danio rerio*) were maintained at 27±1 ºC and a 14 h light/10 h dark cycle. They were housed in closed flow-through colony tanks, with males and females being separated. For fish mating, few adults were placed in small breeding tanks, usually two males and one female. Next morning, one hour after light onset, eggs were collected from breeding tanks and washed with 1:5 diluted ISO-water, according to the ISO standard 7346. Fertilized eggs were placed individually in wells of 96-well plates, together with 300 µL of 0.3 x Danieau's solution and incubated at 27±1 ºC and a 14 h light/10 h dark cycle, for 6 days. Every day in the morning, larvae were examined for malformations and mortality under the stereomicroscope and the 0.3 x Danieau's solution in each well was renewed.

Animal welfare was maintained according to requirements of the Directive 86/609/EEC and Spanish legislation (Real Decreto 1201/2005), by fully implementing their principles regarding the use of animals for experimental purposes.

3.1.2 EXPERIMENTAL REAGENTS

Table I: Reagents, used in experiments.

CHEMICAL NAME	EMPIRICAL FORMULA	MOLECULAR WEIGHT	MANUFACTURER
Sodium chloride	NaCl	58.44	Sigma-Aldrich®, St. Louis, MO
Potassium chloride	KCl	74.55	Sigma-Aldrich®, St. Louis, MO
Magnesium sulfate heptahydrate	$MgSO_4x7H_2O$	246.47	Sigma-Aldrich®, St. Louis, MO
Calcium nitrate tetrahydrate	$Ca(NO_3)_2x4H_2O$	236.15	Sigma-Aldrich®, St. Louis, MO
Hepes	$C_8H_{18}N_2O_4S$	238.30	Sigma-Aldrich®, St. Louis, MO
Calcium chloride dihydrate	$CaCl_2x2H_2O$	147.01	Sigma-Aldrich®, St. Louis, MO
Sodium bicarbonate	$NaHCO_3$	84.01	Sigma-Aldrich®, St. Louis, MO
Agarose			Sigma-Aldrich®, St. Louis, MO
3,4-Dichloroaniline	$Cl_2C_6H_3NH_2$	162.02	Sigma-Aldrich®, St. Louis, MO
d-Amphetamine	$C_9H_{13}N$	135.21	A kindly donation from Dr. J. Camarasa, Laboratory of Pharmacology and Pharmacognosy, Faculty of Pharmacy, University of Barcelona
3,4-Methylenedioxymethamphetamine	$C_{11}H_{15}NO_2$	193.25	A donation from the Government Delegate's Office of Catalonia (Health department) and purified by the Organic Chemistry Laboratory, University of Barcelona

3.1.3 LABORATORY EQUIPMENT

Table II: List of laboratory equipment, used in experiments.

EQUIPMENT	MANUFACTURER
96-well microtiter plates	Sterilin® Limited, UK
24-well plates	VWR International, LLC
6-well plates	VWR International, LLC
Plastic petri dishes	Sterilin® Limited, UK
Plastic droppers	VWR International, LLC
Automatic pipettes	Nichiryo CO., LTD
Flow-through colony tanks	Beta Acuarios, S.L
Breeding tanks	Aquaneering, Inc. San Diego, California
Stereomicroscope SZM-168	Motic®
Weighing scale ST	Gram Precision, S.L
Vortex reax 2000	Heildolph Instruments, KG
Synergy Water Purification System	Merck Millipore, MA
pH meter	Crison Instruments, S.A

3.2 METHODS

3.2.1 PREPARATION OF THE EMBRYO MEDIUM

The 0.3 x Danieau's solution was used in all experiments as the embryo medium. All further stock solutions and final concentrations of test reagents were prepared in embryo medium in order to keep the pH constant during experiments.

Danieau's solution 30 x (stock solution):

1740 mM NaCl (10.17 g/100 mL)
21 mM KCl (0.156 g/100 mL)
12 mM $MgSO_4x7H_2O$ (0.296 g/100 mL)
18 mM $Ca(NO_3)_2x4H_2O$ (0.425 g/100 mL)
150 mM HEPES buffer (3.575 g/100 mL)

All reagents were weighted, placed together, stirred in double distilled water (H_2O_{dd}) until dissolved and diluted with the same solvent up to 100 mL. At the end the pH value was adjusted to 7,4. The resulting stock solution was stored at 4 ºC.

0.3 x Danieau's solution (The embryo medium):

10 mL of stock solution was diluted in 1 L of autoclaved H_2O_{dd}.
Each time the pH of newly prepared medium was checked and adjusted to 7,4 with 1 M HCL or NaOH, if necessary. The 0.3 x Danieau's solution was stored at room temperature.

3.2.2 PREPARATION OF ISO-WATER (according to the ISO standard 7346)

2 mM $CaCl_2x2H_2O$ (11,76 g/40 L)
0.5 mM $MgSO_4x7H_2O$ (4,39 g/40 L)
0.8 mM $NaHCO_3$ (2,59 g/40 L)
0.08 mM KCl (0,23 g/40 L)

$CaCl_2x2H_2O$ was tarred in a beaker, stirred in H_2O_{dd} until dissolved and diluted up to 1 L. $MgSO_4x7H_2O$, $NaHCO_3$ and KCl were also tarred, stirred in H_2O_{dd} until dissolved and diluted up to 1 L. Both solutions were mixed together and the volume was completed with H_2O_{dd} up to final 40 L.

The ISO-water was used for maintaining adult zebrafish in colony tanks and for fish mating in breeding tanks. For cleaning of the eggs 1:5 diluted ISO-water was used.

3.2.3 PREPARATION OF AGAROSE GEL AND AGAROSE PLATES

A 2% agarose gel was prepared from 5g of agarose powder, dissolved in 250 mL of 0.3 x Danieau's solution.

The gel was used for preparation of agarose rings in 6-well plates and "agarose plates" (agarose gel in plastic petri dishes).

3.2.4 OPTIMIZATION OF THE ZEBRAFISH LARVAE BEHAVIOR ASSAY

There are relatively few studies that have assessed behavior effects of acute exposure of zebrafish to psychoactive compounds and many of them are still used today as experimental models to determine alterations in their nervous system functioning (27).

In order to optimize the behavior test, we chose a study in which researchers have examined many responses in behavior of mice, rats and also adult zebrafish to neuroactive compounds, such as ethanol, *d*-amphetamine and cocaine and transferred these findings to a zebrafish larvae model to evaluate if they behave in a similar way (27).
We have used *d*-amphetamine as a psychoactive substance and compared our results to those from reports. In the first part of the optimization only 0.3 x Danieau's solution was used without chemical compounds and the behavior of larvae in light and dark cycles was observed. Since we have introduced some minor changes in extrinsic variables (a shorter duration of light and dark cycles, different size of incubation wells, different material of recording plates), it was necessary to determine whether or not these variables make significant alterations in the already mentioned three-phase pattern of larval activity

(regular acclimatizational activity, decreased movement in light and promoted activity in darkness).

THE PROCEDURE

The optimization of behavior test was divided into two parts:

1. STEADY-STATE: Zebrafish larvae were incubated only in 0.3 x Danieau's solution until day 6 post fertilization and the total distance of movement of individual larvae was analyzed, using automated video-tracking.
2. PSYCHOACTIVE DRUG CHALLENGE: 6 dpf old zebrafish larvae were exposed to a range of nonlethal concentrations of the neuroactive compound *d*-amphetamine and the total distance of movement of individual larvae was analyzed, using automated video-tracking.

Each time, the test was carried out at day 6 post fertilization. The mortality and malformations of larvae were checked each morning. The malformed or dead larvae were removed from the experiment. Two hours before recording, normal larvae were transferred to recording plates (7 larvae/plate), with adequate volume of 0.3 x Danieu's solution and placed in light-tight box in the test room (27±1 ºC) for acclimatization.

In drug challenge tests, three non-lethal concentrations of *d*-amphetamine were used: 0.2 μM, 0.7 μM and 20 μM (concentration/plate). The time of the acute exposure was 20 minutes. As a negative control 0.3 x Danieau's solution was used.

After 2 hours of acclimatization, approximately at 2 pm, each individual plate was placed into recording box, equipped with a camera and a source of infrared (dark) and visible light. The movement of larvae was recorded in five cycles of alternating 4 minutes long light and dark periods, starting with a 6 min light period (2 min needed for acclimatization).

According to respective capabilities of the laboratory, we imposed several changes to improve the performance of the behavior test in terms of rapidity and reliability:

- The duration of alternating light and dark cycles was shortened to 4 minutes.

- Initial glass petri dishes used for incubation (20 mL of 0.3 x Danieau's solution, 25 embryos) were first replaced with 24-well plates (2 mL of 0.3 x Danieau's solution, 1 embryo/well) and finally, 96-well microtiter plates (300 µL of 0.3 x Danieau's solution, 1embryo/well) were used.
- At the beginning, 6-well plates (1 larva/well) in which agarose rings were placed to reduce the shadows and thus improve the accuracy of the automated video analysis of larval movements, were used for recording (34). Introduction of changes continued with newly made "agarose plates" with 7 wells (Figure 7), which were soon replaced by plastic plates with the same size and number of wells.

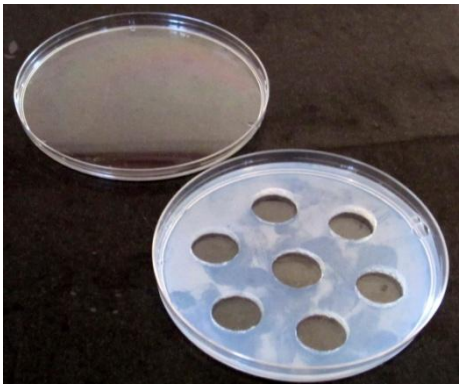

Figure 7: "Agarose plates" used for recording of larval movements, made in our laboratory (44).

ANALYSIS OF RECORDED MOVEMENTS

Recorded videos were cut to clips for each individual plate and for each cycle of the light and dark period. Movements of every individual larva were analyzed using ImageJ software (http://rsb.info.nih.gov/ij/index.html). Stacks were converted to lines (larval trajectory). By using self-made Microsoft Excel spreadsheet "Trajectorias" the lines were converted into total distance of movement (Figure 8). Graphical analysis of data was implemented as a mean distance of movement with the standard error of the mean (SEM). The outlying results (not more than 2) were removed from the analysis.

All data were statistically analyzed with the SPSS program, using a repeated measures analysis of variance test (ANOVA), with a cycle and a plate being independent variables and the locomotor activity (distance/cycle) a dependent one. To compare significant difference between groups (plate/plate in each cycle and cycle/cycle for each plate),

independent-samples T test or single factor ANOVA with post hoc Fisher LSD, Bonferroni or Games-Howell tests, were used. The difference was considered statistically significant at $p < 0.05$.

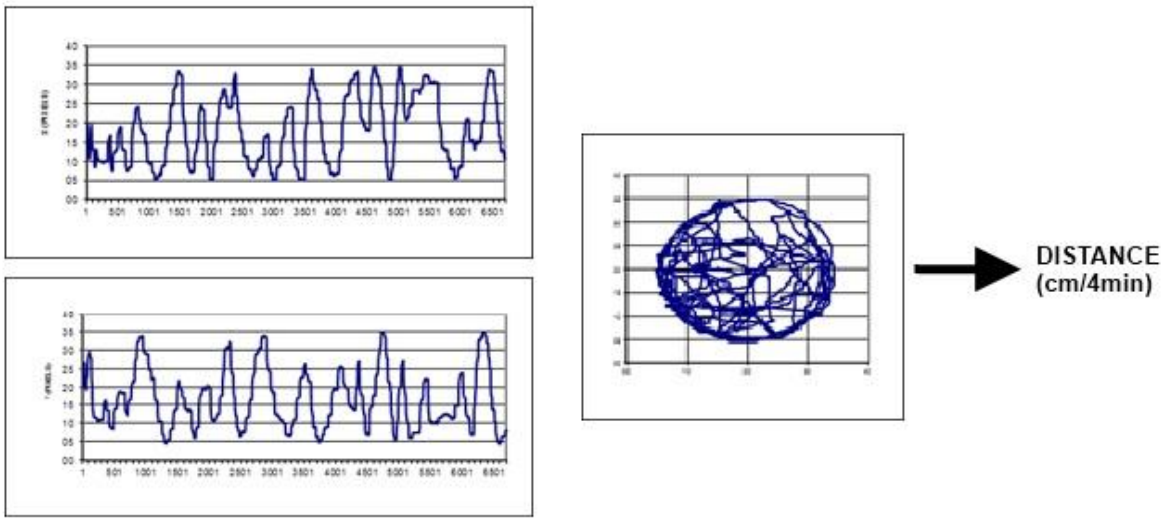

Figure 8: Example of results from "Trajectorias" spreadsheet (Microsoft Excel).

INTERPRETATION OF RESULTS

The obtained results were compared to those found in different published reports, where movement patterns of larvae, untreated or treated with *d*-amphetamine in similar circumstances of light and dark cycles, were determined.

3.2.5 ASSESSMENT OF MDMA-INDUCED DEVELOPMENTAL NEUROTOXICITY

We assessed the developmental neurotoxic potential of MDMA in zebrafish embryos and larvae. Since it is known that MDMA acts on the serotonergic system in humans and other mammals, we used adequate exposure time, taking into account the developmental stages of serotonin system in zebrafish (36, 38). In addition to ZFET, two other methods, based on assessment of locomotor activity were applied in this experiment as well: the evaluation of spontaneous tail coiling in embryos and the evaluation of swimming activity of larvae, using the newly optimized behavior assay.

3.2.5.1 DETERMINATION OF MORPHOLOGICAL EFFECTS OF MDMA

THE PROCEDURE

Before the final experiment, a range-finding test was performed to determine the appropriate range of MDMA concentrations.

The zebrafish embryo toxicity test (ZFET) was performed using five test concentrations of MDMA: 2.59 mM (500 μg/mL), 1.30 mM (250 μg/mL), 0.52 mM (100 μg/mL), 0.26 mM (50 μg/mL) and 0.10 mM (20 μg/mL) and with a negative (0.3 x Daneau's solution) and a positive control (3,4-dichloroaniline; 0.024 mM). Twelve embryos per each concentration and control were used.

Newly fertilized zebrafish eggs were individually distributed in 96-well microtiter plates; one egg per well. Before 4 hpf, the eggs were exposed to various MDMA test concentrations and the two controls, in a volume of 300 μL, for 48 hours. The plates were covered and incubated at 27±1 ºC and a 14 h light/10 h dark cycle. The embryo medium and freshly prepared test solutions of MDMA were renewed after the first 24 hours of exposure.

Appropriate lethal endpoints were determined at defined times of exposure, according to the ZFET SOP (Table III).

Table III: Four lethal endpoints at defined times of MDMA toxicity determination according to the ZFET SOP (31).

	AGE OF EMBRYO / TIME OF EXPOSURE		
	8 hpf / 4h	28 hpf / 24h	52 hpf / 48h
Egg coagulation	+	+	+
Lack of somite formation		+	+
Non-detachment of tail		+	+
Absence of heartbeat			+

At 52 hpf (48 hour after exposure) the embryos, exposed to different test concentrations were transferred into glass petri dishes containing adequate volumes of the 0.3 x Danieau's solution and observed for any kind of developmental disruption by using stereomicroscope and comparing their development with a normal one, as described by Kimmel et al., 1995 (42). Current developmental endpoints, as listed in the draft of the FET test guideline (UBA) and additional selected reports were used (Table IV and Table V) (32).

Subsequently the embryos were individually placed into wells of a new 96-well plate and all tested MDMA solutions were replaced with 300 µL of the 0.3 x Danieau's solution. They were then incubated until day 6 post fertilization, when the behavior assay was performed. Every day, 300 µL of the 0.3 x Danieau's solution in each well was renewed.

At 48, 52 and 72 hpf, hatching of embryos was recorded as additional determination of MDMA-induced developmental neurotoxicity.

Table IV: Developmental endpoints at defined times of MDMA toxicity determination (28, 29, 32, 36).

	EXPOSURE TIME				
	8 hours	24 hours	48 hours	72/96 hours	120/144 hours
Completion of gastrula	+				
Somites formation		+	+	+	+
Eye development		+	+	+	+
Spontaneous movement / Swimming activity		+	+	+	+
Heartbeat/Blood circulation			+	+	+
Skeletal deformities				+	+
Pigmentation			+	+	
Edema			+	+	
Brain necrosis			+	+	

Table V: Teratogenic endpoints at defined times of MDMA toxicity determination (29).

	EXPOSURE TIME				
	8 hours	24 hours	48 hours	96 hours	120 hours
Malformation of head		+	+	+	
Malformation of tail		+	+	+	
Malformation of heart		+	+	+	
Yolk deformation		+	+	+	
General growth retardation		+	+	+	
Length of tail					+

ANALYSIS OF DATA

Incidence of any developmental disruption was recorded as a frequency of affected embryos in a treated or control group. By using Microsoft Excel, the graphical analysis of hatching data was carried out and presented as percentages of embryos with the standard error of the mean (SEM).

All data were statistically analyzed with the SPSS program, using a Chi-square test with the acceptance of statistical significance at $p < 0.05$.

INTERPRETATION OF RESULTS

Any positive outcome in one of the four lethal endpoints indicated mortality and thus toxicity of a tested compound. Any recorded developmental disruption was considered to be a developmental toxicity effect, according to existing endpoints. The hatching data were interpreted according to results obtained in a negative control group of larvae.

3.2.5.2 EVALUATION OF SPONTANEOUS MOVEMENTS OF ZEBRAFISH EMBRYOS

An alteration in spontaneous movements which is the first locomotor activity of embryos is one of the behavioral endpoints that can be used to assess developmental neurotoxicity of a tested compound (19). Moreover, evaluation of spontaneous tail coiling can also be used as an indicator of somite formation when determining lethality in the ZFET (31).

THE PROCEDURE

The test was performed with five different concentrations of MDMA: 2.59 mM (500 µg/mL), 1.30 mM (250 µg/mL), 0.52 mM (100 µg/mL), 0.26 mM (50 µg/mL) and 0.10 mM (20 µg/mL) and with a negative (0.3 x Daneau's solution) and a positive control (3,4-dichloroaniline; 0.024 mM).

Zebrafish eggs were distributed in 96-well microtiter plates, placing one egg in each well. When distributed, the eggs were exposed to MDMA test concentrations and controls in a 300 µL volume, before 4 hpf. The covered plates were incubated at 27±1 ºC and a 14 h light/10 h dark cycle.

At 24 hpf, 10 embryos being exposed to each particular concentration and the negative control, were transferred to 6-well plates containing 3 mL of test solutions per well and placed under stereomicroscope. After 1 minute of acclimatization, 1 minute recording of movements was performed with the stereomicroscope camera.

ANALYSIS OF RECORDED MOVEMENTS

The recorded movies were analyzed with the ImageJ software (http://rsb.info.nih.gov/ij/index.html), where the frequency of movements of embryos in each well was detected by dynamic pixel analysis.
Graphical analysis was performed by using Microsoft Excel, and presented as an average frequency of movements with the standard error of the mean (SEM) for each concentration of MDMA tested.

Data were statistically analyzed with the SPSS program, using single factor analysis of variance test (ANOVA), with MDMA concentration being the independent and the frequency of movements the dependent variable. To compare significant differences between groups, post hoc Games-Howell test was used and the statistical significance was accepted at $p < 0.05$.

INTERPRETATION OF RESULTS

The frequency of movements of MDMA treated embryos was compared to that of control embryos, wherein every specific difference was considered to be a neurotoxic effect of the tested substance.

3.2.5.3 EVALUATION OF SWIMMING ACTIVITY

Alterations in swimming activity represent another behavioral endpoint in neurotoxicity determination (25). According to our knowledge regarding developmental stages of serotonergic innervation in zebrafish, which is fully established between 96 and 120 hpf, we assessed the developmental neurotoxicity of MDMA at different exposure times, using our newly optimized behavior assay (36, 38).

THE PROCEDURE

The behavior assay was performed using 0.3 x Danieau's solution as a negative control and several of the following MDMA test concentrations (depending on a test): 2.59 mM (500 μg/mL), 1.30 mM (250 μg/mL), 0.52 mM (100 μg/mL), 0.26 mM (50 μg/mL), 0.10 mM (20 μg/mL), 0.052 mM (10 μg/mL), 0.026 mM (5 μg/mL) and 0.005 mM (1 μg/mL). The duration of the acute exposure was 2 hours.

Until day 6 post fertilization, the zebrafish embryos were individually incubated in 96-well plates in 300 μL of 0.3 x Danieu's solution/well. On the day of the test, eventual mortality and malformations of larvae were checked, first in the morning. The malformed or dead larvae were removed from the experiment.

Two hours before recording, larvae were transferred into recording plates containing adequate volumes of 0.3 x Danieau's solution (negative control group) and defined test concentrations of MDMA (treated groups). The recording plates were placed in a light-tight box in the test room (27±1 ºC).

After 2 hours of acclimatization (control group) or MDMA exposure (treated groups), each individual plate was placed into recording box, equipped with a recording camera and sources of infrared (dark) and visible light. The movements of larvae were recorded in five cycles of alternating 4 min light and dark periods, starting with a 6 min light period (2 min needed for acclimatization).

The evaluation of swimming activity of 6 dpf old larvae, previously exposed to MDMA from 4 to 52 hpf, was performed as continuation of the ZFET. Testing conditions and procedure of this behavior assay were the same as described above with the only difference being that all larvae were transferred to recording plates with adequate volumes of 0.3 x Danieau's solution.

ANALYSIS OF RECORDED MOVEMENTS

Computer analysis of recorded videos and larval movements, as well as graphical and statistical analyses of obtained data were performed in the same manner as described in optimisation procedure of the zebrafish larvae behavior assay (page 27, 28).

INTERPRETATION OF RESULTS

Results obtained in control groups were compared to those found in published reports, which determined movement patterns of larvae in similar conditions regarding light and dark cycles.

Results from MDMA treated groups were interpreted according to those found in the control group and to a lesser extent compared to published findings from similar studies, where MDMA or other neuroactive chemicals were tested.

3.2.6 STATISTICAL ANALYSIS

All statistical analyses were performed with the IBM® SPSS Statistics 20.0 program:

- Repeated measures analysis of variance test (ANOVA) was used in behavior assays. The cycle and plate were independent variables and the locomotor activity (distance/cycle) was a dependent one. Independent-samples T test or single factor ANOVA with post hoc Fisher LSD, Bonferroni or Games-Howell tests were used to compare significant differences between groups (plate/plate in each cycle and cycle/cycle for each plate).
- Chi-square test was performed for analysis of categorical data obtained in ZFET.
- Single factor analysis of variance test (ANOVA) was used for evaluation of spontaneous movements of zebrafish embryos. Concentration of MDMA was set as independent variable and the frequency of movement was a dependent one. In order to compare significant difference between groups, the post hoc Games-Howell test was performed.

In all analyses the statistical significance was accepted at $p < 0.05$.

4 RESULTS AND DISCUSSION

The first part of our research was aimed at optimization of the zebrafish larvae behavior test at the laboratory of Toxicology unit (Faculty of Pharmacy, University of Barcelona, Spain). We focused on the assessment of general locomotion of larvae in alternating light and dark cycles, to establish the importance of shorter duration of light and dark periods, the size of incubation space, materials of recording plates and age on larval activity (regular acclimatizational activity in dark or light, decreased movement in light and promoted activity on return to darkness). With the use of psychoactive compound *d*-amphetamine that affects behavior in a known manner, we defined the terms and conditions of a newly optimized method and compared the similarity of our results to those found in published reports.

This test was used individually and as a supplement to the ZFET in order to assess developmental neurotoxic potential of the psychoactive substance MDMA in exposed zabrafish embryos and larvae.

4.1 OPTIMIZATION OF THE ZEBRAFISH LARVAE BEHAVIOR ASSAY

4.1.1 DETERMINATION OF THE STEADY STATE

When assessing the toxicity of chemicals through behaviour of living beings, it is important to be aware of different variables and their influences on behavior itself. It has been reported, that the activity of zebrafish larvae increases with age (dpf) and that their movement patterns change accordingly. It is also possible that the size of rearing wells affects the extent of larval activity, which actually increases with the size of wells (46).

The results of larval locomotion testing at different age (a period of few hours), from different lays and in different recording plates, are shown in Figure 9. Since we have initially shortened the duration of light and dark cycles (4 min/cycle), we mainly focused on the behavior patterns of larvae. Embryos used in the test were from three different lays: the control group A was approximately 4 hpf old, when placed in incubation plates; the

control groups B and C were 1 hour younger, while the control group D originated from the same lay as the control group C. Embryos were incubated from time 0 until 6 dpf in 96-well plates in 300 µL of 0.3 x Danieau's solution/well. At 6 dpf larvae were transferred to recording plates with adequate volumes of 0.3 x Danieau's solution, 2 hours before recording. Activities of larvae from control groups A, B and C were recorded in "agar plates", while the larval activity from control group D was recorded in plastic plates. All recordings were performed during five cycles of alternating 4 min light and dark periods, starting with a 6 min light period (2 min needed for acclimatization).

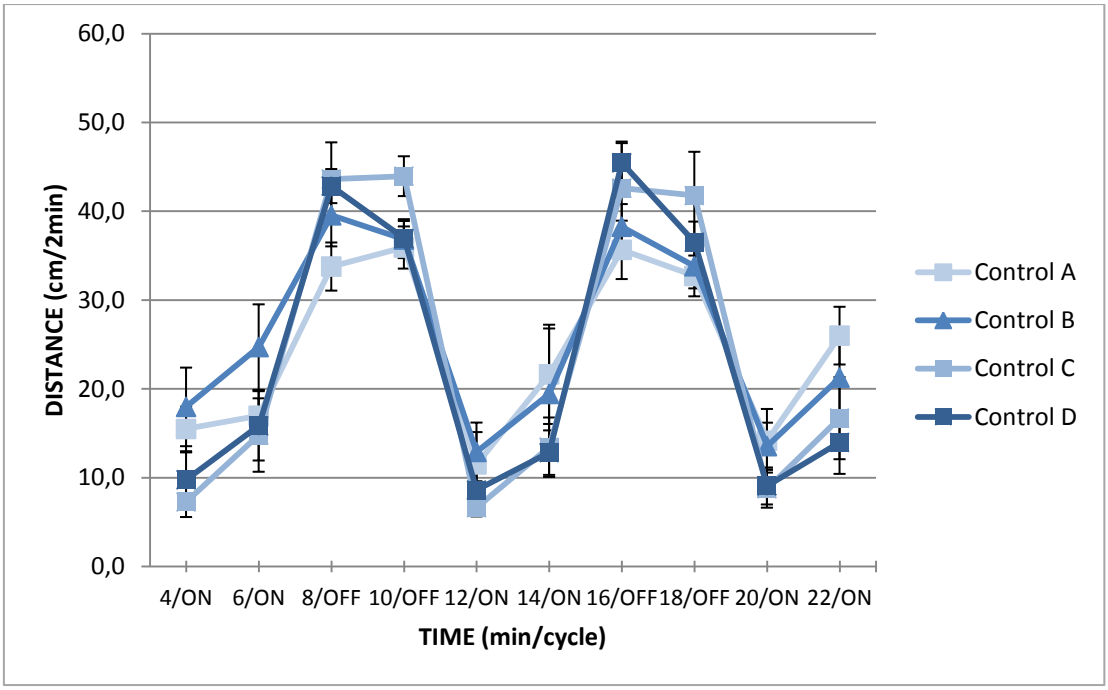

Figure 9: Effects of age and material of recording plates on a general locomotion of zebrafish larvae during alternating 4 minutes light and dark cycles. The results are presented as mean distances of movements in the first and the second half of each cycle (cm/2min) with standard errors of means (SEM), n = 5 - 7 larvae/plate.

It can be very clearly seen that shorter duration of alternating light and dark periods did not affect the basic movement pattern of larvae. At light cycles their activity was considerably low and slowly increasing while it increased rapidly and began to gradually decrease during dark cycles. The movement patterns were not significantly different ($p > 0.05$) regardless if the larvae were not from the same lay or there was a slight difference in their age. We also found that the material of recording plate had no effect on their behavior pattern.

Larval movements were next tested to determine the impact of different size of incubation wells. Embryos used in this test were from two different lays. They were incubated from time 0 until 6 dpf in a 24-well plate with 2 mL of 0.3 x Danieau's solution/well (control group A) and 96-well plate with 300 µL of 0.3 x Danieau's solution/well (control group B). At 6 dpf larvae were transferred to agar recording plates with adequate volumes of 0.3 x Danieau's solution, 2 hours before recording. Activities of both groups were recorded during five cycles of alternating 4 min light and dark periods, starting with 6 min light period (2 min needed for acclimatization).

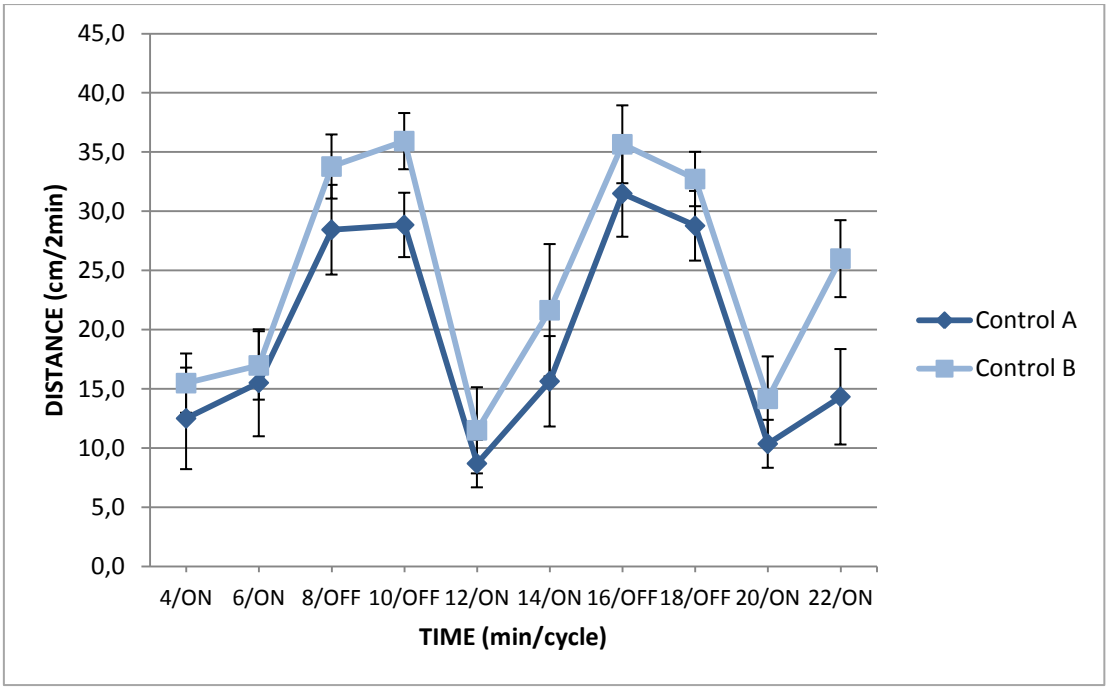

Figure 10: Effects of size of incubation wells on a general locomotion of zebrafish larvae during alternating 4 minutes light and dark cycles. The results are presented as mean distances of movements in the first and the second half of each cycle (cm/2min) with standard errors of means (SEM), n = 7 larvae/plate.

Figure 10 shows that the size of incubation environment had no impact on larval locomotion. The behavior patterns in both groups, as well as mean distances of movements were found to be substantially comparable ($p > 0.05$).

Our results are consistent with a comparative report which had demonstrated the effect of lighting conditions on basic larval locomotion: in extended light, their activity is increasing up to a stable level, while in the dark it increases at first and then decreases to a rather low level. If the light and dark periods are shorter, their activity is low in light and substantially

higher in dark. The cycles of alternating light and dark periods induce alternating levels of low and high activity (43). Another report confirmed this pattern of larval locomotion and defined an even more elaborate one by applying shorter, 10 minutes long light and dark periods which caused ceased at first and then slowly increasing larval movements in light and fast increased activity that slowly lessened in dark periods (27). In our study we used even shorter, 4 minutes long light and dark periods, which did not change the larval activity paradigm and their alternating movements, staying low in light and substantially higher in dark. We have also focused on smaller age difference between larvae and discovered that a few hours age interval does not cause any alterations in behavior patterns nor does the size of incubation wells. Two additional variables were tested, namely the use of larvae from different lays in the same assay and different materials of recording plates, and they both did not cause any significant deviations in larval behavior. Zebrafish larvae were still sensitive to lighting conditions and the well established three-phase pattern was preserved as well.

4.1.2 PSYCHOACTIVE DRUG CHALLENGE

Current studies report that behavioral responses of mammals to acute *d*-amphetamine exposure present a biphasic dose-response or "inverted U" pattern with increased activity at lower doses and decreased activity at higher doses. In humans, the acute exposure to amphetamine related substances has resulted in similar motorical changes (27).

Figure 11 shows the results of testing general locomotion of zebrafish larvae, exposed to psychoactive compound *d*-amphetamine in a concentration range between 0.2 and 20.0 μM. 144 hpf old larvae were transferred into adequate volumes of 0.3 x Danieau's solution (negative control group) and into solutions containing different concentrations of *d*-amphetamine. After 20 min of acute exposure larval activities in each plate were recorded during five cycles of alternating 4 min light and dark periods, starting with a 6 min light period (2 min needed for acclimatization). For recording, "agar plates" were used.

When compared to control group, the 0.2 μM concentration of *d*-amphetamine induced prominent larval hyperactivity during the first and last light cycle and during the second dark cycle, while the 20.0 μM concentration induced their hypoactivity during the second

dark cycle. 0.7 µM concentration of *d*-amphetamine did not produce significant changes in behavior of larvae. Regardless of *d*-amphetamine dose, the behavior pattern of moving less in light and more in dark remained the same in all tested groups.

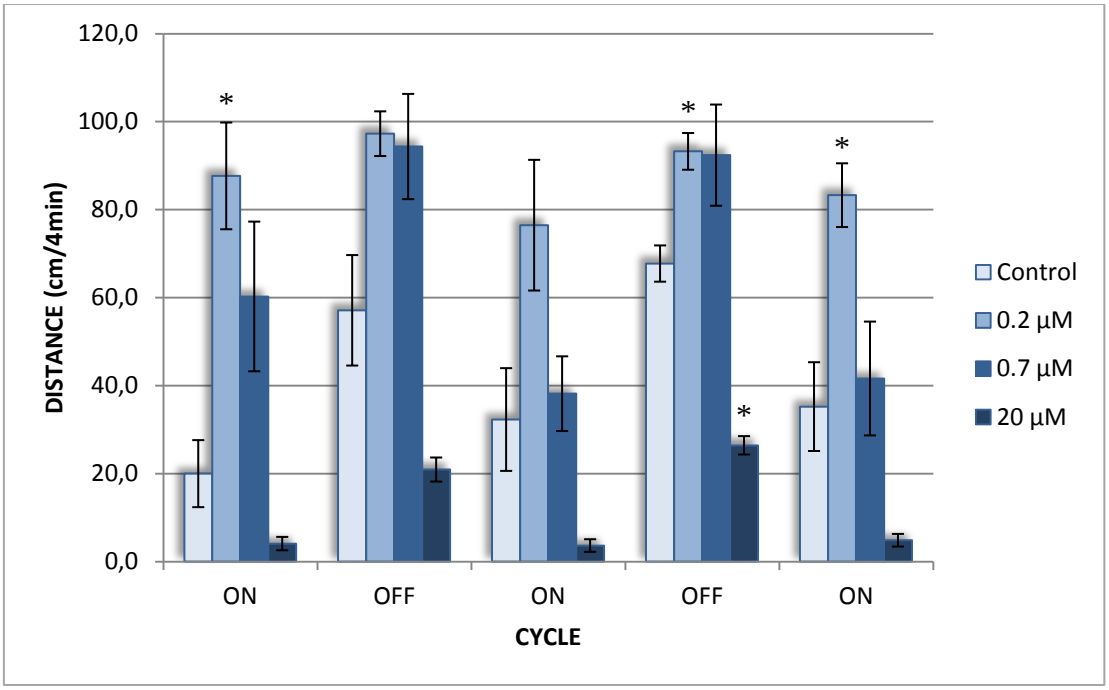

Figure 11: Activity of zebrafish larvae after acute exposure to *d*-amphetamine. The results are presented as mean distances of movement (cm/4min) with standard errors of means (SEM); * p < 0.05; n = 4 larvae/plate in the control and n = 6 - 7 larvae/plate in the treated group.

Although being statistically non-significant, the trend of increasing activity at lower *d*-amphetamine concentrations and decreasing at high concentrations is evident. These results are relatively consistent with current studies which showed the "inverted U" dose-response pattern in behavior of *d*-amphetamine treated zebrafish larvae, which was clearly evident in dark periods. The larval response to alternating 10 minutes light and dark cycles was consistent regardless of *d*-amphetamine dose (Figure 12). In general, lower concentrations increased the locomotor activity in initial cycles of dark or light or sometimes both. Higher concentrations induced larval hypoactivity in the majority of dark cycles and the highest concentration used caused a permanent decrease in activity during all dark cycles (27).

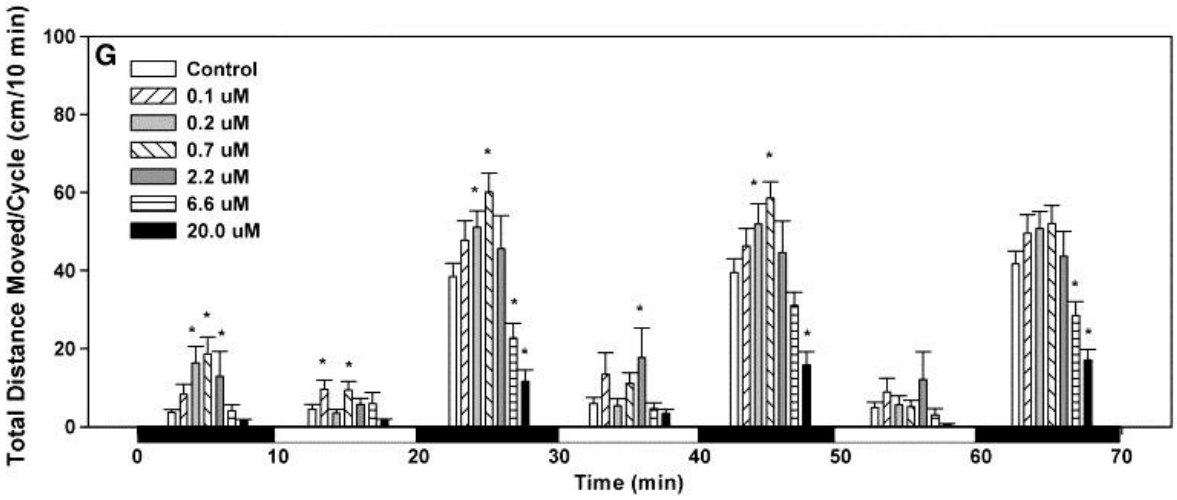

Figure 12: Results of zebrafish larval activity following acute exposure to several different concentrations of *d*-amphetamine. Data were taken from the report which was used for comparison in optimization process of our new behavior assay (27).

Testing of larval locomotion after their acute exposure to 0.2 µM concentration of *d*-amphetamine was repeated to confirm the results obtained from the first assay and to prove that it could be used in a routine as a positive control for further behavior assays. The testing conditions regarding incubation, exposure times and recording were the same as in the test described above. The results are shown in Figure 13.

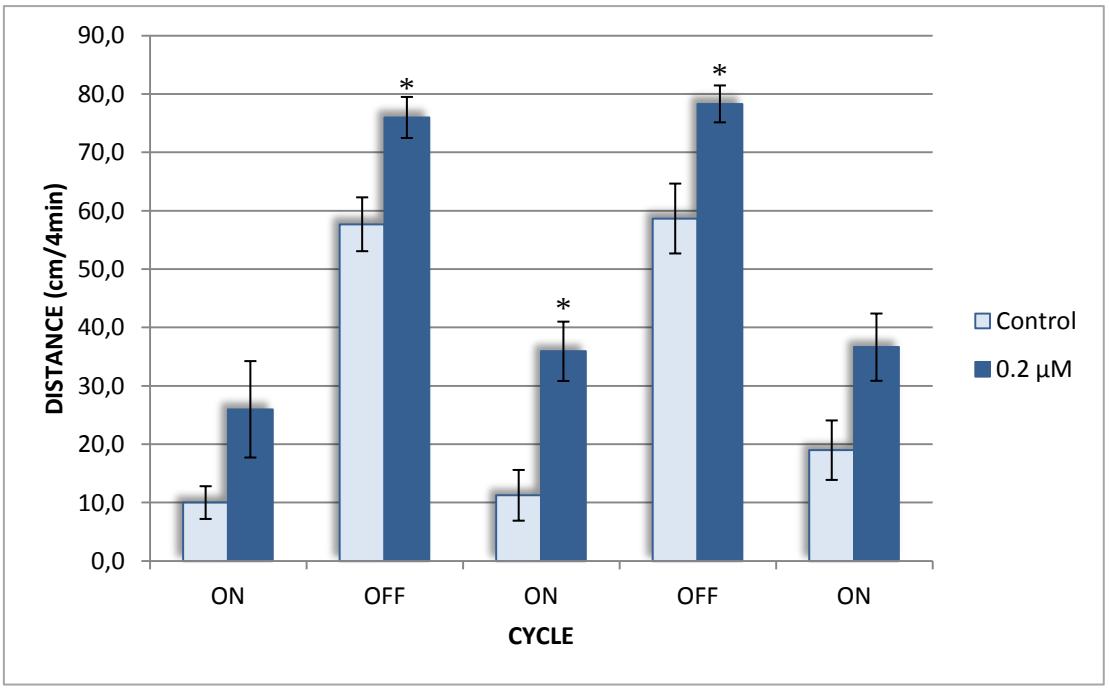

Figure 13: Activity of zebrafish larvae following their acute exposure to 0.2 µM concentration of *d*-amphetamine. The results are presented as mean distances of movement (cm/4min) with standard errors of means (SEM); * p < 0.05; n = 5 - 6 larvae/plate.

The locomotion of treated larvae was significantly higher during both dark cycles and in the second light cycle, as compared to control group larvae and this result is almost completely consistent with a previous one published in report, where the 0.2 μM concentration of *d*-amphetamine caused hyperactivity exclusively during dark cycles (27). Generally speaking, the treatment with *d*-amphetamine did not alter the general pattern of locomotor responses of zebrafish larvae to light or darkness.

However, the experimental conditions of this comparative report were quite different from those used in our study. Namely, larvae were incubated in same 96-well plates that were later also used for recording, the light and dark periods were 10 min long, the recording started with a dark cycle and the total time of recording was 70 min. Despite the fact that we have introduced all described differences, the results obtained in both compared studies were found to be relatively consistent. In summary, our results confirm that larval behavior in a slightly larger, but still small recording environment with shorter lighting periods (4 min) still remains reliable and quantifiable.

4.2 ASSESSMENT OF MDMA-INDUCED DEVELOPMENTAL TOXICITY

4.2.1 MORPHOLOGICAL EFFECTS OF MDMA

Two independent ZFET assays were performed to determine morphological effects of MDMA on developing zebrafish embryos.

Zebrafish eggs from the same lay were transferred to 96-well plates before 4 hpf and exposed to 300 μL of each MDMA test concentrations (2.59 mM, 1.30 mM, 0.52 mM, 0.26 mM and 0.10 mM), 3,4-dichloroaniline (positive control) and 0.3 x Daneau's solution (negative control). The time of exposure was 48 hours and 0.3 x Daneau's solution and freshly prepared MDMA test concentrations were renewed after first 24 hours.

MORTALITY

The mortality was checked at 4, 24 and 48 hours of exposure. According to the ZFET SOP, four lethal endpoints were checked: the egg coagulation, the lack of somite formation (Figure 14), the non-detachment of tail and the absence of heartbeat. Negative and positive control group embryos fulfilled the test acceptance criteria, i.e. > 90% survival in the negative control group and > 30 % mortality in the positive control group at the end of 3,4-dichloroaniline exposure.

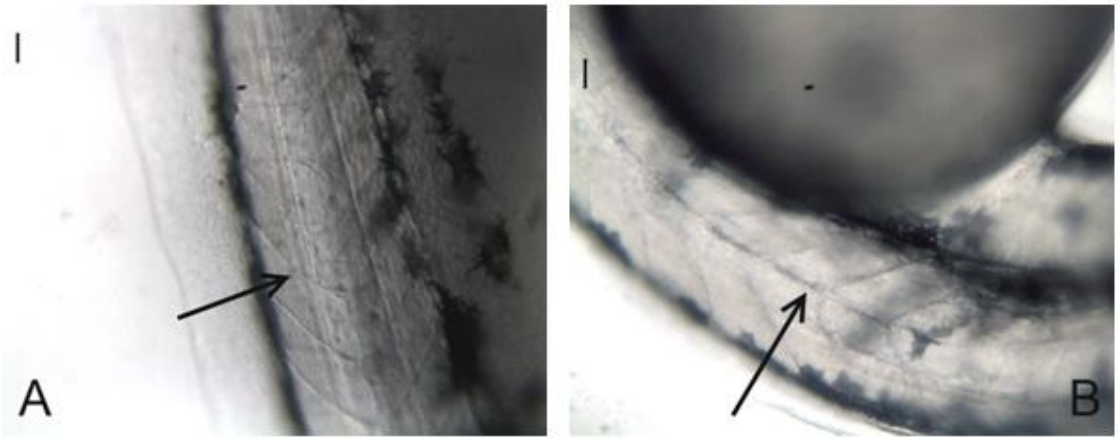

Figure 14: Well developed somites in the MDMA treated group; negative control group embryo (A) and embryo after exposure to 2.59 mM of MDMA (B). Arrows indicate the structure of somites. Scale bar: 100 µm.

Compared to the negative control group we found no significant lethal effects in the group that was exposed to MDMA for 48 hours (4 hpf – 52 hpf).

DEVELOPMENTAL ENDPOINTS

Embryos were checked for any kind of developmental disruption following 48 hours of MDMA exposure. The results are presented in Table VI.

The following observations could suggest the target organs of MDMA-induced developmental toxicity and allow qualitative comparison with similar studies conducted in mammalian experimental models.

Table VI: Incidence of developmental endpoints in zebrafish embryos following 48 hour exposure to different MDMA test concentrations. The results are presented as (–) for negative and (+) for positive outcomes at individual MDMA concentrations; n = 12 embryos/treated group/control in each individual test.

TREATMENT	Developmental endpoints	
	ZFET 1	ZFET 2
Negative control	-	-
0.10 mM	-	-
0.26 mM	-	-
0.52 mM	-	-
1.30 mM	+	+
2.59 mM	+	+

According to normal zebrafish development, as described by Kimmel et al., 1995, two disruptions were noticed in our tests. The embryos that were exposed to the highest MDMA concentrations showed evident opacity in the brain area (Figure 15) (42):

- 1.30 mM MDMA concentration affected 9 out of 12 embryos ($p < 0.05$) in the first and 2 out of 12 embryos ($p > 0.05$) in the second test.
- 2.59 mM MDMA concentration caused brain area opacity in 12 out of 12 embryos in both tests ($p < 0.05$).

This observation could be considered as brain necrosis (developmental endpoint), which is defined as cloudy, brown or white areas in the zebrafish brain tissue, being normally transparent (36).

Also, compared to the negative control group, bigger yolk sac in MDMA exposed embryos was recorded as well:

- 1.30 mM MDMA concentration caused this manifestation in 4 out of 12 embryos ($p > 0.05$) in the first and 3 out of 12 embryos ($p > 0.05$) in the second test.
- 2.59 mM MDMA concentration affected 12 out of 12 embryos ($p < 0.05$) in the first and 9 out of 12 embryos ($p < 0.05$) in the second test.

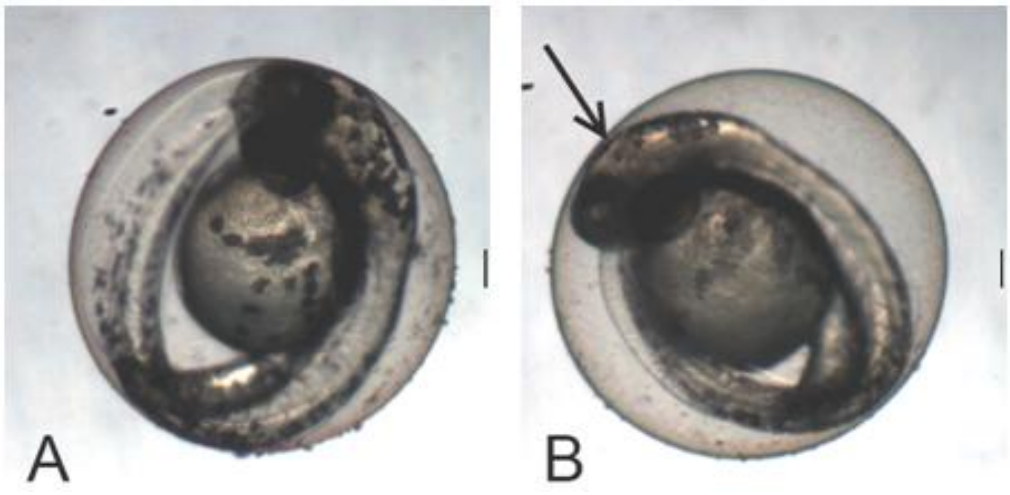

Figure 15: Observations of developmental endpoints in zebrafish embryos following 48 hours of their exposure to MDMA; normally developed negative control group embryo (A) and embryo exposed to 2.59 mM MDMA with the evident opacity observed in the brain area (brain necrosis) (B). Scale bar: 100 µm.

Brain of a developing zebrafish embryo could therefore be suggested as a target organ of MDMA-induced toxicity. This can be confirmed by many research reports defining MDMA as a neurotoxin which, among others, decreases fibre density in forebrain areas and causes oxidative stress in brain mitochondria (7, 8). Previously reported post mortem findings in young humans that have been exposed to MDMA identified perivascular haemorrhages, severe cerebral oedema, neuronal degeneration, disseminated intravascular coagulation or hypoxic changes in the brain, as well (47). Also, a recent study using cultured rat embryos, demonstrated a concentration-dependent effect of MDMA on embryonic development. Namely, an anomaly of head development and an open cranial neuronal pore which both indicate a disturbed neurulation and brain development were observed (48). Zebrafish embryos which had bigger yolk sac at the time of observation turned out to be normal later on with a continuation of the test. This phenomena can be considered as a developmental delay and thus as additional developmental toxic effect of MDMA which is quite consistent with the findings in the rat embryo experimental model, where the highest MDMA concentration used affected yolk sac development as well (48).

After evaluation of developmental disruptions stated above, the embryos were placed in new 96-well plates together with 300 µL of 0.3 x Danieau's solution/well and incubated at 27±1 ºC and a 14 h light/10 h dark cycle until day 6 post fertilization, in order to evaluate their locomotor activity.

HATCHING

At 48, 52 and 72 hpf the hatching of embryos was recorded. The results of tests were more or less consistent and the average results are presented in Figure 16.

In comparison to negative control group, the embryos that have been exposed to 0.10, 0.26 and 0.52 mM MDMA concentrations started their hatching earlier (48 hpf). The hatching of embryos exposed to 1.30 mM MDMA was still substantially consistent with that found in control group, while the highest MDMA concentration (2.59 mM) caused a significant delay of this process. It seems that MDMA elicits a biphasic "inverted U" dose-response pattern in zebrafish embryo hatching.

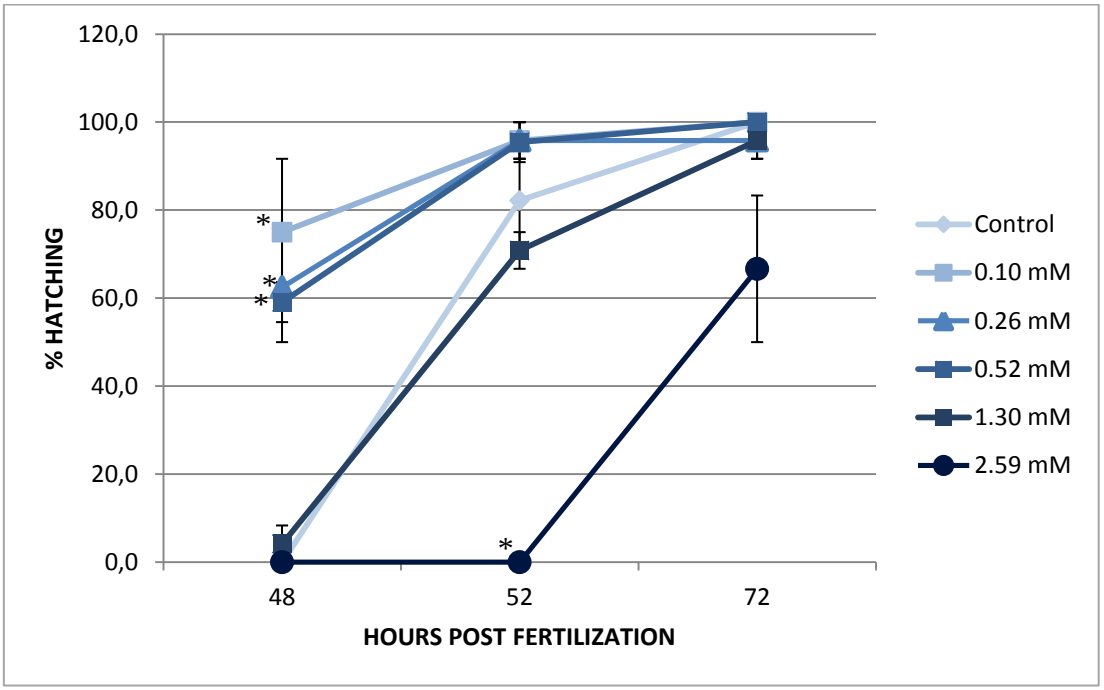

Figure 16: Effects of developmental exposure to MDMA on zebrafish embryo hatching. The evaluations were performed at 48 hpf, 52 hpf and 72 hpf. The results are presented as mean % of hatching of 2 tests (% of hatching/MDMA concentration) with standard errors of means (SEM); * $p < 0.05$; n = 11 - 12 embryos/MDMA concentration in each test (n=2).

In the developmental stage of zebrafish embryo from 24 – 48 hpf, the cells of the hatching gland, which contain cytoplasmic granules with hatching enzymes, play a key role (42). The proteolytic enzymes soften and degrade the chorion after hatching. So, when hatching approaches, the chorion becomes thinner due to protease secretion and the tail coiling of the embryo eventually creates a hole into attenuated chorion (49). Previous studies

proposed that hatching enzyme secretion is regulated by hormones, most probably via the central nervous system, more precisely by dopamine receptors, located in developing nervous system of zebrafish (50, 51).

Normally, the hatching period lasts from 48 up to 72 hpf. Some embryos occasionally hatch later, but certainly all finish up to 96 hpf and that does not mean that they are retarded (42). In our study, all embryos hatched within the 96-hour interval. The fact, that lower doses of MDMA induced premature hatching and that the highest one has delayed it, can be supported by previous reports, presuming that MDMA affects the release of monoamine transmitters (5-HT, DA and NA) while the hatching enzyme secretion is regulated by the developing nervous system, most likely via dopamine receptors, which in zebrafish embryos are already present approximately 24 hpf. On the other hand, we can hypothesize that alterations in hatching could occur due to spontaneous movements, which is another reflection of the developing nervous system function. This kind of correlation was seen when we have compared hatching (Figure 16) and spontaneous movements (Figure 18). Since the zebrafish embryo creates a hole in the chorion by tail coiling, the increased frequency of spontaneous movements stimulates hatching (lower MDMA concentrations) and the reduced frequency of such activity delays it (high MDMA concentrations).

At 72 hpf, deformation of the tails was an additional disruption noticed in smaller percentage of larvae that were exposed to 2.59 mM MDMA in both tests (Figure 17).

Figure 17: Additional observation of tail deformations in larvae at 72 hpf; normally developed larva from the negative control group (A) and larvae exposed to 2.59 mM MDMA (4 - 52 hpf) with evident malformation of their tails (B). Scale bar: 100 µm.

Some studies have shown that incubation of zebrafish larvae in 96-well plates might cause the occurrence of skeletal deformities, particularly larval tail kinks, if they are kept in smaller wells over a period of 144 hpf (28). However, in our experiments the tail deformation was observed in embryos exposed only to one of the tested MDMA concentrations. Therefore this phenomenon cannot be a consequence of incubation space. As the presence of this deformation decreased until 144 hpf, it might be considered as another reversible developmental toxicity effect of MDMA.

4.2.2 EFFECT OF MDMA ON EMBRYONIC SPONTANEOUS MOVEMENT AND LOCOMOTOR ACTIVITY OF ZEBRAFISH LARVAE

The assessment of MDMA developmental neurotoxic potential in zebrafish embryos was performed also by evaluating the zebrafish embryonic and larval locomotor activity. We were observing changes in spontaneous tail coiling in 24 hpf old embryos and the swimming activity of 6 dpf old larvae following different MDMA exposure times, by using the behavioral assay.

4.2.2.1 EVALUATION OF SPONTANEOUS MOVEMENTS

Four independent tests were performed to assess the frequencies of spontaneous movements of embryos. The eggs (before 4 hpf) were exposed to 300 µL of different MDMA concentrations: 2.59 mM, 1.30 mM, 0.52 mM, 0.26 mM, 0.10 mM and 0 mM - 0.3 x Daneau's solution. At 24 hpf the movements were recorded. The results are presented as a concentration-response chart (Figure 18) considering all 4 tests.

When compared to control group, embryos that were exposed to 0.10, 0.26 and 0.52 mM MDMA concentrations showed a pattern of increased activity and those incubated in the presence of 1.30 mM and 2.59 mM MDMA a pattern of decreased activity. However, only the highest MDMA concentration was able to induce significant concentration-dependent alterations in this first motoric activity of 24 hpf old zebrafish embryos.

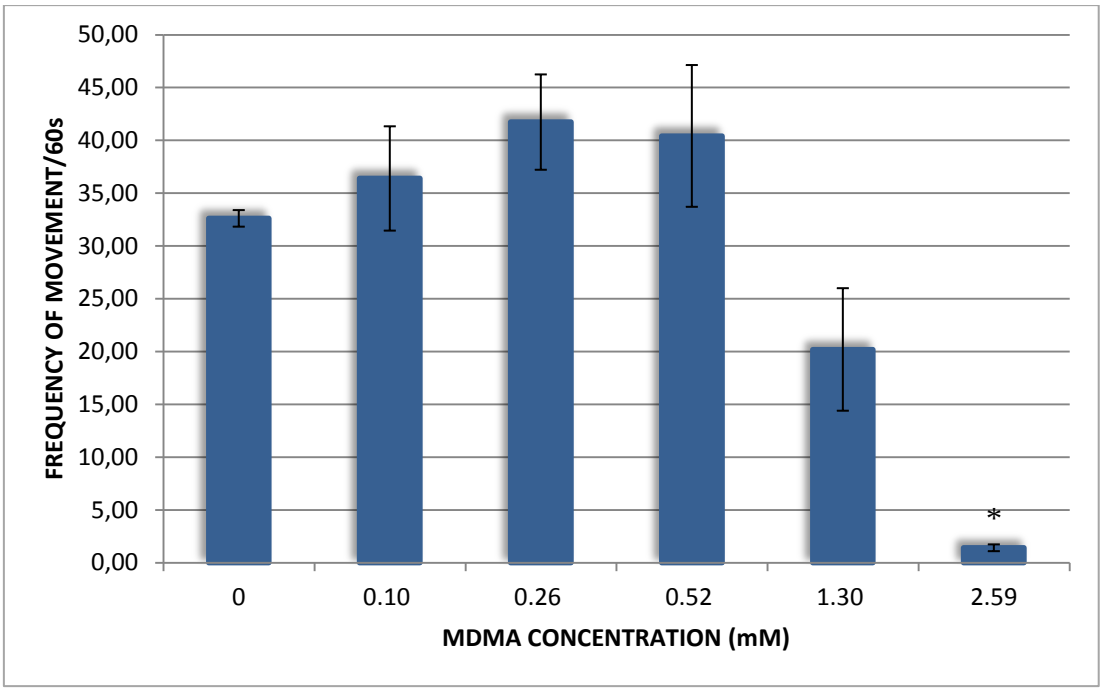

Figure 18: Effects of developmental exposure to MDMA on spontaneous tail coiling in 24 hpf old zebrafish embryos. The results are presented as mean frequencies of movement measured in all 4 tests (frequency of movement in 60s/MDMA concentration) with standard errors of means (SEM); * p < 0.05; n = 8 - 10 embryos/MDMA concentration in each test (n=4).

Nevertheless, we are aware that more test replicas should be carried out to confirm whether or not the activity is significantly different, since just four tests do not give the required statistical potency.

4.2.2.2 EVALUATION OF LARVAL SWIMMING ACTIVITY (exposure from 4 to 52 hpf)

Two independent behavioral assays were performed as a continuation of ZFET, 4 days after the MDMA exposure, in order to evaluate zebrafish larval swimming activity.

Two hours before recording, 144 hpf old larvae were transferred to “agar recording plates” with adequate volumes of 0.3 x Danieau’s solution. Their swimming activity was recorded during five cycles of alternating 4 min light and dark periods, starting with a 6 min light period (2 min needed for acclimatization). The results of general locomotion of larvae, previously exposed to MDMA from 4 to 52 hpf, are presented in Figure 19 and Figure 20.

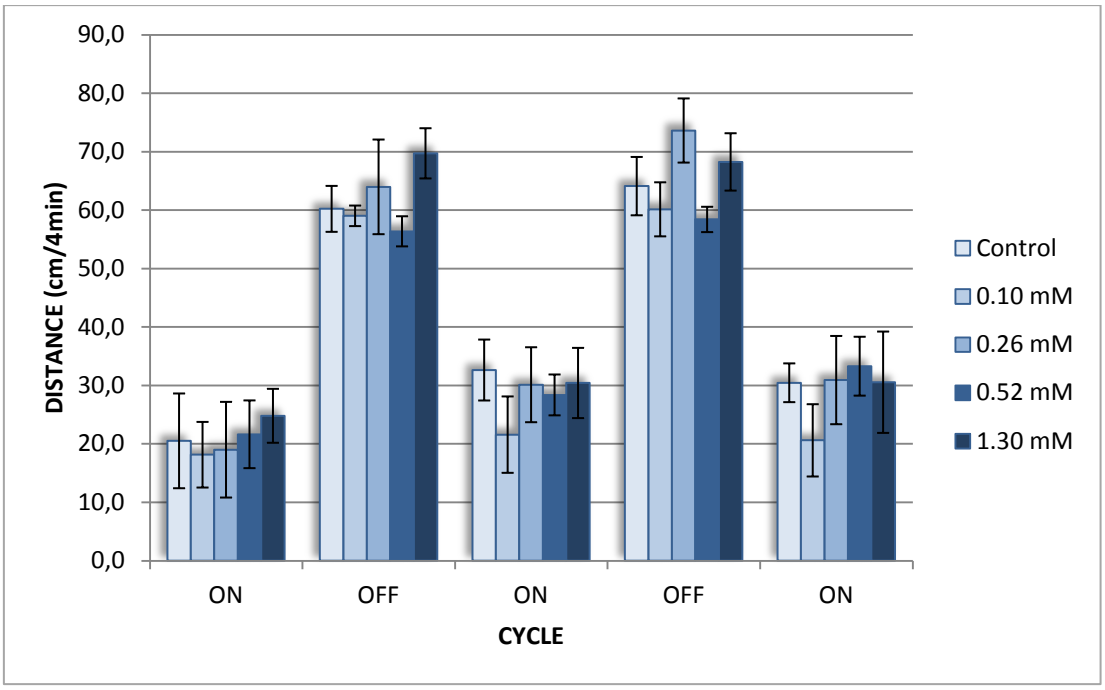

Figure 19: Effects of developmental exposure of zebrafish larvae to MDMA, from 4 – 52 hpf, detected through monitoring their general locomotion at 6 dpf during alternating light and dark cycles (Assay 1). The results are presented as mean distances of movements (cm/4min) with standard errors of means (SEM); * $p < 0.05$; n = 6 larvae/plate in the control group and n = 6 - 7 larvae/plate in the treated group; MDMA concentrations: 1.30 mM, 0.52 mM, 0.26 mM and 0.10 mM.

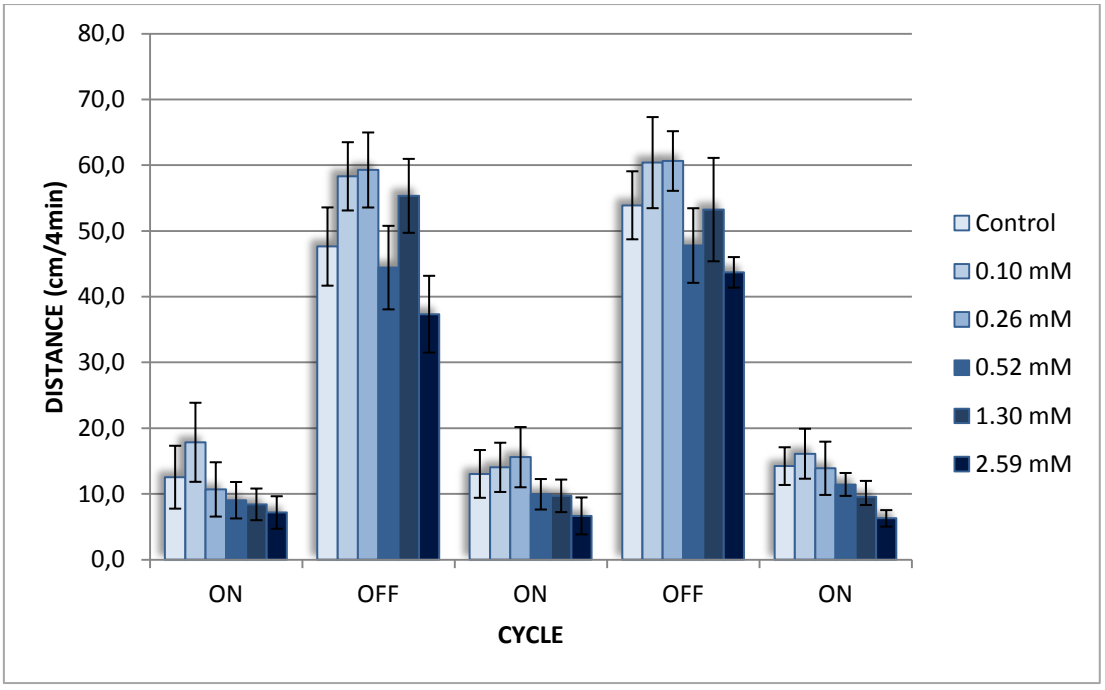

Figure 20: Effects of developmental exposure of zebrafish larvae to MDMA, from 4 – 52 hpf, detected through monitoring their general locomotion at 6 dpf during alternating light and dark cycles (Assay 2). The results are presented as mean distances of movements (cm/4min) with standard errors of means (SEM); * $p < 0.05$; n = 7 larvae/plate in the control group and n = 6 - 7 larvae/plate in the treated group; MDMA concentrations: 2.59 mM, 1.30 mM, 0.52 mM, 0.26 mM and 0.10 mM.

In both assays, larvae exposed to MDMA showed no difference in movements, compared to the control group. Larval activity was lower during light and higher within dark periods, meaning that movement patterns in response to alternating light and dark cycles were preserved, regardless of the MDMA dose used. It seems that 48 hour of MDMA exposure during early development of zebrafish has no significant effect on the subsequent locomotor activity of larvae.

4.2.2.3 EVALUATION OF LARVAL SWIMMING ACTIVITY (exposure at 144 hpf)

In order to determine the MDMA-induced developmental neurotoxicity in 144 hpf old larvae, several replicas of the behavior assay were performed. The exposure times to MDMA differed from the standardized time used in the optimization process of this assay, for which *d*-amphetamine was used. During first few replicas that have been performed with 0.10 – 2.59 mM MDMA concentrations, the tested substance might have reacted with the material of recording plates. Namely, the results obtained in range finding test that was performed later on, showed that the mentioned concentrations of MDMA were too high. Indeed, its effects on larval locomotion were not as expected for such high concentrations used (data not shown). We therefore decided to expose the larvae to more relevant MDMA concentrations in range between 0.005 and 0.052 mM, when no adverse effects were observed.

The results of monitoring basic locomotion of larvae that were exposed to MDMA at 144 hpf, are shown in Figure 21. Larvae were transferred either into adequate volumes of 0.3 x Danieau's solution (the negative control group) or the following MDMA test concentrations (the treated groups): 0.052 mM, 0.026 mM and 0.005 mM. After 2 hours of exposure, larval movements were recorded during five cycles of alternating 4 min light and dark periods, starting with a 6 min light period (2 min needed for acclimatization). 24-well plastic plates were used for recording.

Larvae, exposed to 0.005 mM MDMA concentration, showed no significant difference in their movements compared to the control group. The 0.026 mM and 0.052 mM MDMA concentrations however induced prominently lower activity during both dark periods. We

could therefore conclude that MDMA in the stated concentration range causes concentration-dependent decrease in larval locomotor activity. However, their movement patterns in response to alternating light and dark cycles were preserved, regardless of the dose of MDMA**.**

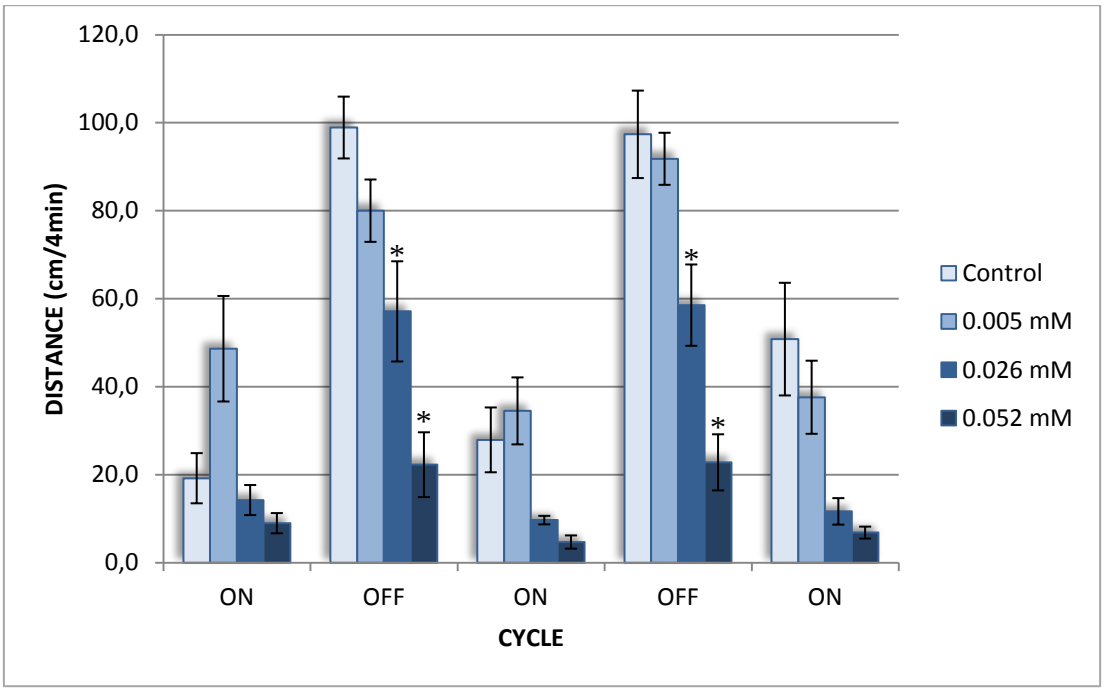

Figure 21: Effects of acute MDMA exposure of zebrafish larvae at 144 hpf, on their locomotion during alternating light and dark cycles. The results are presented as mean distances of movements (cm/4min) with standard errors of means (SEM); * p < 0.05; n = 7 larvae/plate in the control group and n = 8 larvae/plate in the treated group.

There are no reports so far describing acute toxic effects of MDMA in zebrafish embryos or 6 dpf pld larvae.

Concerning spontaneous movement, Kimmel et al., 1995, described, that in zebrafish approximately at 19 hpf individual spinal primary motor axons appear and establish contact with developing myotomal muscles (generated from somites). This causes muscular contractions that are probably associated with neurotransmitter release (42). The organisation of locomotor network and creation of motility patterns is mainly ascribed to serotonin and spinal cord, while the formation of serotonergic innervation temporally correlates with the stages of locomotor development (39). The differentiation of serotonergic circuitry begins at 24 hpf and around 48 hpf the serotonergic neurons are already present in the spinal cord and the hind brain (36, 41). Exposure to the highest MDMA concentration used in our tests produced significant alterations in locomotion of

early life stage zebrafish embryos. Four days after exposure to MDMA, the larvae showed similar behavior than those from the control group, with preserved patterns of movement in response to alternating light and dark periods. The alterations in their behavioral responses can reflect impaired neurological condition (25). In our tests, 48 hour MDMA exposure during 4 to 52 hpf apparently induced short-term neurotoxicity at this particular developmental stage of zebrafish, without drastic changes in formation of serotonergic and motor neuron innervations in later developmental stages.

Acute MDMA exposure produces different responses among species. In rats it typically increases locomotion while in monkeys it does not affect behavior (9, 52). In humans, however the intrasynaptic serotonin excess, following acute MDMA administration, is reflected as behavioral hyperactivity (13). Some authors have demonstrated its stimulant-like "inverted U" dose-response pattern in locomotor activity of mice, with lower doses of MDMA inducing hyperactivity and high ones causing increased activity in the late-phase (53).

According to our results we can suggest that MDMA in general inhibits locomotor activity in 144 hpf old zebrafish larvae without any major effect on their behavioral pattern during alternating light and dark periods. It has also been shown that selective serotonin reuptake inhibitors caused significant decrease of zebrafish swimming activity. It should be noted that MDMA itself inhibits the reuptake of monoamine neurotransmitters as well (7, 54). However, its neurotoxicity that has been shown in our study can be compared to the findings of certain mammal studies, where neurotoxic doses of MDMA induced substantial hypoactivity in rats (8).

To summarize, the zebrafish embryo toxicity test and the assessment of embryonic locomotor activity revealed that acute 48 hours long exposure of 4 hpf to 52 hpf old embryos to MDMA concentrations, ranging from 0.10 to 2.59 mM, was not lethal to them. However, this exposure induced alterations in hatching. The application of 2.59 mM MDMA resulted in decreased spontaneous movements of embryos after 20 hours of exposure, as well as in increased occurrence of brain necrosis which was noticed after their 48 hours exposure. Moreover, the highest MDMA concentration used caused a delay in yolk sac absorption after 48 hours of exposure and possible tail deformations following 24 hours from the termination of exposure. All these phenomena can be considered as

developmental toxicity in general, with a prominent developmental neurotoxicity of 2.59 mM MDMA. On the contrary, the MDMA exposure of zebrafish at developmental stages from 4 to 52 hpf did not cause any significant alterations in larval locomotion at 6 dpf. In addition, it should be mentioned that no teratogenicity in terms of permanent structural abnormalities was recorded. Whereas the re-innervation of serotonin neuronal terminal regions following MDMA exposure in rats and primates has been confirmed, it should be taken into account, that neuronal defects we have observed in zebrafish embryos were recovered until 144 hpf, so that their locomotor activity at that time was no longer affected (52). This phenomenon may clarify the observed evaluation inconsistencies in motion activities of the same embryos/larvae monitored at different ages, which were all exposed to MDMA during the first period of their neuronal development. The acute MDMA exposure of zebrafish larvae at 144 hpf, however induced a concentration-dependent hypoactivity thereby displaying potential neurotoxicity.

In conclusion, MDMA has been proven to cause neurotoxicity at the beginning of neuronal development as well as to affect the already developed nervous system. Nevertheless, our findings suggest that additional assays for determining neurotoxicity of a tested substance should be performed. It would be very useful to establish the effects of MDMA on locomotor activity of zebrafish larvae during the intermediate period of their serotonergic innervation (72 – 144 hpf). Such studies could be accompanied by neuropathological examinations as well, in order to confirm assessed influence of MDMA on neuronal development.

5 CONCLUSION

- The behavior assay, using zebrafish (*Danio rerio*), as an alternative model for detection of behavioral alterations following their specific chemical exposure, was introduced. Additionally, MDMA, a neuroactive drug was tested for its developmental and neuronal toxicity, using the ZFET test and our zebrafish behavior assay.
- The zebrafish larvae behaviour assay has been successfully optimized and standardized, so that it can be used for obtaining reliable information regarding the development of the nervous system, as well as the effects of the toxicants tested, especially their selective behavioral influences by taking advantage of the established paradigm of zebrafish larval behavior. Moreover, this test can simply be implemented for the needs of small laboratories as it does not require expensive equipment and software.
- Our findings on MDMA-induced developmental neurotoxicity in zebrafish indicate that their exposure between 4 hpf and 52 hpf resulted in neurotoxic effects at the highest concentration tested (2.59 mM). However, this exposure did not affect their swimming performance, monitored at 6 dpf (144 hpf).
- The exposure to MDMA at 6 dpf altered zebrafish locomotion already at much lower concentrations than those that have been used in their earlier developmental stages, being reflected in concentration-dependent hypoactivity which is a consequence of its neurotoxicity.
- Nevertheless, it is necessary to perform much more replicas of all the assays that were carried out during our experiments, in order to confirm these findings with a higher statistical potency.
- The zebrafish larvae behavior assay allowed us to classify MDMA as a developmental neurotoxicant. Further studies should be performed to evaluate in-depth if MDMA exposure during interim stages of neuronal development could influence swimming activity of 144 hpf old larvae and thus the maturation of their nervous systems.

- In addition, neuropathological studies could be performed in parallel to confirm the effects of MDMA on zebrafish neuronal development being assessed through the monitoring of their locomotor activity.

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ANNEXES

Annex A: Examples of data for spontaneous embryonic movement (graphical analysis was performed with Microsoft Excel) – the frequency of movement was detected by dynamic pixel analysis by using ImageJ software. The resulting data were transferred into a Microsoft Excel spreadsheet.

PLATE 1 (10.05)											
Control		MDMA 20 µg/ml		MDMA 50µg/ml		MDMA 100µg/ml		MDMA 250µg/ml		MDMA 500µg/ml	
1	1315	1	279	1	375	1	213	1	907	1	9
2	859	2	511	2	266	2	647	2	1332	2	9
3	1323	3	1237	3	678	3	1157	3	1149	3	4
4	1119	4	1269	4	559	4	1036	4	852	4	12
5	650	5	586	5	317	5	1362	5	645	5	11
6	671	6	437	6	199	6	1449	6	997	6	8
7	239	7	308	7	91	7	1074	7	652	7	7
8	154	8	957	8	44	8	1338	8	670	8	5
9	144	9	419	9	25	9	958	9	474	9	9
10	416	10	335	10	23	10	642	10	299	10	11
11	190	11	824	11	38	11	760	11	200	11	6
12	270	12	324	12	15	12	454	12	638	12	10
13	142	13	485	13	14	13	742	13	397	13	18
14	93	14	228	14	35	14	622	14	228	14	9
15	72	15	547	15	23	15	308	15	143	15	8
16	46	16	295	16	432	16	247	16	225	16	15
17	437	17	329	17	468	17	158	17	164	17	8
18	1453	18	295	18	156	18	84	18	130	18	8
19	368	19	86	19	225	19	123	19	547	19	14
20	355	20	44	20	127	20	51	20	466	20	6
21	182	21	182	21	83	21	90	21	316	21	26
22	112	22	673	22	57	22	232	22	278	22	9
23	97	23	198	23	76	23	855	23	175	23	8
24	219	24	265	24	631	24	391	24	127	24	10
25	706	25	186	25	226	25	828	25	68	25	12
26	416	26	433	26	262	26	629	26	73	26	20
27	390	27	172	27	133	27	397	27	238	27	8
28	219	28	1219	28	51	28	222	28	127	28	9
29	137	29	1415	29	27	29	136	29	416	29	9
30	129	30	887	30	215	30	575	30	263	30	6
31	129	31	1041	31	539	31	432	31	225	31	12
32	128	32	865	32	91	32	155	32	515	32	17
33	467	33	550	33	295	33	260	33	243	33	14
34	685	34	205	34	226	34	328	34	171	34	8
35	323	35	101	35	130	35	245	35	40	35	4
36	680	36	50	36	99	36	231	36	53	36	10
37	1000	37	29	37	668	37	233	37	37	37	7
38	479	38	498	38	229	38	752	38	48	38	13
39	352	39	649	39	414	39	345	39	26	39	8
40	192	40	945	40	299	40	908	40	66	40	12
41	122	41	662	41	173	41	864	41	661	41	9
42	107	42	711	42	77	42	578	42	391	42	12
43	769	43	774	43	40	43	926	43	207	43	10
44	180	44	459	44	44	44	1040	44	165	44	4
45	397	45	389	45	16	45	829	45	86	45	15
46	245	46	490	46	25	46	914	46	122	46	1
47	110	47	327	47	26	47	369	47	160	47	12
48	42	48	217	48	656	48	456	48	157	48	5
49	35	49	152	49	227	49	249	49	76	49	9
50	56	50	134	50	395	50	127	50	244	50	8
51	27	51	96	51	732	51	57	51	386	51	6
52	64	52	514	52	249	52	43	52	528	52	5
53	25	53	1707	53	369	53	55	53	446	53	13
54	37	54	327	54	610	54	37	54	609	54	7
55	46	55	628	55	1576	55	65	55	439	55	8
56	14	56	352	56	762	56	215	56	321	56	10
57	57	57	129	57	822	57	157	57	439	57	18
58	16	58	80	58	1278	58	215	58	339	58	3
59	21	59	71	59	1194	59	121	59	233	59	12
60	28	60	44	60	845	60	321	60	79	60	3
61	29	61	175	61	645	61	314	61	476	61	10
62	70	62	527	62	1379	62	480	62	596	62	13
63	473	63	258	63	1023	63	1140	63	208	63	12
64	338	64	588	64	1323	64	639	64	229	64	6

65	248	65	582	65	783	65	663	65	170	65	7
66	49	66	779	66	544	66	686	66	407	66	5
67	41	67	615	67	803	67	262	67	465	67	7
68	34	68	417	68	460	68	242	68	563	68	13
69	22	69	250	69	847	69	152	69	369	69	11
70	16	70	109	70	1319	70	105	70	338	70	11
71	9	71	80	71	1086	71	66	71	522	71	5
72	487	72	68	72	1244	72	48	72	292	72	4
73	282	73	35	73	1254	73	955	73	568	73	11
74	795	74	277	74	793	74	898	74	586	74	8
75	911	75	307	75	898	75	624	75	594	75	12
76	716	76	564	76	1223	76	662	76	150	76	6
77	463	77	385	77	1312	77	866	77	211	77	7
78	422	78	388	78	1473	78	1271	78	135	78	10
79	305	79	1229	79	923	79	692	79	87	79	22
80	524	80	134	80	965	80	639	80	123	80	23
81	514	81	396	81	498	81	300	81	434	81	14
82	443	82	316	82	433	82	396	82	386	82	10
83	502	83	91	83	1570	83	383	83	238	83	17
84	215	84	661	84	830	84	273	84	628	84	19
85	444	85	551	85	1267	85	159	85	120	85	93
86	1402	86	210	86	1731	86	239	86	186	86	64
87	1321	87	265	87	717	87	776	87	547	87	66
88	556	88	164	88	1199	88	220	88	459	88	13
89	528	89	134	89	472	89	399	89	207	89	9
90	249	90	49	90	991	90	269	90	104	90	41
91	57	91	50	91	430	91	126	91	48	91	32
92	39	92	36	92	303	92	250	92	21	92	53
93	53	93	45	93	671	93	557	93	48	93	25
94	334	94	43	94	1026	94	738	94	17	94	18
95	510	95	29	95	648	95	735	95	10	95	12
96	1186	96	454	96	412	96	403	96	10	96	17
97	1054	97	606	97	959	97	435	97	5	97	14
98	847	98	453	98	1069	98	441	98	18	98	9
99	432	99	183	99	790	99	651	99	47	99	7
100	216	100	235	100	635	100	320	100	692	100	7
101	100	101	142	101	652	101	337	101	977	101	14
102	69	102	68	102	431	102	360	102	1268	102	10
103	151	103	373	103	223	103	735	103	952	103	7
104	1027	104	827	104	151	104	188	104	1251	104	8
105	1225	105	541	105	89	105	298	105	1211	105	10
106	996	106	321	106	90	106	205	106	573	106	7
107	1005	107	456	107	145	107	112	107	280	107	11
108	594	108	265	108	282	108	39	108	472	108	5
109	322	109	1015	109	224	109	1161	109	746	109	7
110	346	110	252	110	448	110	1374	110	512	110	8
111	793	111	325	111	979	111	1164	111	264	111	7
112	349	112	328	112	431	112	1601	112	168	112	8
113	225	113	629	113	396	113	521	113	143	113	12
114	189	114	291	114	627	114	803	114	86	114	4
115	209	115	492	115	671	115	436	115	24	115	7
116	167	116	183	116	558	116	273	116	38	116	7
117	93	117	120	117	483	117	290	117	250	117	7
118	107	118	94	118	460	118	344	118	594	118	7
119	34	119	254	119	363	119	300	119	790	119	13
120	31	120	480	120	790	120	224	120	811	120	10
121	19	121	843	121	442	121	450	121	705	121	4
122	21	122	742	122	341	122	662	122	266	122	11
123	39	123	670	123	183	123	571	123	266	123	9
124	20	124	394	124	237	124	916	124	208	124	6
125	25	125	550	125	176	125	519	125	758	125	15
126	36	126	270	126	518	126	579	126	439	126	6
127	614	127	198	127	317	127	733	127	326	127	9
128	672	128	210	128	504	128	889	128	202	128	6
129	1025	129	289	129	871	129	1032	129	146	129	4
130	766	130	339	130	715	130	560	130	312	130	12
131	292	131	293	131	549	131	370	131	98	131	6
132	209	132	136	132	486	132	297	132	111	132	10
133	168	133	49	133	445	133	244	133	75	133	7
134	89	134	68	134	907	134	321	134	55	134	3
135	68	135	536	135	438	135	1060	135	48	135	8
136	46	136	633	136	309	136	703	136	25	136	11
137	543	137	379	137	406	137	542	137	16	137	7
138	377	138	301	138	772	138	1318	138	8	138	4
139	408	139	154	139	1512	139	1743	139	58	139	4
140	183	140	81	140	628	140	702	140	420	140	6
141	162	141	68	141	631	141	698	141	766	141	9
142	110	142	56	142	628	142	513	142	351	142	5
143	58	143	28	143	417	143	238	143	317	143	6
144	275	144	28	144	815	144	385	144	455	144	11
145	963	145	107	145	356	145	734	145	198	145	6

146	662	146	1196	146	935	146	242	146	572	146	9
147	1038	147	780	147	462	147	405	147	452	147	12
148	435	148	600	148	452	148	663	148	695	148	7
149	431	149	473	149	176	149	443	149	362	149	11
150	156	150	263	150	91	150	241	150	267	150	12
151	82	151	98	151	48	151	954	151	604	151	7
152	27	152	31	152	22	152	1097	152	873	152	6
153	20	153	31	153	109	153	714	153	421	153	19
154	18	154	16	154	593	154	647	154	267	154	7
155	11	155	22	155	264	155	772	155	177	155	10
156	23	156	517	156	368	156	222	156	184	156	6
157	24	157	917	157	292	157	440	157	107	157	10
158	30	158	363	158	883	158	503	158	37	158	12
159	27	159	183	159	254	159	185	159	22	159	3
160	7	160	366	160	854	160	367	160	5	160	9
161	34	161	357	161	776	161	107	161	75	161	9
162	62	162	317	162	1114	162	58	162	349	162	9
163	244	163	1391	163	698	163	11	163	83	163	22
164	117	164	1202	164	540	164	20	164	574	164	10
165	256	165	696	165	292	165	447	165	775	165	5
166	359	166	737	166	106	166	1251	166	922	166	16
167	408	167	915	167	67	167	201	167	445	167	5
168	313	168	615	168	37	168	332	168	474	168	12
169	141	169	484	169	36	169	142	169	242	169	11
170	56	170	290	170	350	170	53	170	801	170	15
171	19	171	209	171	926	171	31	171	544	171	7
172	27	172	86	172	850	172	15	172	596	172	14
173	332	173	55	173	876	173	75	173	346	173	8
174	603	174	55	174	693	174	702	174	167	174	12
175	490	175	36	175	473	175	173	175	105	175	9
176	513	176	413	176	273	176	511	176	390	176	14
177	163	177	470	177	348	177	1178	177	408	177	7
178	79	178	228	178	528	178	252	178	261	178	12
179	115	179	176	179	252	179	200	179	117	179	14
180	652	180	291	180	187	180	288	180	153	180	15
181	214	181	766	181	216	181	143	181	128	181	14
182	218	182	194	182	142	182	126	182	74	182	15
183	121	183	74	183	493	183	80	183	50	183	5
184	63	184	107	184	960	184	751	184	33	184	10
185	42	185	111	185	2046	185	1090	185	21	185	8
186	44	186	665	186	1688	186	484	186	23	186	12
187	44	187	420	187	964	187	327	187	442	187	8
188	63	188	123	188	1000	188	151	188	1046	188	14
189	214	189	828	189	454	189	73	189	766	189	92
190	390	190	2226	190	465	190	603	190	670	190	590
191	263	191	1148	191	439	191	243	191	566	191	144
192	308	192	1088	192	1109	192	326	192	1172	192	111
193	407	193	667	193	346	193	224	193	550	193	86
194	598	194	565	194	999	194	162	194	424	194	95
195	352	195	826	195	1217	195	275	195	301	195	75
196	305	196	224	196	555	196	379	196	519	196	69
197	141	197	455	197	454	197	1688	197	851	197	56
198	71	198	267	198	375	198	613	198	461	198	36
199	1123	199	497	199	232	199	875	199	272	199	12
200	783	200	855	200	153	200	474	200	119	200	15
201	1079	201	446	201	111	201	649	201	128	201	10
202	1034	202	428	202	516	202	522	202	138	202	23
203	1404	203	255	203	485	203	545	203	198	203	10
204	1110	204	189	204	532	204	531	204	625	204	10
205	785	205	162	205	266	205	588	205	585	205	11
206	460	206	142	206	187	206	632	206	193	206	15
207	381	207	168	207	493	207	332	207	288	207	19
208	282	208	145	208	607	208	822	208	221	208	30
209	504	209	77	209	335	209	408	209	143	209	12
210	516	210	48	210	384	210	592	210	118	210	13
211	419	211	19	211	253	211	1110	211	134	211	19
212	149	212	19	212	119	212	1046	212	641	212	17
213	200	213	17	213	42	213	790	213	418	213	25
214	88	214	27	214	652	214	835	214	575	214	10
215	62	215	19	215	1526	215	649	215	253	215	3
216	45	216	22	216	655	216	353	216	878	216	24
217	11	217	16	217	599	217	400	217	695	217	28
218	14	218	33	218	304	218	216	218	304	218	46
219	19	219	485	219	135	219	133	219	198	219	11
220	32	220	328	220	109	220	109	220	185	220	13
221	27	221	651	221	98	221	64	221	600	221	11
222	37	222	447	222	617	222	333	222	264	222	18
223	43	223	290	223	328	223	1230	223	1005	223	33

224	24	224	450	224	238	224	609	224	413	224	15
225	21	225	584	225	182	225	1052	225	637	225	7
226	15	226	1265	226	82	226	902	226	1108	226	7
227	13	227	714	227	243	227	616	227	644	227	9
228	327	228	229	228	605	228	212	228	439	228	17
229	1042	229	276	229	1619	229	135	229	374	229	14
230	503	230	274	230	682	230	113	230	479	230	9
231	641	231	374	231	1284	231	95	231	307	231	7
232	336	232	470	232	894	232	95	232	509	232	8
233	204	233	152	233	1523	233	63	233	179	233	15
234	140	234	447	234	884	234	57	234	176	234	11
235	77	235	1080	235	597	235	24	235	175	235	16
236	80	236	921	236	272	236	39	236	336	236	5
237	50	237	700	237	98	237	35	237	721	237	10
238	205	238	583	238	82	238	36	238	372	238	19
239	381	239	249	239	60	239	24	239	390	239	19
240	132	240	116	240	72	240	16	240	354	240	17
241	198	241	88	241	39	241	9	241	323	241	13
242	178	242	69	242	35	242	352	242	309	242	17
243	91	243	48	243	190	243	625	243	564	243	9
244	53	244	53	244	778	244	794	244	1040	244	11
245	34	245	48	245	627	245	479	245	1089	245	22
246	45	246	40	246	511	246	167	246	1485	246	15
247	43	247	649	247	594	247	131	247	491	247	3
248	25	248	1002	248	1360	248	420	248	305	248	5
249	14	249	722	249	697	249	1498	249	170	249	12
250	10	250	436	250	442	250	1408	250	155	250	6
251	574	251	419	251	160	251	708	251	107	251	7
252	795	252	308	252	67	252	538	252	149	252	5
253	484	253	866	253	51	253	221	253	779	253	5
254	672	254	433	254	65	254	191	254	946	254	7
255	801	255	417	255	65	255	152	255	513	255	12
256	993	256	272	256	72	256	145	256	925	256	8
257	736	257	211	257	54	257	110	257	468	257	5
258	616	258	185	258	129	258	585	258	546	258	5
259	305	259	146	259	870	259	1111	259	960	259	11
260	709	260	121	260	689	260	776	260	510	260	9
261	1192	261	94	261	471	261	805	261	384	261	7
262	863	262	86	262	664	262	417	262	233	262	6
263	651	263	75	263	988	263	233	263	91	263	74
264	515	264	64	264	461	264	817	264	48	264	476
265	937	265	48	265	880	265	1416	265	37	265	225
266	460	266	27	266	639	266	585	266	21	266	163
267	155	267	30	267	447	267	407	267	10	267	138
268	191	268	22	268	1116	268	375	268	17	268	186
269	834	269	44	269	509	269	260	269	8	269	191
270	510	270	19	270	446	270	217	270	8	270	115
271	313	271	28	271	297	271	150	271	6	271	89
272	255	272	21	272	126	272	110	272	8	272	76
273	228	273	65	273	66	273	82	273	2	273	54
274	120	274	539	274	191	274	476	274	8	274	38
275	100	275	519	275	611	275	762	275	660	275	26
276	79	276	892	276	953	276	451	276	711	276	24
277	428	277	887	277	1513	277	235	277	466	277	11
278	197	278	525	278	644	278	774	278	235	278	19
279	191	279	1257	279	407	279	2001	279	96	279	9
280	75	280	1403	280	171	280	1004	280	374	280	5
281	68	281	971	281	157	281	785	281	346	281	10
282	174	282	636	282	694	282	390	282	356	282	13
283	692	283	456	283	620	283	662	283	578	283	10
284	431	284	376	284	710	284	293	284	403	284	8
285	287	285	620	285	882	285	621	285	288	285	9
286	79	286	194	286	500	286	560	286	869	286	9
287	140	287	318	287	388	287	227	287	958	287	13
288	512	288	135	288	248	288	258	288	1365	288	9
289	167	289	1200	289	124	289	772	289	654	289	9
290	215	290	615	290	127	290	145	290	333	290	8
291	388	291	337	291	725	291	450	291	234	291	16
292	572	292	265	292	351	292	297	292	269	292	10
293	432	293	487	293	209	293	200	293	92	293	5
294	275	294	629	294	183	294	129	294	121	294	10
295	72	295	1407	295	92	295	155	295	72	295	5
296	41	296	619	296	75	296	100	296	41	296	12
297	644	297	387	297	56	297	99	297	349	297	8
298	1340	298	300	298	51	298	462	298	609	298	10
299	524	299	210	299	35	299	538	299	233	299	7
300	606	300	758	300	451	300	596	300	674	300	6
	340,24		402,12		506,99		487,01		373,07		21,44
	34,02		40,21		50,70		54,11		37,31		2,14

Annex B: The example of data obtained for swimming activity (the graphical analysis was performed with Microsoft Excel) – trajectory lines of larvae from the ImageJ software were converted into total distance of movements in a self-made "Trajectorias" spreadsheet (Microsoft Excel).

Control																	
		Control_1			Control_2			Control_3			Control_4						
	DISTANCE	4MIN	2MIN_1	2MIN_2	4MIN	2MIN_1	2MIN_2	4MIN	2MIN_1	2MIN_2	4MIN	2MIN_1	2MIN_2				
ON	PIXEL	62,38	43,22	19,17	274,97	150,09	124,88	107,33	53,19	54,15	323,85	200,68	123,17				
	CM	3,47	2,40	1,06	15,28	8,34	6,94	5,96	2,95	3,01	17,99	11,15	6,84				
	%NOT MOVING	99,85	99,85	99,88	94,20	92,36	96,06	99,70	99,85	99,58	93,84	91,76	95,94				
	DISTANCE	4MIN	2MIN_1	2MIN_2	4MIN	2MIN_1	2MIN_2	4MIN	2MIN_1	2MIN_2	4MIN	2MIN_1	2MIN_2				
OFF	PIXEL	873,78	470,31	403,47	928,19	521,31	406,89	1008,73	496,09	512,64	1351,10	792,28	558,82				
	CM	48,54	26,13	22,42	51,57	28,96	22,60	56,04	27,56	28,48	75,06	44,02	31,05				
	%NOT MOVING	73,88	71,28	76,50	74,22	70,45	78,02	68,36	68,81	67,94	60,23	53,37	67,10				
	DISTANCE	4MIN	2MIN_1	2MIN_2	4MIN	2MIN_1	2MIN_2	4MIN	2MIN_1	2MIN_2	4MIN	2MIN_1	2MIN_2				
ON	PIXEL	83,73	41,62	42,11	369,44	115,24	254,20	91,49	40,17	51,32	416,54	209,62	206,92				
	CM	4,65	2,31	2,34	20,52	6,40	14,12	5,08	2,23	2,85	23,14	11,65	11,50				
	%NOT MOVING	99,72	99,70	99,76	91,38	95,16	87,62	99,69	99,52	99,88	91,07	90,24	91,92				
	DISTANCE	4MIN	2MIN_1	2MIN_2	4MIN	2MIN_1	2MIN_2	4MIN	2MIN_1	2MIN_2	4MIN	2MIN_1	2MIN_2				
OFF	PIXEL	678,88	400,36	278,52	1329,86	750,02	579,84	1043,38	578,80	464,58	1177,09	635,49	541,60				
	CM	37,72	22,24	15,47	73,88	41,67	32,21	57,97	32,16	25,81	65,39	35,30	30,09				
	%NOT MOVING	78,98	75,67	82,31	63,04	57,76	68,33	67,38	62,99	71,79	63,62	59,61	67,64				
	DISTANCE	4MIN	2MIN_1	2MIN_2	4MIN	2MIN_1	2MIN_2	4MIN	2MIN_1	2MIN_2	4MIN	2MIN_1	2MIN_2				
ON	PIXEL	102,87	61,18	41,69	547,91	212,71	335,20	146,98	91,89	55,09	408,80	119,12	289,68				
	CM	5,71	3,40	2,32	30,44	11,82	18,62	8,17	5,10	3,06	22,71	6,62	16,09				
	%NOT MOVING	99,51	99,52	99,52	86,80	91,04	82,58	98,08	96,30	99,88	91,08	97,10	85,09				

Amphetamine_0.2uM																	
		Amph_1			Amph_2			Amph_3			Amph_4						
	DISTANCE	4MIN	2MIN_1	2MIN_2	4MIN	2MIN_1	2MIN_2	4MIN	2MIN_1	2MIN_2	4MIN	2MIN_1	2MIN_2				
ON	PIXEL	74,64	6,71	67,93	1,11	1,00	0,11	261,63	83,49	178,13	808,38	365,02	443,36				
	CM	4,15	0,37	3,77	0,06	0,06	0,01	14,53	4,64	9,90	44,91	20,28	24,63				
	%NOT MOVING	98,22	99,85	96,63	99,85	99,85	99,88	95,05	97,49	92,63	78,12	80,93	75,34				
	DISTANCE	4MIN	2MIN_1	2MIN_2	4MIN	2MIN_1	2MIN_2	4MIN	2MIN_1	2MIN_2	4MIN	2MIN_1	2MIN_2				
OFF	PIXEL	1526,62	743,89	782,73	1548,92	773,15	775,78	774,75	452,11	322,64	1360,35	714,47	645,87				
	CM	84,81	41,33	43,49	86,05	42,95	43,10	43,04	25,12	17,92	75,57	39,69	35,88				
	%NOT MOVING	54,65	55,25	54,07	57,64	57,73	57,56	78,31	75,16	81,48	60,02	57,76	62,30				
	DISTANCE	4MIN	2MIN_1	2MIN_2	4MIN	2MIN_1	2MIN_2	4MIN	2MIN_1	2MIN_2	4MIN	2MIN_1	2MIN_2				
ON	PIXEL	671,55	392,03	279,51	291,43	204,22	87,21	276,82	94,72	182,10	887,16	426,46	460,70				
	CM	37,31	21,78	15,53	16,19	11,35	4,85	15,38	5,26	10,12	49,29	23,69	25,59				
	%NOT MOVING	81,83	77,94	85,74	95,29	92,21	98,39	97,70	99,19	96,24	74,84	76,15	73,55				
	DISTANCE	4MIN	2MIN_1	2MIN_2	4MIN	2MIN_1	2MIN_2	4MIN	2MIN_1	2MIN_2	4MIN	2MIN_1	2MIN_2				
OFF	PIXEL	1528,52	815,84	712,68	1398,38	751,84	646,53	836,29	492,80	343,49	1299,79	720,99	578,80				
	CM	84,92	45,32	39,59	77,69	41,77	35,92	46,46	27,38	19,08	72,21	40,05	32,16				
	%NOT MOVING	54,22	50,33	58,13	60,49	57,55	63,44	75,42	71,82	79,03	61,01	56,93	65,11				
	DISTANCE	4MIN	2MIN_1	2MIN_2	4MIN	2MIN_1	2MIN_2	4MIN	2MIN_1	2MIN_2	4MIN	2MIN_1	2MIN_2				
ON	PIXEL	643,50	328,47	315,02	222,87	160,32	62,55	265,75	92,65	173,11	886,65	364,16	522,49				
	CM	35,75	18,25	17,50	12,38	8,91	3,47	14,76	5,15	9,62	49,26	20,23	29,03				
	%NOT MOVING	85,34	84,66	86,04	97,27	95,40	99,16	96,94	98,63	95,29	75,06	80,93	69,22				

Control_5			Control_6			Control_7				AVERAGE			SD			S.E.M.		
4MIN	2MIN_1	2MIN_2	4MIN	2MIN_1	2MIN_2	4MIN	2MIN_1	2MIN_2		4MIN	2MIN_1	2MIN_2	4MIN	2MIN_1	2MIN_2	4MIN	2MIN_1	2MIN_2
776,40	300,30	476,10	134,28	109,14	25,15	232,79	93,10	139,69	PIXEL	180,6	111,3	69,3	112,8	66,2	51,7	50,5	29,6	23,1
43,13	16,68	26,45	7,46	6,06	1,40	12,93	5,17	7,76	CM	10,0	6,2	3,9	6,3	3,7	2,9	2,8	1,6	1,3
77,89	83,22	72,59	97,48	95,73	99,25	95,60	97,82	93,41	%NOT MOVI	97,0	95,9	98,1	2,9	3,9	2,0	1,3	1,7	0,9
4MIN	2MIN_1	2MIN_2	4MIN	2MIN_1	2MIN_2	4MIN	2MIN_1	2MIN_2		4MIN	2MIN_1	2MIN_2	4MIN	2MIN_1	2MIN_2	4MIN	2MIN_1	2MIN_2
1528,97	799,46	729,50	1031,45	481,76	549,69	1656,95	726,46	930,48	PIXEL	1038,6	552,3	486,3	185,7	135,5	76,1	83,1	60,6	34,0
84,94	44,41	40,53	57,30	26,76	30,54	92,05	40,36	51,69	CM	57,7	30,7	27,0	10,3	7,5	4,2	4,6	3,4	1,9
54,33	51,40	57,26	66,41	70,42	62,42	55,70	61,19	50,22	%NOT MOVI	68,6	66,9	70,4	5,8	7,6	6,6	2,6	3,4	3,0
4MIN	2MIN_1	2MIN_2	4MIN	2MIN_1	2MIN_2	4MIN	2MIN_1	2MIN_2		4MIN	2MIN_1	2MIN_2	4MIN	2MIN_1	2MIN_2	4MIN	2MIN_1	2MIN_2
978,88	427,57	551,31	53,50	23,91	29,59	559,94	288,79	271,14	PIXEL	202,9	86,1	116,8	174,9	77,6	105,4	78,2	34,7	47,2
54,38	23,75	30,63	2,97	1,33	1,64	31,11	16,04	15,06	CM	11,3	4,8	6,5	9,7	4,3	5,9	4,3	1,9	2,6
71,45	75,10	67,82	99,76	99,67	99,88	85,50	85,04	85,98	%NOT MOVI	96,3	96,9	95,8	4,7	4,2	5,7	2,1	1,9	2,6
4MIN	2MIN_1	2MIN_2	4MIN	2MIN_1	2MIN_2	4MIN	2MIN_1	2MIN_2		4MIN	2MIN_1	2MIN_2	4MIN	2MIN_1	2MIN_2	4MIN	2MIN_1	2MIN_2
1274,28	628,66	645,63	1052,45	510,94	541,51	1772,97	940,32	832,65	PIXEL	1056,3	575,1	481,2	240,9	131,3	120,8	107,7	58,7	54,0
70,79	34,93	35,87	58,47	28,39	30,08	98,50	52,24	46,26	CM	58,7	32,0	26,7	13,4	7,3	6,7	6,0	3,3	3,0
60,40	61,55	59,26	67,47	70,21	64,75	49,91	48,72	51,12	%NOT MOVI	68,1	65,2	71,0	6,4	7,5	6,8	2,9	3,4	3,1
4MIN	2MIN_1	2MIN_2	4MIN	2MIN_1	2MIN_2	4MIN	2MIN_1	2MIN_2		4MIN	2MIN_1	2MIN_2	4MIN	2MIN_1	2MIN_2	4MIN	2MIN_1	2MIN_2
728,40	304,84	423,56	504,07	225,10	278,97	1230,81	566,47	664,34	PIXEL	342,1	142,0	200,1	205,2	73,3	140,2	91,7	32,8	62,7
40,47	16,94	23,53	28,00	12,51	15,50	68,38	31,47	36,91	CM	19,0	7,9	11,1	11,4	4,1	7,8	5,1	1,8	3,5
78,54	82,63	74,47	90,18	91,01	89,38	64,19	65,55	62,84	%NOT MOVI	93,1	95,0	91,3	5,4	3,8	8,1	2,4	1,7	3,6

Amph_5			Amph_6			Amph_7				AVERAGE			SD			S.E.M.		
4MIN	2MIN_1	2MIN_2	4MIN	2MIN_1	2MIN_2	4MIN	2MIN_1	2MIN_2		4MIN	2MIN_1	2MIN_2	4MIN	2MIN_1	2MIN_2	4MIN	2MIN_1	2MIN_2
416,43	146,89	269,53	818,38	284,08	534,30	689,65	303,65	386,00	PIXEL	468,1	184,6	283,5	364,2	157,1	212,6	148,7	64,1	86,8
23,13	8,16	14,97	45,47	15,78	29,68	38,31	16,87	21,44	CM	26,0	10,3	15,8	20,2	8,7	11,8	8,3	3,6	4,8
92,88	96,39	89,41	77,00	84,36	69,67	80,92	83,70	78,17	%NOT MOVI	87,8	90,8	84,8	10,4	8,8	12,3	4,2	3,6	5,0
4MIN	2MIN_1	2MIN_2	4MIN	2MIN_1	2MIN_2	4MIN	2MIN_1	2MIN_2		4MIN	2MIN_1	2MIN_2	4MIN	2MIN_1	2MIN_2	4MIN	2MIN_1	2MIN_2
1183,62	647,25	536,37	1386,40	729,79	656,61	1201,81	598,44	603,37	PIXEL	1368,0	701,2	666,8	154,9	65,5	96,8	63,2	26,7	39,5
65,76	35,96	29,80	77,02	40,54	36,48	66,77	33,25	33,52	CM	76,0	39,0	37,0	8,6	3,6	5,4	3,5	1,5	2,2
62,89	58,36	67,43	57,76	56,51	59,02	64,38	64,30	64,48	%NOT MOVI	59,6	58,3	60,8	3,6	3,1	4,9	1,5	1,3	2,0
4MIN	2MIN_1	2MIN_2	4MIN	2MIN_1	2MIN_2	4MIN	2MIN_1	2MIN_2		4MIN	2MIN_1	2MIN_2	4MIN	2MIN_1	2MIN_2	4MIN	2MIN_1	2MIN_2
658,91	282,54	376,37	863,92	440,72	423,19	508,81	195,33	313,48	PIXEL	647,0	323,6	323,4	224,1	110,8	133,7	91,5	45,2	54,6
36,61	15,70	20,91	48,00	24,48	23,51	28,27	10,85	17,42	CM	35,9	18,0	18,0	12,4	6,2	7,4	5,1	2,5	3,0
84,67	87,34	82,02	74,66	74,36	74,98	89,56	95,04	84,10	%NOT MOVI	83,5	83,8	83,1	8,2	8,9	8,9	3,3	3,6	3,7
4MIN	2MIN_1	2MIN_2	4MIN	2MIN_1	2MIN_2	4MIN	2MIN_1	2MIN_2		4MIN	2MIN_1	2MIN_2	4MIN	2MIN_1	2MIN_2	4MIN	2MIN_1	2MIN_2
1219,64	671,11	548,52	1595,28	854,49	740,79	1417,04	719,87	697,17	PIXEL	1409,8	755,7	654,1	139,2	67,9	77,0	56,8	27,7	31,5
67,76	37,28	30,47	88,63	47,47	41,16	78,72	39,99	38,73	CM	78,3	42,0	36,3	7,7	3,8	4,3	3,2	1,5	1,7
61,50	57,97	65,05	52,82	50,84	54,82	58,10	57,61	58,60	%NOT MOVI	58,0	55,2	60,9	3,7	3,6	4,3	1,5	1,5	1,7
4MIN	2MIN_1	2MIN_2	4MIN	2MIN_1	2MIN_2	4MIN	2MIN_1	2MIN_2		4MIN	2MIN_1	2MIN_2	4MIN	2MIN_1	2MIN_2	4MIN	2MIN_1	2MIN_2
708,13	313,31	394,82	924,56	449,10	475,47	572,45	249,95	322,50	PIXEL	659,7	310,9	348,8	254,0	98,6	162,5	103,7	40,3	66,4
39,34	17,41	21,93	51,36	24,95	26,41	31,80	13,89	17,92	CM	36,6	17,3	19,4	14,1	5,5	9,0	5,8	2,2	3,7
80,67	83,04	78,32	73,03	73,64	72,44	86,58	89,43	83,75	%NOT MOVI	83,0	84,5	81,5	8,8	7,4	10,8	3,6	3,0	4,4