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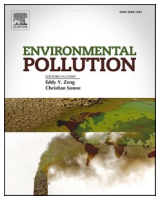

Daphnia magna an emerging environmental model of neuro and cardiotoxicity of illicit drugs.☆

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ABSTRACT

Cocaine, methamphetamine, ectasy (3,4-methylenedioxy amphetamine (MDMA)) and ketamine are among the most consumed drugs worldwide causing cognitive, oxidative stress and cardiovascular problems in humans. Residue levels of these drugs and their transformation products may still enter the aquatic environment, where concentrations up to hundreds of ng/L have been measured. In the present work we tested the hypothesis that psychotropic effects and the mode of action of these drugs in *D. magna* cognitive, oxidative stress and cardiovascular responses are equivalent to those reported in humans and other vertebrate models. Accordingly we expose *D. magna* juveniles to pharmacological and environmental relevant concentrations. The study was complemented with the measurement of the main neurotransmitters involved in the known mechanisms of action of these drugs in mammals and physiological relevant amino acids. Behavioural cognitive patters clearly differentiate the 3 psychostimulant drugs (methamphetamine, cocaine, MDMA) from the dissociative one ketamine. Psychostimulant drugs at pharmacological doses (10–200 μM), increased basal locomotion activities and responses to light, and decreased habituation to it. Ketamine only increased habituation to light. The four drugs enhanced the production of reactive oxygen species in a concentration related manner, and at moderate concentrations (10–60 μM) increased heartbeats, diminishing them at high doses (200 μM). In chronic exposures to environmental low concentrations (10–1000 ng/L) the four drugs did not affect any of the behavioural responses measured but methamphetamine and cocaine inhibited reproduction at 10 ng/L. Observed effects on neurotransmitters and related metabolites were in concern with reported responses in mammalian and other vertebrate models: cocaine and MDMA enhanced dopamine and serotonin levels, respectively, methamphetamine and MDMA decreased dopamine and octopamine, and all but MDMA decreased 3 MT levels. Drug effects on the concentration of up to 10 amino acids evidence disruptive effects on neurotransmitter synthesis, the urea cycle, lipid metabolism and cardiac function.

1. Introduction

The continuous consumption of illicit drugs combined with their incomplete removal during wastewater treatment means that residues of these psychoactive compounds are constantly introduced into the aquatic environment, where they have the potential to affect non-target organisms. Among the most consumed illicit drugs cocaine (COC), methamphetamine (mAMP), ecstasy (3,4-methylenedioxymethamphetamine (MDMA), ketamine (KET) and their transformation products are often found in surface waters at ng/L but can reach values of several μg/L (Verovšek et al., 2023).

Cocaine, probably the most consumed illicit drug worldwide, is a psychostimulant affecting human behaviour and brain physiology by the alteration of dopamine (DA) release from dopaminergic neurons (Jeon et al., 2008). Methamphetamine, a central nervous system stimulant, induces euphoria and sense of well-being in humans by increasing the neuronal release of monoamines, mainly dopamine (Jeon et al., 2008)., MDMA, with both stimulant and hallucinogenic properties, acts as a powerful releasing agent of serotonin (5-HT), norepinephrine (NE) and dopamine (DA), and also promotes reuptake inhibition of their

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high-affinity transporters (Rothman et al., 2001). Ketamine acts as a dissociative anaesthetic by blocking of the N-methyl-D-aspartate (NMDA) receptor (Freo et al., 2021), but it may also act such as an antidepressant (Freo et al., 2021), although its mechanisms are still unknown. There is also evidence that these drugs have undesired detrimental side effects causing oxidative stress and cardiac problems (De Felice et al., 2020; Moritz et al., 2003; Vollenweider et al., 1998; Waxman et al., 1980; White et al., 1996).

Neurological signalling pathways are highly conserved across phylogenetically different species (Arendt et al., 2016). These means that the psychotropic, cardiac, and oxidative stress effects of these drugs are likely to affect non target organisms such as the ecotoxicological model crustacean species *Daphnia magna*, despite that is phylogenetically distant from humans. Likewise in humans and vertebrate species, dopaminergic, serotonergic, adrenergic and NMDA receptor signalling pathways and associated neurotransmitters regulate life-history traits and behaviour in *D. magna* (Bedrossiantz et al., 2020; J. Bedrossiantz et al., 2021a,b; Campos et al., 2016; Fuertes and Barata, 2021; Jeong et al., 2018; Rivetti et al., 2018). Indeed there is evidence that at environmental relevant concentrations cocaine, its major transformation product benzoylecgonine (BZ) and methamphetamine affect the swimming activity, reproduction and/or promote oxidative stress in *D. magna* individuals (De Felice et al., 2020, 2019; Parolini et al., 2018). The above-mentioned studies, however, did not assess the molecular targets of the studies drugs, neither assessed key cognitive effects of the studied drugs such as *Daphnia* responses to light stimuli, which is a primary antipredator response. This means that the mechanisms of action by which illicit drugs may affect phylogenetically distant non-target species such as *Daphnia* are still largely unknown. In this study we tested the hypothesis that psychotropic effects and the mode of action of the four selected illicit drugs on *D. magna* are equivalent to those reported in humans and other vertebrate models. Accordingly in the present work we provide a comprehensive study of the psychotropic effects of cocaine, methamphetamine, MDMA and ketamine on behavioural *D. magna* responses linked with cognition, oxidative stress and cardiovascular effects upon acute exposures to pharmacological doses and chronic exposures to environmental relevant ones. For the latter we also assessed effects on reproduction. The study was complement with the measurement of the main neurotransmitters involved in the known mechanisms of action of these drugs in mammals and physiological relevant amino acids. Exposure to pharmacological doses equivalent to those used by illicit drug consumers (10–200 μM (Drummer, 2004; Elliott, 2005; Festa et al., 2004; Melega et al., 2007; Peiró et al., 2013; Peltoniemi et al., 2016),) will allow to compare *Daphnia* acute responses to those reported in human drug addicts. Chronic exposures to low environmental concentrations will inform about potential environmental hazardous effects.

2. Methods

2.1. Chemicals and reagents

Cocaine·HCl and MDMA·HCl were generously provided by the Spanish National Institute of Toxicology. Methamphetamine·HCl was generously provided by Dr. Elena Escubedo laboratory from Faculty of Pharmacy and Food Science at University of Barcelona. Ketamine and benzoylecgonine were purchased as hydrochloride and tetrahydrate salts from Sigma-Aldrich, respectively. Further information is on Supplementary Material.

2.2. Experimental animals and culture conditions

Parthenogenetic cultures of the *D. magna* clone F were used for this study. This clone has been maintained for over 20 years in our lab (Barata and Baird, 1998). Animals were cultured under a 16 h light:8 h dark photoperiod cycle, and at 20 ± 1 °C. Several bulk cultures of 10 adult *Daphnia* females were maintained in 2 L of lab water, i.e. ASTM hard synthetic water (APHA-AWWA-WEF et al., 1995) using a food ratio of 5 x10^5 cells/mL of *Chlorella vulgaris* that was cultured in semi-axenic conditions (Barata and Baird, 1998). Hard ASTM water has a pH around 8, which is convenient to increase the bio-availability of the studied drugs that are weak bases (Chang et al., 2021; Fontes et al., 2020). Culture media were changed every other day. Groups of 50 third brood neonates collected within the first 12 h of being release by their mothers from the adult bulk cultures were reared in 1.5 L of media as previously described, during 4 days (hereafter referred as 4-day old juveniles) before initiate the experiments.

2.3. Exposures and sample collection

2.3.1. Acute exposures

D. magna juveniles of 4 days old cultures as decribed in the previous section were exposed to pharmacological concentrations of 10, 60 and 200 μM of the four studied drugs for 24 h without food in groups of 10 individuals in 300 mL of media (4 replicates per treatment). Following exposures, 24 and 10 individuals were used to determine behavioural responses to light stimuli and the heartbeat frequency, respectively, and then used to measure the content of reactive oxygen species (ROS). Experiments were performed twice. The concentrations tested in this study (10–200 μM) include concentrations in the range of those described in humans after drug consumption (Elliott, 2005; Karch et al., 1998; McIntyre et al., 2013; Peiró et al., 2013). Experimental drug concentrations were freshly prepared each day from their respective hydrochloride salts in ASTM water.

2.3.2. Chronic exposures

Effects of chronic exposures to environmental concentrations of 10, 100 and 1000 ng/L of the studied drugs on *D magna* behavioural and reproductive responses were studied as follows. New born neonates (<12 h old) were exposed to environmental concentrations of 10, 100 and 1000 ng/L of the studied drugs for 5 days in groups of 10 individuals in 300 mL of media (3 replicates) with the addition of food (5 x 10^5 cells *Chlorella vulgaris*), after which behaviour and heartbeat responses were determined as above. At day five, 10 *D. magna* animals randomly chosen from each treatment were exposed to the same drug and food concentration for an additional 12 days in 100 mL of media to determine effects on total offspring production of at least three broods and growth in length. Offspring production was counted daily and removed and at the end of exposures the length of adult females was measured from the top of the head until the bottom of the tail to the nearest 0.1 mm using a GigE camera (UI–5240CP-NIR-GL, Imaging Development Systems, Germany) mounted onto a stereomicroscope (Motic SMZ-171, Wetzlar, Germany). In chronic exposures, acetone was used as a carrier (<0.1 ml/L) for preparing drug stock and experimental solutions. The same amount of acetone was used in all treatments including controls. Cultures were renewed with fresh media and contaminant every other day.

2.4. Stability assurance

The stability of the four drugs of abuse at 10 ng/μL in ASTM water was studied for 24 h using ultra-high performance liquid chromatography (UHPLC) coupled to a hybrid triple quadrupole detector with ion trap spectrometer (Shimadzu, Sciex QTrap 7500). Three different solutions were prepared and analyzed after 48 h, for each compound. As cocaine its known to be less stable in water solution than the other target drugs (Bijlsma et al., 2013), its main metabolite, benzoylecgonine (BZ), was analyzed during the exposition time too. Further methodological details are in Supplementary Material.

2.5. Behaviour assays

The *Daphnia* Photomotor Response Assay (DPRA) was performed as

described in (Bedrossiantz et al., 2020). Details of the assay are in Supplementary Material. The assay measured the distance moved after a sudden increase in light intensity across 30 repetitive light stimuli of 1 s followed by 4 s of darkness. Following a previous study, the "maximal photomotor response" (Max), was defined as the greatest distance moved during the first 10 stimuli. "Habituation or non-associative learning" was computed as the area under the curve (AU) for the decreasing responses to stimuli (Bedrossiantz et al., 2020). To better characterize the swimming activity under darkness and upon continuous light, basal locomotor activity (BLM) and visual-motor response (VMR) analyses of 5 d old *D. magna* juveniles were also assessed using the *Daphnia* visual-motor response (DVMA) assay described in (Bellot et al., 2021), and also in Supplementary Material. In both assays 24 individuals per treatment were monitored using 24 well plates and 1 mL of media per well. Each treatment was monitored in two separate plates and treatments were randomized across plates.

2.6. Heartbeat determination

Daphnia individuals were directly positioned in lateral view in methylcellulose and the cardiac activity of each daphnia was video recorded for 30 s with a GigE camera (UI–5240CP-NIR-GL, Imaging Development Systems, Germany) mounted onto a stereomicroscope (Motic SMZ-171, Wetzlar, Germany), basically as reported by (Faria et al., 2022). Video analyses of each individual Daphnia were performed using a recently developed MATLAB algorithm (Duran-Corbera et al., 2022).

2.7. ROS measurement

Intracellular oxidative stress was measured using 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA, Sigma-Aldrich, St. Louis, MO, USA), which oxidizes to the fluorescent product 2′,7′-dichlorofluorescein (DCF) (Holovská et al., 1998; Barja, 2002). Procedures are described in Supplementary Materials.

2.8. Metabolomic determinations

Up to 20 neurotransmitter and related metabolite changes following acute exposures to the highest concentrations of drugs (200 μM) were assessed using the same conditions as in acute exposures (Table S2, Supplementary Material). Following exposures, animals were frozen with liquid N_2 in pools of 5 in an Eppendorf and kept at −80 °C until processed for metabolites' extraction. Procedures are described in Supplementary Materials.

3. Results

3.1. Chemical stability

Three out of the 4 tested drugs were stable during the 48 h exposure period before renewal (Table S3). Freshly prepared solutions of COC, however, had trace levels of its main metabolite benzoylecgonine, and decreased by 50% after 48 h. Benzoylecgonine showed the opposite behaviour. Mass balance indicates that the concentration sum of both compounds was stable in time.

3.2. Behavioural responses

There were two different patterns of response between the psychostimulant drugs and the dissociative KET one at pharmacological concentrations. Fig. 1 shows the behavioural effects of the studied drugs in *D. magna* juveniles. Further details for the entire locomotion trajectories are depicted in Fig. S1 (Supplementary Materials). mAMP, MDMA and to a greater extent COC increased significantly ($P < 0.05$, see Table S5, Supplementary Material for stats) basal locomotor activity and decreased or even completely abolished visual responses to light in a concentrated related manner, whereas KET only diminished the latter response. The three psychostimulant drugs also compromise habituation to repetitive light stimuli in a concentration related manner, whereas KET enhanced it (Fig. 1). Results for Max responses to first light stimuli flashes varied across drugs and experiments but for COC were consistent across them, diminishing at 60 μM (Fig. 1).

Behavioural responses to chronic exposures to low environmental concentrations of the studied drugs that are shown in Fig. S2 (Supplementary Material), did not significantly affect any trait ($P > 0.05$, Table S5, Supplementary Material). Further details for entire locomotion trajectories are depicted in Fig. S3 (Supplementary Materials).

3.3. Heartbeat and ROS

The four tested drugs dosed at pharmacological concentrations had a bimodal effect on heartbeats, increasing them at low and intermediate concentrations and decreased them at the highest concentration (Fig. 2). The production of ROS was enhanced in a concentrated related manner upon exposure to the pharmacological concentrations of the four drugs (Fig. 2).

Chronic exposures to the tested drugs increased significantly ($P < 0.05$, Table S5) heart rates except at low concentrations of COC and KET (Fig. 3).

3.4. Life history effects in chronic exposures

Total offspring production was reduced significantly ($P < 0.05$, Table S5) at low concentrations of mAMP and COC and low concentrations of MDMA reduced growth (Fig. 3).

3.5. Metabolites

Target metabolites presented great correlation (R2>0.99) in the concentration range of study (5–500 ng/mL). Quantification was performed using isotopically labelled internal standards. The instrumental detection limits (IDLs) were ranged from 0.40 pg (Chol) to 138.0 pg (NE), while MDLs varied from 0.4 (Chol) to 23.8 (Tyra) pg/Daphnia. Intra-day precision ranged from 0.4% to 5.5% and inter-day precision values were from 1.8% to 25.5%. Regarding matrix effect, compounds with values below 70% indicated signal suppression due to the matrix (Phe), whereas values above 130% suggested a signal enhancement (GABA). Table S4 summarizes the quality parameters obtained for each target metabolite.

The concentrations of 16 out of 21 metabolites analyzed, which are depicted in Fig. 4, varied significantly ($P < 0.05$, ANOVA results are in Table S7) across pharmacological concentrations of the four studied drugs. From the 7 metabolites directly related with the mode of action of the studied drugs (DA, octopamine [Oct], 3-Methoxytyramine [3-MT], levodopa [LD], NE, 5-HT, 5-Hydroxyindoleacetic acid [5-HIAA]), the concentrations of 4 of them were significantly ($P < 0.05$) affected upon exposure to drugs: DA levels increased upon COC exposure and decreased following exposures to mAMP, whereas concentrations of the DA degradation product (3-MT) decreased in animals exposed to mAMP, COC and KET; OCT levels decreased under mAMP and MDMA exposures; 5-HT concentrations increased upon exposure to MDMA. The concentrations of other neurotransmitters like ACh increased following exposures to mAMP and KET, and those of GABA decreased in daphnids exposed to COC. The concentrations of ten amino acids showed also significant differences ($P < 0.05$, Table S7) across the tested drugs, with two clear patterns: a moderate reduction of concentrations upon exposure to COC, mAMP and MDMA (phenylalanine [Phe], tyrosine [Tyr], tryptophan [Trp], methionine [Met], valine [Val]) and a strong reduction by the four drugs (arginine [Arg], citrulline [Citr], proline [Pro]). Levels of Chol decreased upon exposure to mAMP and MDMA and those of tyramine (Tyra) upon mAMP and KET exposure. Metabolites that did

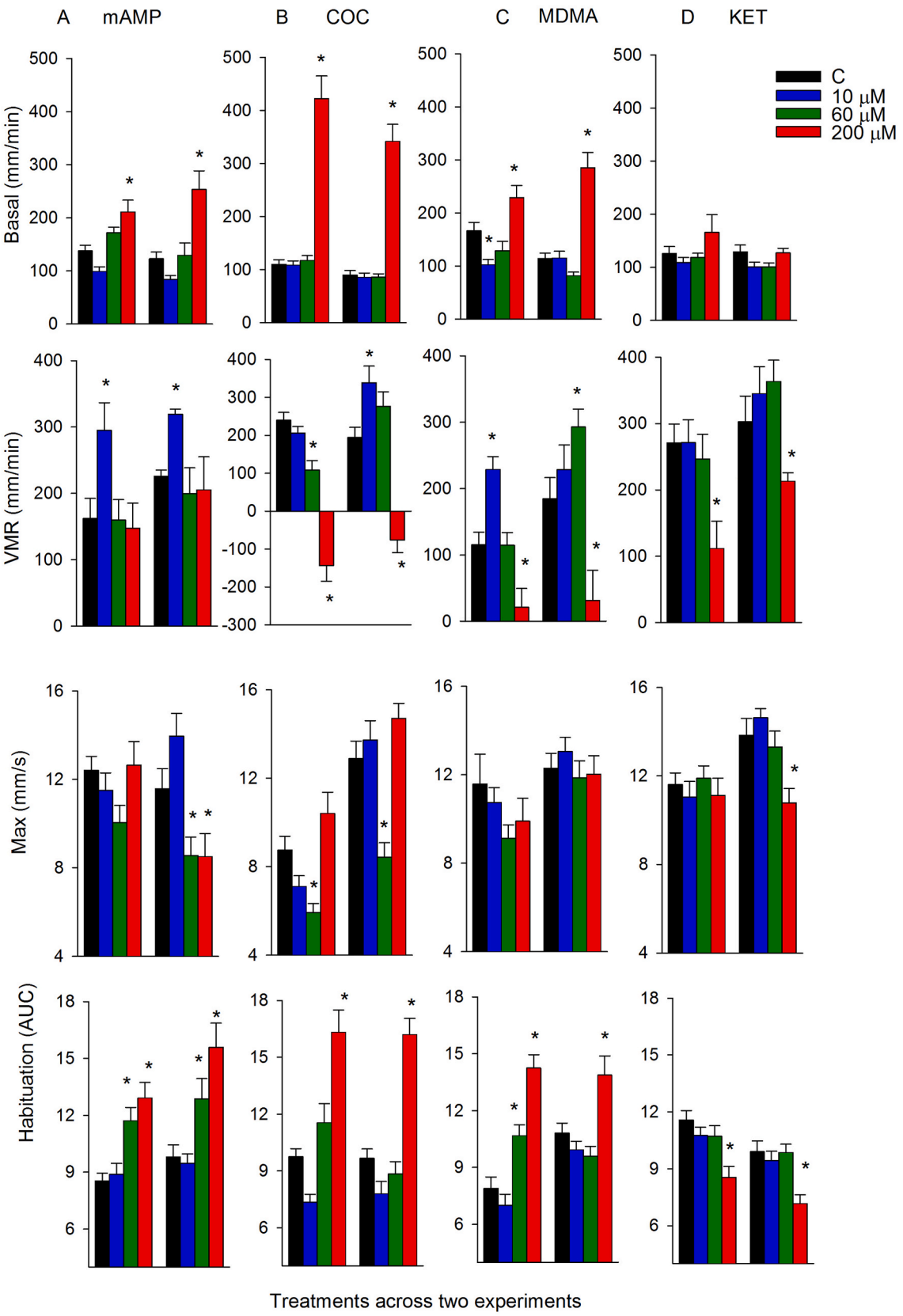

Fig. 1. Behavioural effects of the studied drugs in *D. magna* juveniles acutely exposed to pharmacological concentrations across duplicated experiments. Responses included basal locomotion activity, visual motor response (VMR), maximal response to light stimuli (Max) and habituation (Mean ± SE, N = 12–24). *means significant different from controls following ANOVA and Dunett's tests. Graphs within column panels A, B, C, D belong to mAMP, COC, MDMA and KET, respectively.

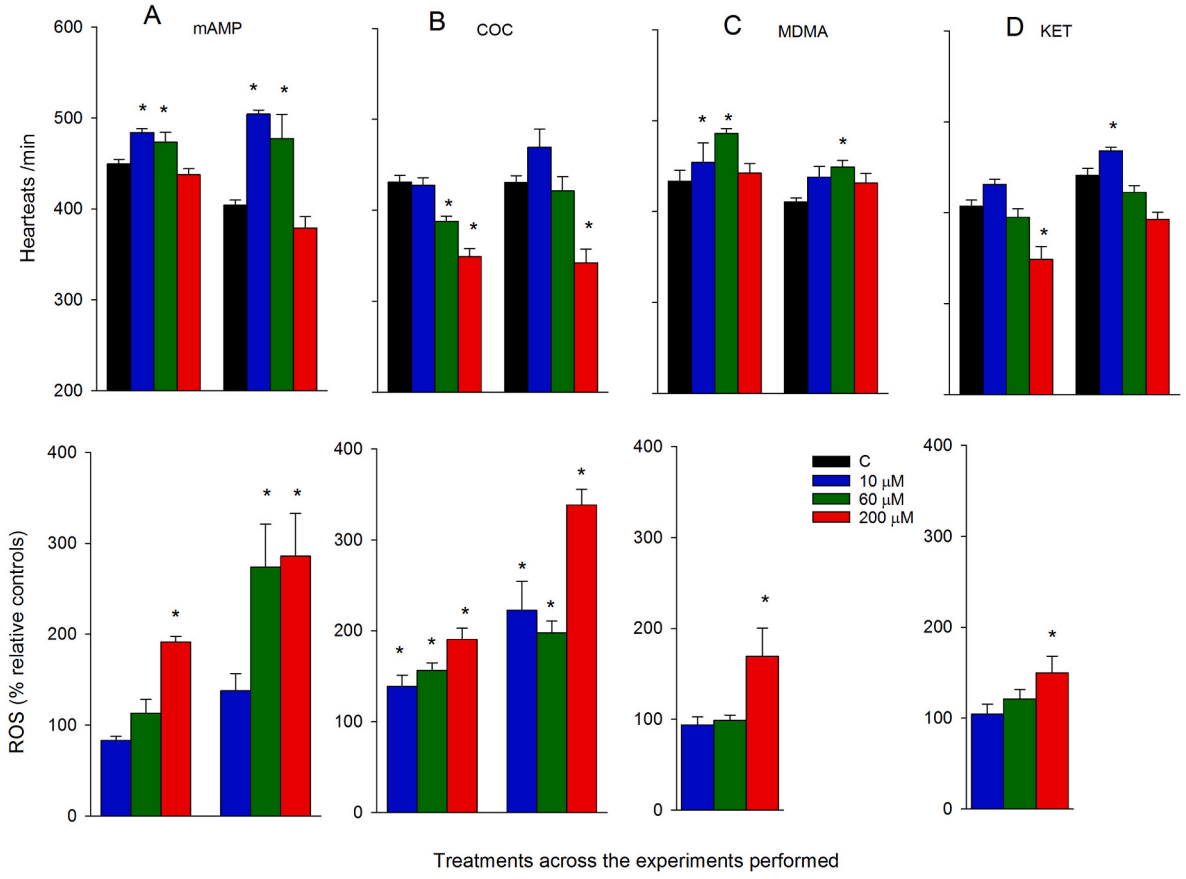

Fig. 2. Drug effects on heartbeats and oxygen reactive species (ROS) in *D. magna* juveniles acutely exposed to pharmacological concentrations across duplicated experiments (Mean ± SE, N = 10). Graphs within column panels A, B, C, D belong to mAMP, COC, MDMA and KET, respectively. *means significant different from controls following ANOVA and Dunett's tests.

not changed across drugs are depicted in Fig. S4 (SI).

4. Discussion

The aims of this study were to study the mode of action (MoA) of four illicit drugs in *Daphnia* following acute exposures to pharmacological doses and to assess potential chronic effects at environmental relevant concentrations ≤1 µg/L. The concentrations of 3 out of the 4 drugs tested were stable in water during experiment showing negligible changes within 48 h. The exception was COC whose concentrations in water were not stable being in part degraded to its main metabolite benzoylecgonine after 48 h. COC and its main metabolite benzoylecgonine are often found in both waste and surface waters (Fontes et al., 2020). Previous studies found that both compounds have similar concentration defects on *D. magna* reproduction and swimming activity (De Felice et al., 2019; Parolini et al., 2018). This means that from the toxicological point of view their combined effect is interchangeable (Altenburger et al., 2003).

Behavioural cognitive patters clearly differentiate the 3 psychostimulant drugs (mAMP, COC, MDMA) from the dissociative one KET. Psychostimulant drugs, in particular COC and MDMA, at the highest concentrations (200 µM), increased dramatically basal locomotion activities abolishing *D. magna* responses to light and its habituation to it. Alternative KET did not impair basal locomotion activity and increased habituation to light. At lower pharmacological concentrations (10–60 µM) psychostimulant drugs increased responses to light and except COC, decreased habituation, whereas KET did not have any effect on the measured responses. In chronic exposures to environmental low concentrations (10, 100, 1000 ng/L) the 4 drugs did not affect any of the behavioural responses measured. These results indicated that the four drugs were able to disrupt key *D. magna* cognitive responses but only at pharmacological doses. Previous reported studies found that cocaine and its transformation product benzoylecgonine at 50–1000 ng/L altered *D. magna* swimming activity (De Felice et al., 2019; Parolini et al., 2018), but mAMP at similar concentrations did not (De Felice et al., 2020). This disparity of results is likely related to the use of different behavioural assay procedures aimed to measure locomotion activity rather than basal swimming activity and responses to a visual stimulus as in our case. Also the use of non standardized and rather undefined behavioural set ups (i.e. light intensity and camera frames were not depicted), in the previous studies (De Felice et al., 2019; Parolini et al., 2018) precluded a proper comparison.

The observed *D. magna* cognitive responses at the highest concentrations (200 µM) upon exposure to the mAMP, MDMA, COC indicated a high degree of excitability depicted as high locomotion activity under darkness combined with an almost absent response to light stimuli. Responses to light in *D. magna* is an anxiety like response related to anti-predatory fish responses since fishes are visual predators. The observed diminished response to light at 200 µM is related to both, a decrease response of light and an already enhanced basal locomotion activity (BLM). Results obtained at 10–60 µM, however, showed a normal BLM but a greater response to light. Habituation to a repetitive stimuli, which is a primary trait for short term non associative memory and learning (Bedrossiantz et al., 2020), was also impaired by the drugs at high concentrations, which means that the central nervous system of the exposed *D. magna* individuals lost its capacity to adapt to the surrounding environment. In humans, mice, monkeys and even in zebrafish, there is huge information showing that acute exposures to

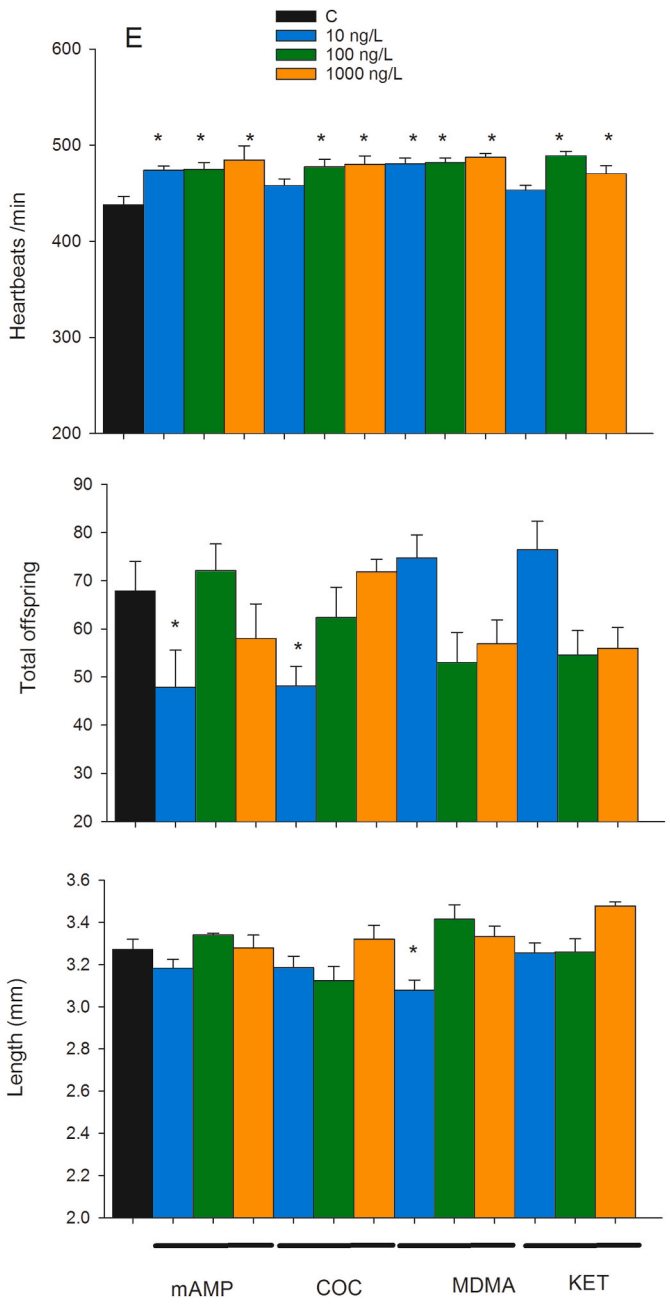

Fig. 3. Effects of the studied drugs on heartbeats, cumulative offspring production and final length in *D. magna* individuals exposed to environmental relevant concentrations. (Mean ± SE, N = 10). *means significant different from controls following ANOVA and Dunett's tests.

pharmacological and/or drug abuse doses of mAMP, MDMA and COC increase locomotion activity, enhanced the response to visual stimuli, anxiety-like behaviours and impaired learning (J Bedrossiantz et al., 2021a,b; Jentsch et al., 2002; Kalivas et al., 1998; Oliveri and Calvo, 2003; Ortman et al., 2021; Pum et al., 2011; Saddoris and Carelli, 2014; Shukla and Vincent, 2021; Singh et al., 2012; Stewart et al., 2011; Strickland et al., 2016; Wagner et al., 2015; Zombeck et al., 2010). This means that the observed drug mediated disruptive behavioural defects of mAMP, COC and MDMA on *D. magna* are equivalent to those reported in vertebrates.

On the other hand, KET, only at high concentrations decreased responses to light but surprisingly decreased habituation to repetitive light stimuli. There is also reported information that KET at sub-anaesthetic drug abuse doses may impair responses to stimuli in humans (Schwertner et al., 2018). However, reported studies on KET effects on learning in rodents are contradictory (Li et al., 2020; Shi et al., 2021). Interestingly in adult zebrafish KET evoked anxiolytic-like responses in the novel tank and light–dark box tests at concentrations of 80–160 μM (Riehl et al., 2011). In our study the observed decrease response to light at 200 μM can also be interpreted as anxiolytic-like responses. Decreased habituation to stimuli has also been reported in zebrafish larvae exposed to anti-depressants fluoxetine and deprenyl (Faria et al., 2021) and it may be related to the reported anti-depressive side effects of KET (Freo et al., 2021).

Effects on heartbeats were quite consistent across concentrations. At low pharmacological doses (10, 60 μM) and at environmental concentrations of 100 and 1000 ng/L the four drugs enhanced heartbeats, only inhibited them at the highest exposure levels studied (200 μM). These results indicate that the four drugs have a great potential to disrupt the cardiovascular system in exposed *D. magna* even at low exposure levels. Cardiovascular problems such as increasing heart rates upon exposures to the 4 studied drugs are known in humans and also in other vertebrate species such as fish (De La Torre et al., 2004; Frishman et al., 2003; Goddard et al., 2021; Idvall et al., 1979; Martinez-Raga et al., 2013; Mersereau et al., 2015; Sinha et al., 2000; Zhang et al., 2021).

The four drugs were also able to enhance ROS production in a concentration related manner upon exposure to pharmacological doses, which also agree with reported results in *D. magna* for COC, its main metabolite benzoylecgonine and mAMP (De Felice et al., 2020, 2019; Parolini et al., 2018). There is also huge information indicating that in humans and mammalian model species the four studied drugs are able to cause oxidative stress leading to neurotoxicity and cardiotoxicity (Bai et al., 2013; Cerretani et al., 2012; Kovacic, 2005; McDonnell-Dowling and Kelly, 2017; Moritz et al., 2003; Song et al., 2010).

Chronic exposures to environmental concentrations of mAMP and COC diminished reproduction but only at 10 ng/L. MDMA at 10 ng/L impaired growth. Previous studies reported that COC inhibited reproduction at 50, 500 ng/L (De Felice et al., 2019), its metabolite benzoylecgonine did so at 500, 1000 ng/L (Parolini et al., 2018), but mAMP increased it at 50, 500 ng/L (De Felice et al., 2020). Our results, thus, only partly agree with those obtained for cocaine and its metabolite benzoylecgonine since both compounds inhibited reproduction at 10 but not at 100 or 1000 ng/L. Bi-modal non monotocic effects of neuroactive contaminants on behaviour and reproduction responses have been often reported in *D. magna*, amphipods and fish (Bedrossiantz et al., 2023; Campos et al., 2012; Guler and Ford, 2010). Desensitization of neurotransmitter receptors and feedback mechanism in related signalling pathways could explain the reduced behaviour effect we found at concentrations higher than 10 ng/L. There is reported evidence that the chronic use of cocaine can produce tolerance due to the functional desensitization of dopamine Dl-like receptors (Hammer Jr. et al., 1997). Similarly mAMP exposure caused long-term reductions in all of the dopaminergic markers assayed in rats (Crawford et al., 2003). Moreover, repeated MDMA exposure causes neural and behavioral adaptations in mice by inhibitory feedback mediated by adrenergic and serotonin autoreceptors (Lanteri et al., 2014).

The drug's neurotransmitter profile targets of the *D. magna* individuals exposed to the highest doses of the studied drugs indicated that COC and MDMA enhanced DA and 5HT levels, respectively, that of mAMP and MDMA decreased DA and OCT and all tested drugs but MDMA decreased 3 MT levels. Both COC and MDMA have psychostimulant effects in humans acting as powerful releasing agents of DA and 5HT, respectively (Jeon et al., 2008; Liechti et al., 2000), thus, both drugs altered the same neurotransmitters in *D. magna* and humans. Studies on mammalian species reported that after an initial release of DA, acute high doses of mAMP causes damage to dopaminergic axon terminals in the brain. Consequently, a decrease in the DA in the brain is used as a neurochemical marker of mAMP neurotoxicity (Yu et al., 2015). Similarly, a concentration- and time-dependent decrease in the levels of DA, NE and 5-HT have been reported in the brain of zebra fish

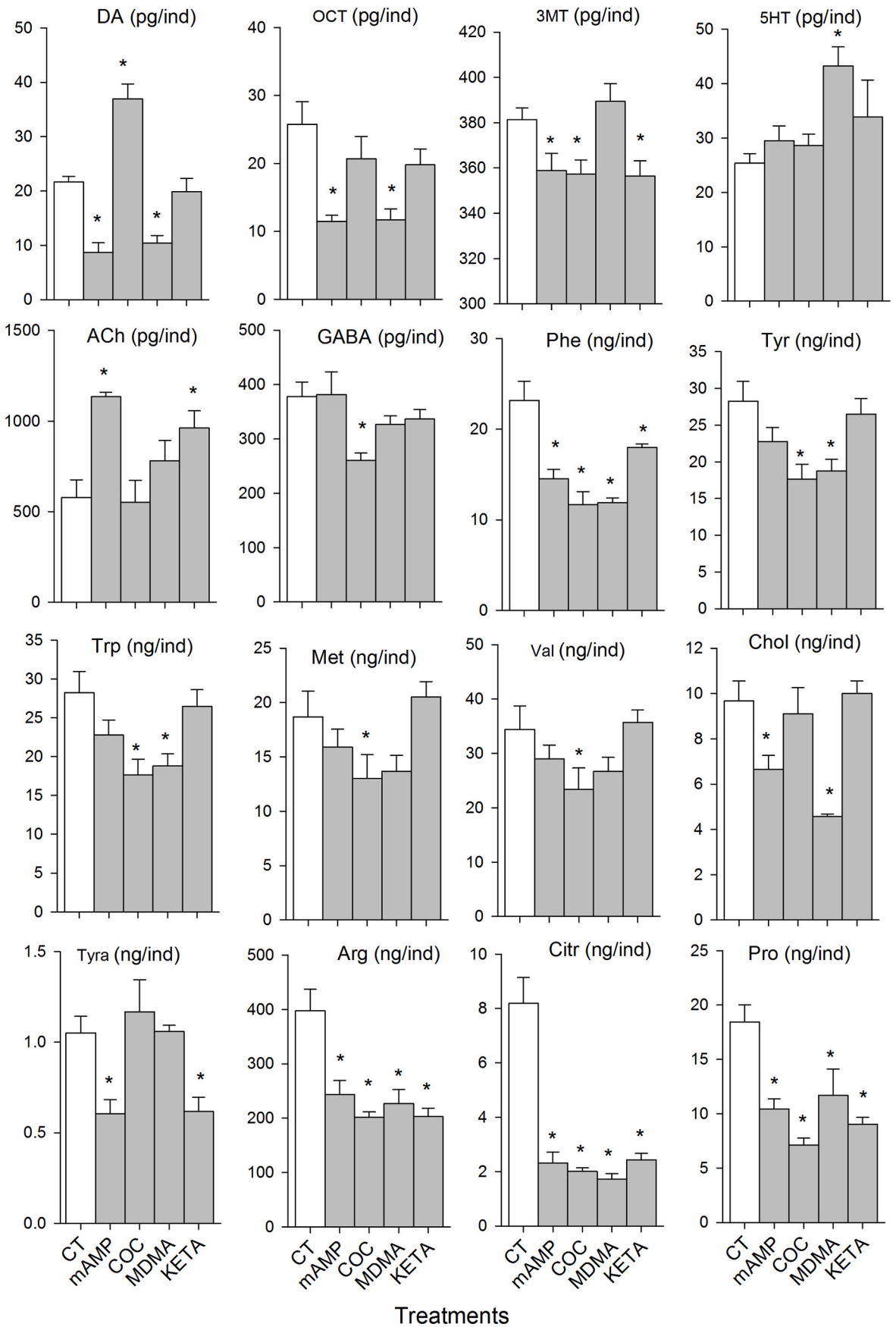

Fig. 4. Effects of the studied drugs on selected metabolic profiles measured in whole *D. magna* individuals exposed top 200 μM. Results are depicted as Mean ± SE, N = 5–6). * means significant different from controls following ANOVA and Dunett's tests.

treated with MT (J Bedrossiantz et al., 2021a,b). Moreover, the previous study also found that levels of the DA metabolite 3-MT decreased after 48 h exposure to 40 mg/L mAMP, an effect that may be related with the decreased DA levels. In insects and likely in *D. magna* octopamine is the monohydroxylic analog of norepinephrine (Zhou et al., 2008). This means that for mAMP the results found in our study also agree with those reported for mammalian and fish models.

Ketamine primary molecular mode of action is blocking the NMDA receptor (Freo et al., 2021), but it may also inhibits the uptake of serotonin increasing its concentration (Martin et al., 1982). In male rats it was reported that KET may decrease the concentrations of 3-MT in specific brain areas (Rao et al., 1989). Thus the observed decreased levels of 3-MT and relative increases of 5HT in *D. magna* individuals exposed to KET are in line with previous studies.

There is also evidence that behavioural response of mouse models to acute exposures of mAMP and KET are associated to an increased release of ACh and that exposures to high doses of COC may also inhibit the release of GABA (Ferrucci et al., 2019; Goitia et al., 2016; Nelson et al., 2002). Thus, the observed increased levels of ACh in *D. magna* exposed to mAMP and KET and decreased levels of GABA upon exposure to COC also agree with previously reported findings in mammals.

Little is known about how changes of serotonergic, dopaminergic, cholinergic and GABAergic neurotransmitters affect *Daphnia* behaviour. The lack of serotonin in tryptophan hydrolase (TRH) knock-out *D. magna* clones or those treated with the TRH enzyme inhibitor chloro-DL-phenylalanine (PCPA) showed anxiety like behaviour such as increased responses to light stimuli, high basal locomotion activities and impaired habituation (Bedrossiantz et al., 2020; Gómez-Canela et al., 2023), but an excess of serotonin also enhanced responses to light stimuli and habituation (Bedrossiantz et al., 2020). Increased levels of both serotonin and dopamine following deprenyl exposure, increased visual motor responses (VMR) (Bellot et al., 2021) as in the present study did COC and MDMA at moderate acute exposures. There is also information that compounds that increased cholinergic signalling like mAMP did, impaired habituation to light stimuli (Bedrossiantz et al., 2020). Furthermore, the results obtained for COC together with those reported for picrotoxin indicated that compounds that antagonize or reduce GABA signalling enhanced the responses to light (Bedrossiantz et al., 2020). Ketamine, despite of increasing cholinergic signalling reduced the responses to light. This is likely to be related to the KET mode of action blocking the NMDA receptor (Freo et al., 2021), since previously we reported that NMDA receptor antagonists like memantine also decreased responses to light (Bedrossiantz et al., 2020).

We also found that the studied drugs decreased the concentrations of 10 amino acids, some of them precursors of the studied neurotransmitters, but also involved in many other metabolic functions. Cocaine, MDMA and mAMP were able to decrease the concentration of 3 out of 5 amino acids directly involve in the neurotransmitter synthesis (phenylalanine, tyrosine, tyramine, tryptophan, choline) (Fernstrom and Fernstrom, 2007; Finetti et al., 2023; Grünewald, 2013; Richard et al., 2009), whereas KET did so for two of them; the four drugs dramatically decreased the concentrations of amino acids involved in the urea cycle (arginine, citrulline), neurology (proline), and in immunity, lipid metabolism, cardiac function (methionine, proline) (Draper et al., 2018; Wu, 2009). In addition COC reduced the levels of valine, which is involved in glucose metabolism (Wu, 2009).

5. Conclusions

In summary the findings found in this study for six neurotransmitters and related transformation products provides additional evidence for the mechanisms of action of the tested drugs in *D. magna*, indicating that behavioural, neurotransmitter, oxidative stress and cardiac disruptive effects of the studied drugs were quite similar to those reported in vertebrate models. Psychostimulant drugs at pharmacological doses increased basal locomotion activities and both psychostimulant and dissociative drugs altered cognitive responses to external stimuli. The four drugs enhanced the production of reactive oxygen species in a concentration related manner, at moderate concentrations increased heartbeats, diminishing them at high doses. Effects on neurotransmitters and related metabolites were in concern with reported responses in mammalian and other vertebrate models. We also found that the studied drugs have detrimental side effects on amino acids involved in the urea cycle, immunity, lipid metabolism and cardiac function.

CRediT authorship contribution statement

Marina Bellot: Investigation, Methodology, Writing – original draft. **Fernando Soria:** Investigation. **Raul López-Arnau:** Conceptualization. **Cristian Gómez-Canela:** Conceptualization, Supervision, Writing – original draft, Methodology. **Carlos Barata:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Supervision, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2024.123355.

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