**TITLE**

**Characterization of neurotransmitters and related metabolites in *Daphnia magna* juveniles deficient in serotonin and exposed to neuroactive chemicals that affect its behavior: a targeted LC-MS/MS method**

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**ABSTRACT**

Neurotransmitters are endogenous metabolites that play a crucial role within an organism, at the chemical synapses. There is a growing interest in their analytical determination for understanding the neurotoxic effect of contaminants. *Daphnia magna* represents an excellent aquatic model for these environmental studies, due to its similarities with vertebrates in several neurotransmitters and related gene pathways and because of its wide application in ecotoxicological studies. Within this study, an accurate and sensible method of analysis of 17 neurotransmitters and related precursors and metabolites was developed. The method was validated in terms of sensitivity, reproducibility, precision, and accuracy, and also matrix effect was evaluated. As an independent probe of method validation and applicability, the method was applied to two different scenarios. First, it was used for the study of neurotransmitter levels in genetically mutated tryptophan hydrolase *D. magna* clones, confirming the absence of serotonin and its metabolite 5-HIAA. Additionally, the method was applied for determining the effects of chemical compounds known to affect different neurotransmitter systems and to alter *Daphnia* behavior. Significant changes were observed in 13 of the analyzed neurotransmitters across treatments, which were related to the neurotransmitter systems described as being affected by these neurochemicals. These two studies, which provide results on the ways in which the neurotransmitter systems in *D. magna* are affected, have corroborated the applicability of the presented method, of great importance due to the suitability of this organism for environmental neurotoxicity studies.

**Keywords:** Neurotransmitters · *Daphnia magna* · LC-MS/MS · Quality assurance · neurochemicals · Behavior ·

1. **Introduction**

Neurotransmitters (NTs) are endogenous metabolites elementary for neurotransmission. NTs play a fundamental task in the organism in response to stress, motor coordination regulation, control of psychomotor, gastrointestinal and homeostatic function and inter-neuronal communication (Matys et al., 2020). In humans, imbalance in neurotransmitters are known to produce a wide range of neurological disorders, such as neuropsychiatric disorders, Alzheimer's disease, attention deficit hyperactivity disorder (ADHD), hyperserotonemia or autism spectrum disorder (ASD) (Engert and Pruessner, 2008; Heyer and Meredith, 2017; Khachaturian, 1985; Kurian et al., 2011). Drugs, pharmaceuticals, chemotherapeutic agents, radiation, food additives, pesticides and heavy metals can affect neurotransmission (Andersen et al., 2000; Horzmann and Freeman, 2016). Thus, there is a growing interest in the analytical determination of neurotransmitters for understanding the effect of contaminants on them and how this imbalance disrupts key organism functions in different biological models (Gómez-Canela et al., 2019; Kim et al., 2020; Rivetti et al., 2019; Tufi et al., 2016; Wirbisky et al., 2015).

Analysis of neurotransmitters in biological matrices, and particularly in small organism such as *Daphnia*, requires efficient extraction methods and precise, reliable, accurate and sensitive analysis techniques, due to their low concentration. The development of different analytical methods for metabolomics studies has been widely expand in recent years (Burgess et al., 2014; Emwas et al., 2019; Fiehn, 2002; Labine and Simpson, 2020; Liu et al., 2019), but only a few of them have detected and investigated neurotransmitter and related metabolites. Some studies have reported gas chromatography analysis techniques, having to deal with derivatization steps of the metabolites (Hong et al., 2013; Zhang et al., 2020), which makes the use of liquid chromatography (LC) preferred. LC coupled to triple quadrupole mass spectrometry (LC-MS/MS) is particularly suitable for the analysis of targeted metabolites because its extraordinary sensitivity and selectivity (Gómez-Canela et al., 2013). In last years, some researchers have applied different LC-MS/MS methods for NTs analysis (Gómez-Canela et al., 2018; Konieczna et al., 2018; Pan et al., 2018; Wang et al., 2019). In this context, and due to the high polarity of neurotransmitters, the use of hydrophilic interaction liquid chromatography (HILIC) has the advantage to retain very polar compounds without applying any derivatization step to increase their retention, as required in reversed-phase LC applications (Park et al., 2013; Tufi et al., 2015). Furthermore, due to its selectivity, HILIC provides greater freedom from matrix effects, compared to reverse phase LC (Van Eeckhaut et al., 2009). For all these reasons, HILIC couple to MS/MS is a promising and reliable technique, which is increasingly being applied in more studies of neurotransmitter analysis in different organism (Danaceau et al., 2012; Olesti et al., 2019; Rivetti et al., 2019; Sardella et al., 2014; Tufi et al., 2015).

The crustacean and aquatic ecotoxicological model organism *Daphnia magna* is a suitable model to study the effects and toxicological consequences of environmental contaminants that produce neurotransmitter disorders. This invertebrate share with vertebrates several neurotransmitters and related gene pathways (Dircksen et al., 2011; McCoole et al., 2011, 2012a; Weiss et al., 2012a). However, there are few studies that focus on analyzing neurotransmitter levels in *D. magna*. Neurotransmitter-related studies in this model specie have been mainly focus in the transcriptomic disruption of neurological pathways (An et al., 2018; Christie and McCoole, 2012; Fuertes et al., 2019; McCoole et al., 2011, 2012b, 2012a) or about the anatomic or functional characterization of its brain (Barry, 2002; Kress et al., 2016; Weiss et al., 2012b). In recent years, studies relating *Daphnia* behavior and the effect of neuroactive chemicals in NTs have been developed (McCoole et al., 2011; Ren et al., 2015; Rivetti et al., 2016; Simão et al., 2019). Only a scarce number of studies have been focused on analyzing the amounts of neurotransmitters in *Daphnia* matrices with analytical techniques, using ion-pair reversed phase liquid chromatography with electrochemical detection (Ehrenström and Berglind, 1988) or by liquid chromatography-mass spectrometry (Gómez-Canela et al., 2019; Rivetti et al., 2019). The previous mentioned studies had certain limitations. The study of Rivetti et al (2018) was only limited to 8 key neurotransmitters and neglected related metabolites and precursors, and thus provided limited information of metabolomic neurological pathways. Furthermore, in terms of sensitivity, it had relatively high detection limits. The study of Gómez-Canela et al. (2019), despite of characterizing a large number of metabolites, was unable to quantified serotonin, one of the targeted neurotransmitter of many neurochemicals, being only partial validated across pharmaceuticals whose mode of action in non mammalian species is uncertain. Pharmacological treatments are difficult to interpret because they are generally not specific and can have unwanted side effects on other neurotransmitters. Thus, the use of reverse genetic models deficient in specific neurotransmitters offers a more robust way to validate them. Reverse genetics have been widely used to validate gene metabolic pathways (Perkins et al., 2006; Yamada et al., 2003) and to a lesser extent metabolite pathways (Begolo and Clayton, 2016; Bringaud et al., 2015). Recently, using CRISPR genome editing methods, we obtained *D. magna* clones having bi-allelic mutations on the serotonin synthesis rate limiting gene/enzyme tryptophan hydrolase (Rivetti et al., 2018). These mutated clones showed lack of brain serotonin immuno-reactivity and were also deficient in serotonin.

The aim of this study is to develop an appropriate, accurate and sensible method of analysis of a high number of neurotransmitters in *D. magna*, which allows the characterization of the vast majority of metabolite pathways related to neurotransmitters, thus having a fast and reliable tool to be able to understand the effects of neuroactive chemicals. For that purpose, hydrophilic interaction liquid chromatography (HILIC) was applied as reported in previous studies (Rivetti et al., 2019) but developed for a much higher number of targeted metabolites, characterizing the vast majority of neurotransmitter systems, and coupled to a more sensible and reliable MS/MS analyzer.

The neurotransmitters analyzed in this study belong to the most important neurotransmitter systems, as well as some of their precursors and metabolites. These include the major NT of the cholinergic system, acetylcholine, and its precursor choline, implicated in arousal, reward, and learning and memory (Robinson et al., 2011). Histamine is released from histaminergic neurons and is associated with wakefulness, feeding, learning and memory (Horzmann and Freeman, 2016). In the serotonergic neurons, amino acid L-tryptophan is converted to 5-hydroxytryptophan (5-HTP) by the action of the enzyme tryptophan hydroxylase, and subsequently converted to the neurotransmitter serotonin by the enzyme aromatic amino acid decarboxylase (AAAD). Serotonin can be then metabolized to 5-hydroxyindoleacetic acid (5-HIAA). Serotonin has been associated with motor function, circadian rhythms, arousal and depression (Horzmann and Freeman, 2016). Phenylalanine is the precursor of tyrosine, and thus of dopamine, norepinephrine, epinephrine and octopamine. In the dopaminergic neurons, tyrosine hydroxylase produces 3,4-dihydroxyfenilalanina (L-DOPA), that is converted by AAAD to the catecholamine dopamine, that can be metabolized to 3-methoxytyramine. In adrenergic neurons, dopamine is converted to norepinephrine by dopamine beta-hydroxylase and can be further converted by phenylethanolamine-N-methyltransferase to epinephrine, or by catechol O-methyltransferase to normetanephrine (Horzmann and Freeman, 2016). Octopamine is closely related to norepinephrine, and synthesized by an homologous pathway in the biosynthetic pathways for [catecholamines](https://en.wikipedia.org/wiki/Catecholamine) and [trace amines](https://en.wikipedia.org/wiki/Trace_amine), and it has been implicated in regulating aggression in invertebrates as *Drosophila* (Zhou et al., 2008). In some invertebrates, adrenergic signaling is consider absent and analogous functions being performed by octopamine and its precursor tyramine (Bauknecht and Jékely, 2017). Norepinephrine and epinephrine have not been unequivocally identified in *Drosophila*, although low concentrations have been detected in some other insect species, meanwhile crustaceans have been reported to use both signaling pathways (Adamo, 2008; Gallo et al., 2016). GABA is the major inhibitory neurotransmitter in the CNS reducing excitability (Horzmann and Freeman, 2016). Taurine is a β-amino acid present in high concentrations in different areas of the CNS, participating in processes as signal transduction, modulation of calcium movement or neurotransmission. It is also an agonist of GABAA receptors (Ochoa-de la Paz et al., 2019).

The performance of the LC-MS/MS method was evaluated in terms of comprehensive mass spectral characterization, selectivity, linearity, accuracy, precision and sensitivity. The application of this technique for the analysis of serotonergic metabolites was validated, after performing behavioral assays, by their analysis in genetically mutated CRISPR tryptophan hydrolase (TRH) *D. magna* individuals, which should be deficient in serotonin (Rivetti et al., 2018). Furthermore, the response of the remaining neurotransmitters and also of serotonergic ones was studied in *D. magna* individuals exposed to 12 chemicals known to alter the behavior of mammals and fish and modulate the cholinergic, serotonergic, dopaminergic, adrenergic, histaminergic and GABAergic systems (details of selected neurochemicals, their mode of action and reported studies are in Table S1, Supplementary Material).

1. **Experimental**
   1. Chemicals and materials

Pure analytical standards of neuroactive chemicals used for the exposition experiments were purchased from Sigma-Aldrich (USA/Netherlands): apomorphine (APO; CAS 41372-20-7; purity ≥98.5%), caffeine (CAFF; CAS 58-08-2; purity ≥99%), chloro-DL-phenylalanine (PCPA; CAS 7424-00-2; purity ≥98.5%), cimetidine (CIM; CAS 51481-61-9; purity ≥98%), 6-hydroxydopamine (6OH; CAS 28094-15-7; purity ≥97%), imidacloprid (IMI; CAS 138261-41-3; purity ≥98%), memantine (MEM; CAS 41100-52-1, purity ≥97%), N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine (DSP4; CAS 40616-75-9; purity ≥98%), nicotine (NIC; CAS 54-11-5, purity ≥99%) and scopolamine (SCO; CAS 55-16-3; purity ≥90%); except diphenhydramine (DIPH; CAS 147-24-0; purity ≥98%), that was purchased from Santa Cruz Biotechnology (Dallas, TX, USA) and mecamylamine hydrochloride (MEC; CAS 826-39-1, purity ≥98%), that was purchased from Tocris Bioscience (Minneapolis, MN, USA). Detailed information about the studied neurochemicals is depicted in Table S1 (Supplementary Material).

Neurotransmitter and isotope-labelled analytical standards, solvents and acids used for chemical analyses and procedures to prepare stock solutions are described in Supplementary Material and Table S2.

* 1. Experimental animals

Parthenogenetic cultures of a single clone of *D magna* (clone F) were used in the neuroactive chemicals study and also as a wild type control in the mutated clone studys. This clone has been described to have a marked negative phototactic behavior (Simão et al., 2019). Three additional genetically modified CRISPR *D. magna* clones originated from clone F were used to test how mutations in the tryptophan hydrolase (TRH) gene enzyme modulates responses to repetitive light stimuli. These included two clones presenting bi-allelic mutations in the TRH gene enzyme that should lack serotonin (hereafter TRHA-, TRHB-) and a mono-allelic mutant (TRH+) that should have normal levels of serotonin (Rivetti et al., 2018). Further details of cultured conditions are in Supplementary Material.

* 1. Exposures and sample collection

Two sets of experiments were carried out. One with the three mutated clones plus the wild type one (clone F) and the other with *D. magna* clone F exposed to different neuroactive chemicals. *D. magna* juveniles were pre-exposed to the selected chemicals for 24 h, in groups of 12 individuals in 300 mL of test medium plus algae prior to behavioral assays. Following exposures, 10 animals were distributed randomly among 24 well plates (two treatments per plate) without food. Selected concentrations for each tested chemical within this study were far below those impairing survival or swimming. Neurochemicals were initially screened for light stimuli motile responses using the concentrations reported in other studies, detailed in Table S1. Those concentrations having the greatest effect in light stimuli motile response assays were selected for neurotransmitter determination. Detailed information about the studied neurochemicals across experiments and concentrations used is depicted in Table S1 (Supplementary Material). Non-exposed samples (controls) were used in all the experiments. Acetone at 20 µL/L was used as the carrier chemical, and was also added to the controls. Just after behavioral assays, juveniles were collected in groups of five animals, snap-frozen dry in liquid nitrogen and stored at −80 °C until extraction. Additional control samples for blanks, quality controls and matrix standard calibration were also sampled. Similar culturing conditions were used in the experiment with mutated clones but animals were not exposed to any chemical.

* 1. Behavioral assays

The behavioral assay was based in the automatized delivery of visible light stimuli using a DanioVision Observation Chamber (DVOC-0040). Locomotor response of *Daphnia* to these light stimuli was video tracked and analyzed using the EthoVision XT 9 software (Noldus, Wageningen, The Netherlands). All testing was performed at 20 °C. Light intensity of the stimuli was selected at 50% in DanioVision setting (290 lux), and then, sequences of the stimuli (light) were delivered at 5 s interstimulus interval (ISI). The period of time between stimuli was in darkness (near infrared light). Trials were conducted in 24 well plates, with one 4 day old *D. magna* juvenile in each well containing 1 mL of exposure medium. Before delivering the first stimulus, *Daphnia* individuals were left in the DVOC in darkness (near infrared light) for 20 min to acclimatize. Videos were recorded at 30 frames per second and the locomotion response was analyzed for each individual *Daphnia* by measuring the maximal distance moved per second (mm) within the light period after first stimulus.

* 1. Sample extraction

Samples were extracted following previous studies (Rivetti et al., 2019) with minor modifications, which are described in Supplementary Material.

* 1. UPLC-MS/MS analysis

Targeted neurotransmitters were measured using Ultra Performance Liquid Chromatography couple to tandem mass spectrometry (UPLC-MS/MS), consisting of an Acquity UPLC system (Waters, USA) connected to a Xevo TQ-S triple quadrupole mass spectrometer (Waters, USA). Chromatographic conditions were as reported in previous studies (Rivetti et al., 2019) with minor modifications, which are described in Supplementary Material. Since much higher number of neurotransmitters were measured simultaneously in this study, a first analytical approach with the analysis of a solution containing all the analytical standards at 1 mg/L in ACN:H2O (50;50, v/v) was analyzed in order to check the peak resolution. Neurotransmitters were measured under positive electrospray ionization (ESI+). Mass spectrometer conditions were similar for the common optimized metabolites to those reported in some previous studies for zebrafish samples (Gómez-Canela et al., 2018) with minor modifications, optimized as described in Supplementary Material. Table S2 (Supplementary Material) displays final optimized conditions. Experimental data were acquired and processed using the MassLynx v4.1 software package (Waters, USA).

* 1. Quality assurance and method validation

Calibration was performed over a concentration range from 0.005 to 1mg/L, using seven calibration points. Calibration standard curves were prepared into *D. magna* neurotransmitter samples to account for matrix effects (Zhou et al., 2015). Further details are in Supplementary Material. To evaluate the quality and validate the method, linearity, sensitivity, reproducibility and repeatability (inter- and intra-day precision), carry over, recovery and matrix effect parameters were determined as it is indicated in Supplementary Material.

* 1. Data analysis

Likewise it has been described in fish (Faria et al., 2019a, 2019b), *D. magna* responses to repetitive light stimuli are bi-phasic, increasing the response to light stimuli until a maximum response is reached, and then the response decreases due to habituation (Fig S1A). The relative (versus controls) motile response of *D. magna* juveniles exposed to the tested chemicals varied largely before the first light stimuli (basal activity) and the last 5 light stimuli (late habituation phase), and was quite consistent between the 5 to the 20 stimuli (Fig S1 B, C,D, E). Accordingly, for each individual replicate per tested neurochemical, the gross mean response before light was determined, from 5-20 and from 25-30 light stimuli, and compared by one way ANOVA followed by Tukey’s post hoc tests and also by Student’s t-test. Prior to analyses data was tested to meet the ANOVA assumptions of normality and variance homoscedasticity (Zar, 1996).

Levels of neurotransmitters per *D. magna* samples were normalized per individual (pg/animal) and were compared with those of controls or wild type clone using one-way ANOVA followed by Dunnett’s post-hoc tests (P < 0.05). Neurotransmitter levels in *D. magna* samples exposed to the studied neuroactive chemicals were normalized with respect to the control of each of the experiments and expressed as fold-change.

Overrepresentation (enrichment) analysis of categorical KEGG annotations (Kanehisa, 2000) for the set of analyzed neurotransmitters was performed using MBrole (López-Ibáñez et al., 2016). KEGG number annotation of each neurotransmitter (Table S2) was submitted as compound set and enrichment analysis was computed using the full MBrole database as background set. Only categories related to KEGG pathways and with false discovery rate (FDR) <0.05 were considered. Categories with just one hit in set were also excluded.

1. **Results and discussion**
   1. Behavioral assays

Figure 1 shows behavioral responses for TRH mutated clones and *Daphnias* exposed to the tested neuroactive chemicals. Individuals from mutated clones lacking serotonin (TRHA-, TRHB-) moved significantly more (P <0.05) under darkness relative to the wild type clone (F 2, 27 = 7.1) (Figure 1B) and were habituated to a lower extent (F 2, 27 = 7.1) (Figure 1F). These results indicate that knocking down TRH gene increases basal activity and decreases habituation to light. Greater locomotor activity in darkness for the same clones lacking serotonin has been previously reported (Rivetti et al., 2018). Reduced habituation in organisms having diminished serotonin levels agrees with reported responses in rodents, fish and invertebrates such as *Aplysia* (Carlton and Advokat, 1973; Conner et al., 1970; Faria et al., 2019a, 2019b; Glanzman et al., 1989).

The tested chemicals affected significant (P <0.05) the motile response of *D. magna* juveniles in dark upon light stimuli (F 11, 108 = 66.0) and later on during habituation (F 11, 108 = 48.7) (Figure 1). MEM, 6OH, MEC and PCPA treatments increase basal motile responses (Figure 1A); 6OH, PCPA, NIC and SCO enhanced the response to light (Figure 1C) and 6OH, PCPA, MEC and APO decreased habituation to light (Figure 1E). The opposite behavior was observed in IMI, MEM and CIM exposed samples. Contrasting effects of the two tested agonists of the nicotinic acetylcholine receptor (nAChR), NIC and IMI, enhancing and decreasing the response to light, respectively, are in line with reported higher affinity of neonicotinoid insecticides (i.e. IMI) for arthropod nAChRs (Tomizawa and Casida, 2003) and results for the zebrafish embryo vibrational startle response assay (Faria et al., 2019a, 2019b). Effects of SCO, antagonist for muscarine AChR, decreasing habituation to light stimuli have also been reported for the rabbit nictitating membrane responses (Harvey et al., 1983) Contrasting effects of the nAChR antagonist MEC and its agonist IMI, decreasing and enhancing habituation, is also in line with effects found in rabbit eye blink (Woodruff-Pak, 2003). Results also indicate that 6OH increased motile activity and decrease habituation, which is in concern with reported findings in rats (Adams and Geyer, 1981; Luthman et al., 1989). CIM inhibitory effects on the *D. magna* response to light were also reported elsewhere in *D. pulex* (McCoole et al., 2011).

* 1. LC-MS/MS conditions

Optimization of mass spectrometer conditions were performed by the flow injection analysis (FIA) of standards at 1 mg/L. In order to maximize the method sensitivity, mass spectrometer analysis was carried out in Multiple Reaction Monitoring (MRM) detection mode. Table S2 displays MRM parameters, final transitions used as well as optimized cone voltages and collision energies for each fragment of the 17 targeted neurotransmitters and their 7 isotope labelled internal standards analyzed, that were similar for those reported in Gómez-Canela et al. (Gómez-Canela et al., 2018) for the common optimized metabolites. In most cases, the protonated molecule was observed as precursor ion, except for dopamine and serotonin that present the loss of an ammonia molecule, and norepinephrine and normetanephrine that formed the protonated ion with the loss of a water molecule as base peak. The entire analyzed compounds showed a good fragmentation pattern. For each one, two fragment ions were optimized and detected following the choice of precursor ion, in accordance with the Commission Decision 2002/657/EC recommendations (European Commission, 2002), except for 5-hydroxy-L-tryptophan (5-HTP). As previously mentioned, chromatographic conditions were those reported in Rivetti et al. (Rivetti et al., 2019), but taking into account the good chromatographic separation of the much larger number of analyzed metabolites. Figure S2 shows the LC-MS/MS extracted ion chromatograms of the 17 targeted metabolites in a mixed solution at 1 mg/L, showing good resolving peaks for all them.

* 1. Quality parameters and method validation

In order to correct mass analyzer responses and to ensure exact quantification performance, external standard calibration was conducted, correcting the obtained signal per calibration point by an internal standard. Calibration curves were prepared with *D. magna* neurotransmitter extracts with a standard mixture of the targeted metabolites and the isotope labelled internal standards, quantified by subtracting the contribution from the endogenous neurotransmitters within the matrix. Table 1 displays all the quality and validation parameters obtained by LC-MS/MS.

Correlation coefficients (R2) were equal or higher than 0.99 for most of the studied metabolites in a range from 0.005 to 1 mg/L, except for epinephrine, histamine, serotonin, 5-HIAA and 5-HTP, that was until 0.5 mg/L. Carryover effects were investigated by injecting solvent blanks throughout the sample analytical sequence, and no effect was observed, indicating no carryover during LC-MS/MS runs. Instrumental detection limit (IDL) values ranged between 0.0015 pg and 8.60 pg, being lower than those reported in previous studies for the common targeted metabolites (Gómez-Canela et al., 2018; Rivetti et al., 2019). Method detection limit (MDL) ranged from 0.05 pg/daphnia for 5-HTP and 51.56 pg/daphnia for taurine. . These values were within the range reported by Gómez-Canela et al. (2018) but lower than those reported by Rivetti et al. (2019)for the common metabolites. As an example, in the previour study, norepinephrine MDL was reported as 18.56 pg/daphnia whereas here is 0.52 pg/daphnia. Method quantification limit (MQL) range between 0.15 pg/daphnia for 5-HTP and 171.85 pg/daphnia for taurine, and values were also lower to those reported in previous mentioned studies. Intra- and inter-day precision values were lower than 20%, thus indicating a robust method. Method performance was tested using five replicates of *D. magna* samples spiked with 100 µg/L of the neurotransmitter standard mixture and 50 µg/L of the internal standard mixture, recovering the 17 neurotransmitters in the range 51.3% ± 9.1 % to 91.7% ± 7.6%. In addition, matrix effect (ME), which may cause suppression or enhancement of the analytes ionization, was also evaluated by comparing for each analytes the peak area from the spiked *D. magna* calibration curve with the signal obtained from the standard solution at the same concentration in solvent. In most cases, no matrix effect was observed, with values between 74.1% and 118.6%, meaning that signal suppression or enhancement was much lower than around 25%, except for 5-HIAA and GABA that showed values a little bit lower (58.6 and 65.0%, respectively). Therefore, quantification with the external procedure, correcting the obtained signal per point by an internal standard, seems adequate to avoid over or underestimation of the calculated analytes concentration (Van Eeckhaut et al., 2009).

* 1. Method suitability and D. magna TRH mutants neurotransmitter profiles

Enriched analyses of the measured neurotransmitters showed a good coverage of some of the most relevant pathways related to neurotransmitters systems, as those related to neuroactive ligand receptor interaction, tyrosine metabolism, biosynthesis of alkaloids, gap junction, tryptophan metabolism or ABC transporters, among others (Figure 2). Obtained enriched pathways are listed in Table S3 (Supplementary Material).

The presented analytical method was used for determining differences in the neurotransmitter profiles of wild type and genetically mutated CRISPR tryptophan hydrolase (TRH) *D. magna* individuals, including one mono-allelic mutant (TRH+) that should have normal levels of serotonin and two bi-allelic mutants (TRHA- and TRHB-) were no presence of serotonin should be detected. Obtained results, represented in Figure 3, provide an independent probe of method validation. Raw data and ANOVA results for the response of the quantified neurotransmitters and related metabolites or precursors across clones are detailed in Table S4 and S5 (Supplementary Material). Significant differences (P <0.05) were detected in 9 of the 17 analyzed metabolites. Serotonin was measured in similar amounts in wild type and TRH+ samples, and no serotonin was detected in either TRHA- or TRHB- samples. A similar pattern was observed for 5-HIAA and 5- HTP, which are respectively, the immediate metabolite and precursor of serotonin. These results are in line with previous studies. Rivetti et al. (2018), who despite of using a less sensitive method found similar serotonin levels for the wild and TRH+ clone and did not detect serotonin in individuals from TRHA- and TRHB- clones. Campos et al. (2019) reported down-regulated molecular processes related to serotonergic metabolism for TRHA- and TRHB- *D. magna* clones*.*

Contrary to the study of Rivetti et al (2018) significant differences were detected in 8 metabolites (acetylcholine, choline, tryptophan, epinephrine, dopamine, phenylalanine, 3-MT and taurine; Figure 3), suggesting that the disruption in tryptophan metabolism by serotonin may have affected the metabolism of other related neurotransmitters, as the biosynthesis of catecholamines and the tyrosine metabolism, where dopaminergic and adrenergic neurotransmitters are found.

* 1. Neurotransmitter profiles in D magna exposed to neurochemicals

This analytical method was also applied to determine the effects of neuroactive chemicals that were known to affect different neurotransmitter systems and were previously tested to produce changes in *Daphnia* behavior. Of the 17 neurotransmitters analyzed, 13 showed significant differences (P <0.05) across treatments (Figure 4). Raw data and ANOVA results for the response of the quantified neurotransmitters and related metabolites or precursors across treatments are detailed in Table S4 and S5 (Supplementary Material). *Daphnia* individuals were exposed to two neurochemicals affecting dopaminergic system: APO and 6OH. APO, known to be a non-selective agonist of dopamine receptor activating D2-like receptors and to have affinity for serotonin receptors and α-adrenergic receptors (Bownik et al., 2018; Jenner and Katzenschlager, 2016), decreased epinephrine, normetanephrine, tryptophan, taurine and choline. 6OH is used in research for the selective destruction of dopaminergic and noradrenergic neurons (Breese et al., 2005; Feng et al., 2014). In line with that, 6OH exposed *D. magna* individuals showed significant lower levels of dopamine and of norepinephrine, which is synthetized directly from dopamine by dopamine beta-hydroxylase. Also, lower levels of octopamine, neurotransmitter analogous to norepinephrine in invertebrates, and 3-MT, a direct metabolite of dopamine by the action of the enzyme catechol O-methyltransferase, were detected in individuals exposed to 6OH. To a lesser extent and despite not being significantly affected, the concentrations of two possible metabolizing products of norepinephrine, normetanephrine and epinephrine, were reduced. These results are in line with the destruction of the dopaminergic and noradrenergic neurons reported for 6OH (Breese et al., 2005; Feng et al., 2014) , showing lower levels in most of the neurotransmitters related with this dopaminergic and noradrenergic system through the tyrosine metabolism and the biosynthesis of catecholamines. Diphenhydramine (DIPH) and cimetidine (CIM), histamine H1 and H2 receptor antagonist, respectively (Berninger et al., 2011; McCoole et al., 2011), decreased epinephrine levels. These results are in line with expectations, since epinephrine is one of the neurotransmitters release upon stress and one of the effects of anti-histaminic compounds is to attenuate the neuroendocrine response to stressors (Carrasco and Van De Kar, 2003). Referring to the cholinergic systems, no significant changes were observed in the neurotransmitter profile of *D. magna* samples exposed to SCO, although L-DOPA levels decreased more than 50%. *D. magna* exposed to MEC had enhanced levels of L-DOPA and decreased levels of phenylalanine, tryptophan and taurine, whereas those exposed to NIC and IMI showed reduced levels of 3-MT and taurine, respectively. The decrease in 3-MT, the metabolite of dopamine catalyzed by catechol O-methyltransferase (as represented in Figure 2), suggest down regulation in the activity of this enzyme, whose low activity has been associated with nicotine in humans (Beuten et al., 2006). Observed effects of the studied cholinergic active chemicals on metabolites from the dopaminergic system (phenylalanine and L-DOPA) are in line with the reported cross talk between these two systems in their involvement in cognitive functions (Levin and Simon, 1998). The almost absence of effects in the neurotransmitter profiles of SCO, IMI and NIC, despite the obtained effects on behavior; are in line with reported results for zebrafish embryos (Faria et al., 2019a).

*Daphnias* were also exposed to DSP4, a compound known to destroy noradrenergic neurons (Castelino and Ball, 2005). No significant changes were observed in neurotransmitters related to adrenergic or noradrenergic neurons, although a slight decrease in epinephrine and, to a lower extent, in norepinephrine and normetanephrine were observed. On the other hand, DSP4 decreased 3-MT, increased acetylcholine and although no significant, enhanced the levels of histamine more than 50%. Enhanced levels of acetylcholine by DSP4 have also been reported in in rats (Magnani et al., 1985) and there is reported information that DSP4 can also affect negatively dopaminergic neurons and hence the associated metabolism (Ross and Stenfors, 2015). The little effects observed of DSP4 on the adrenergic system are in line with the few behavioral effects found for this compound. Despite the observed effects of MEM on *D. magna* behavior (Figure 1), no effects were observed in the analyzed neurotransmitters. This is probably related to the fact that the primary effect of MEM is to regulate the release of glutamate (Rogawski and Wenk, 2006), which was no analyzed in this study. Caffeine (CAFF), known to stimulate the CNS, just produced a significant increase in epinephrine. This result is in agreement with the reported increase in epinephrine levels produce by caffeine in healthy humans (Flueck et al., 2016). Finally, PCPA decreased serotonin levels, which agrees with its known effect inhibiting TRH activity. TRH inhibition and thus inhibition on serotonin synthesis after exposure to PCPA has also been reported in zebra fish studies (Airhart et al., 2012). Moreover, an increase in epinephrine and a decrease in norepinephrine were also observed. This decrease in norepinephrine is in line with some results obtained in rat studies upon exposure to PCPA (Reader, 1982).

1. **Conclusions**

A comprehensive optimization of a method for the determination of 17 neurotransmitters belonging to some of the most important pathways related to neurotransmitter systems in *D.magna* samples has been developed and validated in terms of sensitivity, reproducibility, precision, selectivity and accuracy. The improvement of this method in terms of sensitivity with respect to previously developed methods (Rivetti et al., 2019) has been probed. Furthermore, matrix effect was also evaluated for all the analyzed neurotransmitters, which was not observed for practically any compound. This method was further validate by applying it for the study of neurotransmitter levels in genetically mutated TRH *D.magna* samples, confirming the absence of serotonin and its metabolite 5-HIAA in knock out individuals that should not contain serotonin (TRHA- and TRHB- samples), together with lower levels of tryptophan and 5-HTP, which is in concordance with transcriptomic and immunohistochemistry results reported in previous studies (Campos et al., 2019; Rivetti et al., 2018).

Additionally, the method was applied for determining the effects of neuroactive chemicals known to affect different neurotransmitter systems and that altered *Daphnia* behavior. Neurotransmitter profiles in *D.magna* exposed to all these chemicals helped to reaffirm the applicability of the method presented in this work, and shed some light on the mode of action of the tested neurochemicals in *D. magna*. The measurement of not only neurotransmitters but also of their main metabolites and/or precursors provided a robust analytical method that helped to elucidate the mechanisms of action of neuroactive chemicals, one of the major advantages of the proposed methods relative to the previous ones (Rivetti et al., 2019).

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