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Linking chemical exposure and fish metabolome: discovering new biomarkers of environmental exposure of *Argyrosomus regius* to the antidepressant venlafaxine

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Abstract

In this study, a non-target metabolomic approach was used to investigate changes in the metabolome of juvenile meagre (*Argyrosomus regius*) exposed to venlafaxine (20 µg/L). A total of 24, 22 and 8 endogenous metabolites tentatively identified in liver, brain and plasma, respectively, were significantly changed in venlafaxine exposed meagre, showing tissue-dependent variations in the metabolic profile. The amino acids tryptophan, tyrosine and phenylalanine, which are related to the synthesis, availability, and expression of neurotransmitters (e.g., serotonin, dopamine, epinephrine), showed to be dysregulated by venlafaxine exposure. A high impact was observed in fish brain metabolome that showed a trend of up-regulation for most of the tentatively identified metabolites. In conclusion, the identification of possible biomarkers of exposure in fish metabolome to environmental stressors such as venlafaxine is crucial to assess early signal changes at molecular level, enabling the prevention of deleterious effects at the organism and population levels.

Keywords:

Non-target metabolomics; aquatic organisms; pharmaceuticals; ecotoxicology; molecular effects

1. Introduction

Venlafaxine, a selective serotonin-norepinephrine reuptake inhibitor (SNRI) antidepressant, is among the most prescribed for depression treatment (Magalhães et al., 2014). For instance, its consumption registered an increment of around 400% from 2005 to 2015 in Germany (Boulard et al., 2020). Its high human consumption allied to its incomplete removal in wastewater treatment plants (WWTPs) (Golovko et al., 2021; Lopez et al., 2022) led to a ubiquitous occurrence of venlafaxine in the aquatic environment (Čelić et al., 2019; Lopez et al., 2022), including marine waters at concentrations up to 291 ng/L (Fernández-Rubio et al., 2019). Its detection in wild fish species has also been reported, reaching levels up to 22.9 ng/g (Moreno-González et al., 2016; Grabicova et al., 2017; Huerta et al., 2018; Rojo et al., 2019).

There is a growing concern on the potential negative effects of pharmaceuticals to non-target organisms, including marine species, representing a current research challenge. Laboratory exposure experiments have shown that venlafaxine may adversely affect fish (Painter et al., 2009; Best et al., 2014; Maulvault et al., 2018a; Tang et al., 2022). These studies mainly focused on endpoints related to the mode of action of venlafaxine in humans, which acts by inhibiting the reuptake of the neurotransmitters serotonin (also known as 5-hydroxytriptamine (5-HT)) and norepinephrine (or noradrenalin) from the synaptic cleft, increasing the levels of these neurotransmitters (Magalhães et al., 2014). Although the mechanism of action of venlafaxine in non-target organisms has not been established, the dysregulation of serotonin levels has been reported as a response to venlafaxine exposure in fish (Salahinejad et al., 2022). This dysregulation can cause several adverse effects at different levels, since serotonin can be related with different neuronal and hormonal mechanisms in fish, participating in several regulatory and endocrine functions related to appetite, reproduction, immune system and fish behaviour (Santos et al., 2010).

For instance, the metabolic response of rainbow trout to stress was compromised after exposure to venlafaxine (0.2 and 1.0 µg/L for 7d) by attenuating the plasma glucose levels and lowering the levels of glycogen in the liver, suggesting a disruption in liver metabolism by increasing the liver energy demand. The capacity of gluconeogenesis and amino acid catabolism were also enhanced (Best et al., 2014), while the antioxidant defences of meagre tissues were impaired by the exposure to 20 µg/L of venlafaxine for 28d, reducing their capacity to prevent the induced oxidative stress (Maulvault et al., 2019). Venlafaxine is also able to affect fish behaviour, by decreasing the anxiety in fish and, thus increasing their exploratory behaviour (Maulvault et al., 2018a), slowing their escape response (Painter et al., 2009), or increasing their time to capture preys (Bisesi Jr et al., 2014). Additionally, exposure to venlafaxine can also impact social interactions between fish by lowering the initiative to pursue fish of the opposite sex (Tang et al., 2022) or decreasing the tendency to stay within the shoal formation, which can compromise their chances of survival and make fish more vulnerable to predators (Maulvault et al., 2018a). Fish reproduction, development and survival can also be jeopardized due to exposure to venlafaxine. For instance, zebrafish showed a reduction in embryo production when exposed to 10 µg/L of venlafaxine for 72h (Galus et al., 2013), while a higher concentration (88 µg/L for 167-168d) induced an increase in egg production of fathead minnows (Parrott and Metcalfe, 2017).

Besides the detection of adverse effects to aquatic organisms at the phenotype level, changes at molecular level are even more pronounced since they will allow an early signalling of environmental exposure to pharmaceuticals. In this context, metabolomic studies appear as an interesting approach, since they allow getting an overview of endogenous metabolites resulting from chemical reactions that occurs in a cell, tissue or biofluid of a living organism in response to external stressors or stimuli (Courant et al., 2014). Metabolomic studies to assess the effect of

pharmaceuticals in aquatic organisms have been described in literature following either target (David et al., 2018) or non-target approaches (Bonnefille et al., 2018; Serra-Compte et al., 2019; Ziarrusta et al., 2019; Brew et al., 2020; Ramirez et al., 2022). The former is more sensitive and very specific, focusing on particular target compounds or metabolic pathways, while the latter is an open analysis that allows the identification of undocumented metabolites, having the potential to find new biomarkers (Courant et al., 2014).

A few studies have assessed the perturbations in the metabolome of aquatic organisms (e.g., fish, oysters, mussels) exposed to antidepressants (Mishra et al., 2017; Ziarrusta et al., 2019; Brew et al., 2020; Ramirez et al., 2022), though little information is available on the metabolites disrupted by venlafaxine exposure (Ramirez et al., 2022). In the present study, a non-target metabolomic approach using liquid chromatography coupled to high-resolution mass spectrometry (LC-HRMS) was applied to evaluate changes in the metabolic fingerprint of juvenile meagre (*Argyrosomus regius*) exposed to 20 µg/L of the antidepressant venlafaxine. Environmental concentrations of venlafaxine generally do not exceed 1 µg/L (Fernández-Rubio et al., 2019; Wilkinson et al., 2022). However, in highly impacted rivers this antidepressant can reach levels up to 55.0 µg/L (Voloshenko-Rossin et al., 2015). Therefore, a nominal concentration of 20 µg/L was selected as a representative concentration of a worst-case exposure scenario and based on previous studies that showed that levels of venlafaxine ranging from 1 to 500 µg/L affect fish behaviour (Bisesi Jr et al., 2014; Wigner et al., 2020) and the levels of neurotransmitters in fish (50-500 µg/L) (Bisesi Jr et al., 2014; Thompson and Vijayan, 2020). Therefore, we can guarantee the identification of endogenous metabolites impacted by the exposure to venlafaxine. Indeed, in our previous study such nominal concentration showed to affect fish behaviour, making fish more susceptible to predators and impairing their chances of survival in wild environment (Maulvault

et al., 2018a). Overall, variations in the metabolome of brain, liver and fish plasma were evaluated to identify outcomes related either with venlafaxine mode of action (monoamine neurotransmitters reuptake inhibition) or off-target effects (interaction with receptors unrelated to monoamines reuptake e.g., 5-HT receptors), which may affect other biochemical pathways, such as energy metabolism, amino acid metabolism and hormone signalling (Webhofer et al., 2011; Ziarrusta et al., 2019; Costa et al., 2021).

2. Materials and Methods

2.1. Chemicals and reagents

Analytical standards of venlafaxine and ibuprofen of high-grade purity (> 98%) were purchased from Sigma-Aldrich and LGC Standards, respectively. Methanol, acetonitrile and water were provided by Fisher Scientific (Geel, Belgium). All solvents were MS grade. Formic acid 98–100% was purchased from Merck (Darmstadt, Germany) and ammonia 30% (as NH3) was purchased from Panreac (Barcelona, Spain).

2.2. Experimental design

The experimental design is described in detail in our previous work (Santos et al., 2020). Briefly, meagre (*A. regius*) specimens were raised at the aquaculture pilot station of the Portuguese Institute for the Sea and Atmosphere (EPPO-IPMA, Olhão, Portugal) until reaching the juvenile stage. Then, fish with similar biometric characteristics (total length: 6.8 ± 0.4 cm; total weight: 2.6 ± 0.5 g) were transported to the aquaculture facilities of Laboratório Marítimo da Guia (MARE-FCUL, Cascais, Portugal), where they were randomly distributed in 50 L glass tanks filled with natural UV-disinfected seawater for acclimation.

Juvenile meagre (*A. regius*) was selected as biological model because: i) it is a predator fish species that tends to accumulate organic chemical contaminants (Bodin et al., 2014), being more susceptible to the impact of environmental contaminants; and ii) its commercial value has been growing in the last years, especially in Mediterranean area (FAO, 2022), thus the data obtained can contribute to the protection of the specie by allowing the assessment of early signal changes at molecular level, which will enable the prevention of deleterious effects at the organism and population levels, which might compromise the survival of the specie. Furthermore, the early life stages of fish species (such as juvenile) are more susceptible to deleterious effects due to exposure to environmental chemical contamination.

The exposure experiment to venlafaxine (nominal concentration of 20 µg/L) was performed using 10 individuals per tank. Two treatments (control and exposure to venlafaxine) were performed in triplicate. For the exposure to venlafaxine, seawater of the exposure tanks was daily spiked with an aqueous stock solution of venlafaxine to obtain a nominal concentration of 20 µg/L. Control and exposed tanks were maintained under the following conditions: i) dissolved oxygen (DO) > 5 mg/L; ii) temperature (19.0 ± 0.5 ºC); iii) pH = 8.0 ± 0.1; iv) salinity = 35 ± 1 ‰; and v) photoperiod = 12h light: 12h dark. There was a 25% daily renewal of seawater. The exposure time was 28 days, followed by 7 additional days (until 35 days), during which the spiking of venlafaxine was stopped. Measured concentrations of venlafaxine in seawater are indicated in Supplementary Material (Table S1). In total, ten fish were randomly sampled from each treatment (c.a. 3-4 fish per tank) at days 28 and 35. Then, two composite samples were prepared (5 individuals in each of them) for brain and liver tissues and for fish plasma. Detailed description of sample collection and preparation (including fish and seawater from each replicate tank, as well as liver, brain and plasma) are described in our previous work (Santos et al., 2020).

2.3. Sample preparation

Freeze-dried liver and brain (50 and 25 mg, respectively) were extracted with a mixture methanol:water (75:25, *v/v*) using ultrasonic assisted extraction (Santos et al., 2020). Briefly, tissues were extracted with 1 mL of methanol:water (75:25, v/v) in an ultrasonic bath for 15 min in ice bath. Then, samples were centrifuged (7500 rpm, 15 min, 4 °C) and the supernatant was collected in a glass tube. This protocol was repeated three times and all supernatants were combined and evaporated under a gentle stream of nitrogen to remove the organic solvent. Then, evaporated extracts were reconstituted in 50 mL Milli-Q water with 0.1% formic acid and a clean-up step was performed by solid phase extraction (SPE) using Oasis® MCX (3 cc, 60 mg) (Waters, Ireland). Extracts were reconstituted in 1 mL of methanol:water (50:50, v/v). For fish plasma, 50 µL of methanol were added to 50 µL of fish plasma, centrifuged (5000 rpm, 10 min, 4 ºC), and 60 µL of supernatant were transferred to an insert for further analysis.

2.4. LC-HRMS analysis for non-target metabolomics

Non-target metabolomics analysis was performed using a LC-LTQ-OrbitrapVelos™ from Thermo Fisher Scientific Inc., equipped with a heated electrospray ionization (HESI-II) operating both in positive and negative mode. An analytical methodology adapted from Serra-Compte et al. (2019) was used. Briefly, chromatographic separation was performed on a Hypersil GOLD PFP column (100 x 2.1 mm; 1.9 µm) (Thermo Scientific™, USA). For positive mode, the mobile phase consisted in 0.1% formic acid in water and acetonitrile, while for negative mode, the mobile phase was water and acetonitrile. A flow rate of 0.5 mL/min was used for both ionization modes, the column was kept at room temperature and the autosampler was operated at 10 ºC. An injection

190 volume of 10 µL was used. For positive mode, ESI parameters were set up as follow: spray voltage
191 at 3.5 kV, source heater temperature at 300 ºC, capillary temperature at 350 ºC, sheath gas flow at
192 40 and auxiliary gas flow at 20 (arbitrary units). For negative mode, spray voltage was kept at 3.0
193 kV, source heater temperature at 450 ºC, capillary temperature at 450 ºC, sheath gas flow at 35
194 and auxiliary gas flow at 10 (arbitrary units). Data were acquired through Data Dependent
195 Acquisition (DDA) mode: a full-scan mode from a mass-to-charge (*m/z*) range of 100 to 700 at a
196 resolving power of 60,000 FWHM, followed by a MS/MS full-scan fragmentation from a *m/z*
197 range of 50 to 700 at 30,000 FWHM of the three most intense ion masses selected in each MS scan
198 event.
199
200 **2.5. Post-acquisition metabolic fingerprint data processing**
201 **2.5.1. Quality control**
202 Due to limited availability of sample material, LC-LTQ-Orbitrap performance during batch
203 analyses was monitored using two independent standards (venlafaxine and ibuprofen for positive
204 and negative ionization mode, respectively) instead of composite samples. The variation of
205 retention time was less than ±0.01 min for positive quality control (venlafaxine) and less than
206 ±0.02 min for the negative one (ibuprofen). For both ionization modes, the variation of mass
207 accuracy was less than ±4 ppm. Quality controls were injected at the beginning, middle and end
208 of the analytical batches and there was no difference in the mean response values between batches,
209 guaranteeing a little drift in the instrument sensitivity. Instrumental blank samples (methanol and
210 a mixture methanol:water, 50:50 (*v/v*)) were also injected every 4 samples for monitoring possible
211 carryover.
212

2.5.2. Metabolomics data processing

Non-target metabolomics data analysis was processed using Compound Discoverer 3.0 software (Thermo Scientific^{TM}). An automated data processing workflow was used for the identification of endogenous metabolites in fish tissues and plasma, which consisted of peak alignment, peak feature extraction, peak identification, compound grouping across all samples, and differential analysis. Background subtraction was also performed using blank samples. Data pre-treatment was run separately in positive and negative ionization modes in fish tissues (liver and brain) and plasma, for the two exposure times (28 and 35 days), to identify the endogenous metabolites that displayed significantly changes between the control and the exposed conditions (28 days – end of the exposure; 35 days – 7 days after stopping venlafaxine spiking).

First, computational data files (chromatograms and mass spectra) were loaded into the software. Then, automated data processing started with data filtering in the *m/z* range of 50-700 and by setting a peak intensity threshold at a signal-to-noise (S/N) ratio of 1.5. Chromatographic alignment was performed using a mass tolerance error of 5 ppm and a maximum retention time shift of 0.3 min. For peak extraction, a mass tolerance of 5 ppm was set, as well as an intensity tolerance of 30%, a S/N ratio of 3, a minimum peak intensity of 10,000, a maximum peak width of 0.5 min, and a minimum of 5 scans per peak. The identification of metabolites was automatically performed using compound exact masses and MS/MS ion fragmentation spectra, which were compared with mzCloud, mzVault and ChemSpider databases for metabolite annotation. From the list of features automatically generated only those with a best match (with databases) ≥ 65% and a statistically significant difference (p-value < 0.1) between control and exposure to venlafaxine were considered. Then, the list of significant features was manually checked, and those peaks that presented a poor chromatographic shape were discarded for further data processing. Finally, from

the tentatively identified metabolites, only those that had a positive finding in open-source databases, such as KEGG Pathways or the Endogenous Metabolites database with 4400 compounds (both integrated in Compound Discoverer 3.0 software (Thermo Scientific^{TM})), or the Human Metabolome database or LIPIDS MAPS (which were manually checked), were considered for further statistical analysis.

2.6. Data treatment

Data were processed using Compound Discoverer 3.0 software and 4 datasets for each tissue were generated, namely $POS_{28\ days}$, $POS_{35\ days}$, $NEG_{28\ days}$ and $NEG_{35\ days}$, that were merged in a single dataset and were analysed together. To avoid statistical misreading of the data, peaks of venlafaxine and its metabolites were discarded (Santos et al., 2020).

For identification purposes only those features with a statistically significant difference (p-value < 0.1) between control and exposure to venlafaxine were considered. Although a p-value < 0.05 is statistically more powerful, the low number of fish per group may lead to a low statistical power, that could be partial surpassed using a higher p-value (p-value < 0.1). Using a threshold limit of p-value < 0.1, a trend in the down- or up-regulation of several metabolites of the same metabolic pathway could be identified, allowing the assessment of biologically relevant modulation trend due to the exposure to venlafaxine. The same approach was followed by Bonnefille et al. (2018) in the evaluation of the effect of diclofenac exposure on mussels (*Mytilus galloprovincialis*) metabolome, allowing the identification of a trend in the three main impacted biological pathways, namely down-regulation of tyrosine metabolism, up-regulation of tryptophan metabolism, and up-regulation of hormone biosynthesis pathway.

Principal Component Analysis (PCA) was performed to cluster samples. For annotated metabolites, fold change (FC) values were calculated for fish liver, brain and plasma, at both exposure times (28 and 35 days), according to Ziarrusta et al. (2019) (Equation 1):

$$\text{Fold Change (FC)} = \frac{\text{Average area metabolite in the exposed sample at day 28 or 35}}{\text{Average area metabolite in control sample at day 28 or 35}} \quad \text{(Eq. 1)}$$

The identification of the metabolic pathways potentially affected based on the tentatively identified metabolites was performed using the integrated web-based platform MetaboAnalyst 5.0. Based on the number of metabolites identified belonging to the same pathway and on the role of each metabolite in a specific pathway, a p-value for each metabolic pathway was assessed.

3. Results and Discussion

3.1. Analytical performance

Following a non-target screening approach, a total of 24,265, 15,510, and 12,533 features were detected in fish liver, brain and plasma, respectively, in both positive and negative ionization modes. From these, a total of 3,812, 2,296 and 2,279 features in fish liver, brain and plasma, respectively, showed statistically significant differences between control and exposure to 20 µg/L of venlafaxine, allowing the tentatively identification of 24, 22 and 8 endogenous metabolites in fish liver, brain and plasma, respectively, with confidence level 2 according to the classification defined by Schymanski et al. (2014).

3.2. Impact of venlafaxine in fish metabolome

Changes in the metabolome of juvenile meagre (*A. regius*) exposed to the antidepressant venlafaxine were evaluated after 28 days of exposure and following a period of 7 days (until 35 days), during which the spiking of venlafaxine was stopped. The concentration of venlafaxine in seawater was kept constant through the 28 days of exposure phase (19.2 ± 1.6 µg/L at day 28) (Table S1). However, due to a limitation of the semi-static system used to perform the exposure experiment, it was not possible to completely remove venlafaxine from seawater, since only 25% of the total tank volume was renewed in a daily basis. Additionally, venlafaxine could be excreted by fish as well, since it was shown the ability of meagre to accumulate, metabolize and eliminate venlafaxine (Maulvault et al., 2018b; Santos et al., 2020). Therefore, at the end of the experiment (day 35) it was still detected venlafaxine in the seawater (17.6 ± 1.4 µg/L) (Table S1).

The metabolic fingerprint of exposed fish was assessed separately in different tissues (brain and liver) and in plasma. A Principal Component Analysis (PCA) using all the tentatively identified metabolites was performed to assess the overall effect of venlafaxine on fish metabolome in the different organs (Figure 1). Brain and liver PCAs revealed a clear separation between control and both times (28 and 35 days) (Figure 1A and 1B), while fish plasma PCA only showed a separation between control and exposed fish and no differences were detected between both exposure times (Figure 1C).

Insert Figure 1

In total, 24, 22 and 8 endogenous metabolites, that showed statistically significant differences between control and exposure to venlafaxine, were tentatively identified in fish liver, brain and plasma, respectively, comprising a variety of metabolites of classes such as amino acids,

purines, carboxylic acids and derivatives, fatty acids and hydroxy fatty acids, and lipids (Figure 2 and Table S2). Alterations in the same classes of metabolites were also described in fish species exposed to other antidepressants, such as fluoxetine (Mishra et al., 2017) and amitriptyline (Ziarrusta et al., 2019).

Exposure to venlafaxine showed tissue-dependent variations in the metabolome of fish (Figure 2). After 28 days of exposure, most metabolites (15 out of 22) detected in the brain showed up-regulation, being more pronounced at day 35. On the other hand, an opposite trend was seen in fish liver after 28 days, where most metabolites (14 out of 24) were down-regulated. However, at day 35, there was a reversion in the expression of liver metabolites that revealed a marked up-regulation (21 out of 24). In the case of fish plasma, most metabolites were down-regulated at both times (28 and 35 days). Tissue-dependent variations were also observed in the liver and brain metabolome of gilthead seabream (*Sparus aurata*) exposed to 0.2 µg/L of the antidepressant amitriptyline (Ziarrusta et al., 2019).

Insert Figure 2

The tentatively identified metabolites are involved in different metabolic pathways that may be altered due to the exposure to venlafaxine. Figure 3 shows the most significantly impacted metabolic pathways by the exposure to venlafaxine in the different fish organs. Considering a threshold limit of p-value < 0.05, a pathways analysis using the web-based platform MetaboAnalyst revealed a total of 5 metabolic pathways significantly altered in brain, 1 in liver and 2 in fish plasma (Tables S3-S5), mainly associated with amino acids metabolism, signalling pathway and lipid metabolism. In the brain, aminoacyl-tRNA biosynthesis and several metabolic

pathways involved in the biosynthesis and metabolism of different amino acids, such as phenylalanine, tyrosine, tryptophan, lysine, methionine, arginine, and leucine, were among the most altered metabolic pathways due to the venlafaxine exposure (Table S3). In liver, arginine biosynthesis was the only metabolic pathway that was significantly altered by the exposure to venlafaxine (Table S4), while in fish plasma, the most altered metabolic pathways are involved in lipid metabolism (biosynthesis of unsaturated fatty acids; and linolenic acid metabolism) (Table S5).

Insert Figure 3

3.2.1. Fish brain

The present study showed that fish brain was among the organs with a higher number of endogenous metabolites affected by the exposure to venlafaxine, being noteworthy several amino acids, including some related to the synthesis of neurotransmitters. This is not surprising, since venlafaxine belongs to the family of SNRI antidepressants and acts by inhibiting the reuptake of the neurotransmitters serotonin and norepinephrine from the pre-synaptic nerve cleft (Salahinejad et al., 2022), by targeting transporters of serotonin, norepinephrine and even dopamine, when present at high concentrations (Thompson and Vijayan, 2020). Nevertheless, contrarily to what should be expected, a decrease in brain serotonin levels of fish exposed to venlafaxine (Bisesi Jr et al., 2014; Thompson and Vijayan, 2020) and even to other antidepressants such as fluoxetine (Gaworecki and Klaine, 2008) has been described. In the present study, a down-regulation of tryptophan, the precursor amino acid of serotonin, was observed in meagre brain (Figure 2 and Table S2), which might indicate an increase in serotonin synthesis as an attempt to overcome the

depletion of this neurotransmitter in fish. However, in Thompson and Vijayan (2020) study, the spatial distribution of the gene *tph2*, a key gene encoding a protein essential for the biosynthesis of serotonin in fish brain, was not affected by the exposure of zebrafish embryos to venlafaxine, as well as it was not altered the transcripts abundance of the gene *serta*, which encodes for serotonin transporter that it is inhibited in humans by venlafaxine, leading to a higher content of serotonin in the synaptic cleft. Nevertheless, the expression of the gene *mao* that encodes an enzyme responsible for the breakdown of serotonin, was lower in zebrafish exposed to venlafaxine. Therefore, Thompson and Vijayan (2020) suggested that the decrease in serotonin levels might not be associated with the disruption of serotonin turnover in the brain, but rather to a disruption in its reuptake into nerve fibres.

The amino acids phenylalanine and tyrosine were also altered in meagre brain, showing both up-regulation (Figure 2 and Table S2). Phenylalanine is the precursor of tyrosine, which in turn leads to the formation of dopamine, norepinephrine and epinephrine, neurotransmitters related with different monoaminergic systems where venlafaxine acts, being highly phylogenetically conserved between mammals and fish (Gould et al., 2021). Our results are in agreement with findings of Costa et al. (2021) who described alterations in pathways that regulate dopamine and tyrosine level in the brain of European seabass (*Dicentrarchrus labrax*) exposed to 1 µg/L of venlafaxine. The synthesis of dopamine in the pre-synaptic neuron is reduced due to the action of pre-synaptic dopamine receptors that recognize the concentration of dopamine in the synaptic cleft, which in turn should be increased due to the exposure to venlafaxine (Costa et al., 2021), eventually leading to an increase in its amino acid precursors (phenylalanine and tyrosine) as observed in the present study. Costa et al. (2021) also observed a significant decrease in the expression of dopamine *d2* and *d3* receptors in the brain of venlafaxine exposed fish. An up-

372 regulation of the precursor tyrosine in mice brain was also reported after chronic exposure to
373 paroxetine (Webhofer et al., 2011). Conversely, zebrafish embryos exposed to fluoxetine (12 ng/L
374 to 700 µg/L) showed a down-regulation of phenylalanine and tyrosine (Mishra et al., 2017). These
375 contradictory responses may be attributed either to differences in the developmental stage of both
376 species and/or to species-specific responses to antidepressants exposure.

377 The amino acid arginine was also up-regulated in fish brain after exposure to venlafaxine
378 (Figure 2 and Table S2). Antidepressants can induce their effect by inhibiting the synthesis of nitric
379 oxide (Wigner et al., 2020), a by-product of the transformation of arginine to citrulline by the
380 enzyme nitric oxide synthase (NOS). Antidepressants might bind to this enzyme, preventing the
381 formation of nitric oxide (Ziarrusta et al., 2019), and leading to an increase in arginine levels in
382 the brain, as seen in this study. Ziarrusta et al. (2019) also observed alterations in arginine level in
383 the brain of gilthead seabream (*Sparus aurata*) exposed to amitriptyline. Likewise, venlafaxine
384 induced a decrease in the generation of nitric oxide in the brain of rats by reducing the *NOS1*
385 mRNA expression in the brain (Wigner et al., 2020).

386 Finally, up-regulation of vitamin choline in fish brain was observed as a consequence of
387 venlafaxine exposure (Figure 2 and Table S2). Antidepressants may affect the activity of
388 acetylcholinesterase (AChE), which is an enzyme responsible for hydrolysing the neurotransmitter
389 acetylcholine into acetic acid and choline in the synaptic cleft and neuromuscular junctions
390 (Ziegler et al., 2020), by either inhibiting (Duarte et al., 2019) or inducing (Xie et al., 2015) AChE
391 activity in fish. An increase in choline levels in fish brain exposed to venlafaxine might indicate a
392 potential increase in the AChE activity. However, while two previous studies did not observe a
393 significant effect in AChE activity in the brain of fish exposed to venlafaxine via water
394 (concentrations ranging from 1 to 1000 µg/L) (Maulvault et al., 2019; Ziegler et al., 2020), another

study reported a significant increase in AChE activity in the brain of fish exposed to venlafaxine via contaminated feed (concentration = 161.7 ± 17.1 ng/g, dw) (Maulvault et al., 2019).

3.2.2. Fish liver

Although the main mechanism of action of antidepressants like venlafaxine is associated with the dysregulation of monoaminergic systems, especially the serotonergic one in brain, serotonin can also be involved in different signalling pathways outside the brain. Antidepressants can inhibit serotonin transporters existing in peripheral fish tissues, increasing circulatory levels of serotonin, which may alter several physiological processes (Salahinejad et al., 2022). However, the mechanisms involved in modifications of non-brain serotonin levels are not clearly understood, and further research is still needed. In fish, the synthesis of serotonin predominantly occurs in liver, followed by brain (Salahinejad et al., 2022); thus, it was not surprising that levels of its precursor, tryptophan, were also altered in fish liver (Figure 2 and Table S2). A down-regulation of tryptophan was observed in liver during meagre exposure to venlafaxine, indicating a possible increase in serotonin production. However, at the end of the experiment (day 35), this trend was reversed, and tryptophan become up-regulated (Figure 2 and Table S2). The amino acid tyrosine that is the precursor of different neurotransmitters, including epinephrine (also known as adrenalin), was also down-regulated in liver (Figure 2 and Table S2), indicating a possible decrease in the noradrenergic transmission (Webhofer et al., 2011). This could be attributed to the fact that venlafaxine might also act as beta-blocker by blocking the β-adrenoceptor signalling in hepatocytes, reducing the epinephrine-stimulated glucose production and interfering with energy metabolism (Ings et al., 2012). Arginine also showed a similar trend to tryptophan, i.e., it was down-regulated in fish liver at day 28, whereas was up-regulated at the end of the experiment (day

35), but in this last case the difference was not statistically significant relatively to the control (Figure 2 and Table S2). Moreover, arginine biosynthesis was the only metabolic pathway significantly affected in meagre liver (Figure 3 and Table S4). These results suggest that arginine synthesis was reduced, lowering its level in liver and, consequently, decreasing the enzymatic generation of nitric oxide.

3.2.3. Fish plasma

Venlafaxine enhances the generation of reactive oxygen species (ROS) in fish, inducing oxidative stress (Ziegler et al., 2020), a common adverse effect due to the exposure to xenobiotics. In a previous study, Maulvault et al. (2019) described changes in the response of several oxidative stress biomarkers of meagre exposed to venlafaxine, namely the enhancement of catalase (in liver) and glutathione-S-transferase (in muscle) activities, which are enzymes usually associated to the antioxidant defence system of organisms to overcome the formation of ROS induced by stressors. In addition, an increase in lipid peroxidation was reported in different fish tissues, including liver and brain. In the current study, most altered metabolites detected in fish plasma were related to lipid metabolism (Figure 2 and 3, Table S2), suggesting the occurrence of oxidative stress. In general, lipid metabolites were down-regulated in fish plasma. Changes in the expression of genes related to lipid metabolism were also reported in the brains of European seabass (Costa et al., 2021) and zebrafish larvae (Rodrigues et al., 2020) exposed to venlafaxine. Alterations in lipid metabolism were also described in fish exposed to other antidepressants such as amitriptyline (Ziarrusta et al., 2019). Other effects of oxidative stress caused by the exposure to venlafaxine were also observed in fish brain, particularly a down-regulation of oxidized glutathione (Figure 2 and Table S2). Low levels of oxidized glutathione might impact the production of reduced

glutathione by decreasing the substrate of glutathione reductase, potentiating the risk of lipid peroxidation due to a decrease in the production of reduced glutathione (Dringen, 2000).

4. Conclusions

Understanding the effect of environmental stressors such as venlafaxine in fish metabolome can be a very challenging task, since different tissues show distinct biomarkers response and trends. This is related to the fact that antidepressants act in different receptors, which may be differently expressed in cells and organs, thus exerting varying influence on a variety of biochemical, physiological and behavioural processes associated with basic functions of fish (Rodrigues et al., 2020; Gould et al., 2021). Using non-target metabolomics, it was possible to identify changes in endogenous metabolites of fish exposed to venlafaxine, revealing that brain, liver and plasma possess different metabolic profiles, which can be related to their sensitivity to venlafaxine and the different physiological functions they are involved. Due to the mechanism of action of SNRI antidepressants, a high impact in brain metabolome was observed, since venlafaxine dysregulates the levels of serotonin, which is the most widely distributed neurotransmitter in brain. Additionally, phenylalanine, tyrosine and tryptophan biosynthesis were among the most significantly altered pathways in brain, indicating a significant impact on the nervous system. The exposure of meagre to venlafaxine caused perturbations in several metabolites that are related to the synthesis, availability and expression of neurotransmitters (e.g., serotonin, dopamine, epinephrine) that can affect fish behaviour, as it was shown in a previous study (Maulvault et al., 2018a). Thus, amino acids related to the synthesis of neurotransmitters, such as tryptophan, tyrosine, or phenylalanine, that showed to be dysregulated in fish tissues, can be further used as biomarkers of venlafaxine exposure and impact. Assessing the alterations in

464 specific biochemical pathways in an early stage of development of fish can be crucial to prevent
465 irreversible damage of their biological systems and to guarantee their survival. Furthermore,
466 differences in the developmental stages of fish and species-specific response to the contaminant
467 might also affect fish metabolome and should be considered in further studies.
468

Figure Captions

Figure 1 – Principal component analysis score plots and component loadings for fish A) brain, B) liver, and C) plasma, using all the tentatively identified metabolites statistically significant ($p < 0.1$). Each dot represents a composite sample corresponding to a pool of 5 individuals.

Figure 2 – Fold change values, expressed as log2, of the endogenous metabolites significantly changed in fish brain (A), liver (B) and plasma (C) after exposure to venlafaxine (p-value < 0.1). Identity of the metabolites is indicated in the *y* axis of each graphic.

Figure 3 – Most relevant impacted metabolic pathways due to the exposure to venlafaxine in fish: A) brain; B) liver; and C) plasma. 1 – Aminoacyl t-RNA biosynthesis; 2 - Arginine and proline metabolism; 3 - Phenylalanine, tyrosine and tryptophan biosynthesis; 4 - Phenylalanine metabolism; 5 - Arginine biosynthesis; 6 - Biosynthesis of unsatured fatty acids; 7 - Linoleic acid metabolism. Only the pathways with a p-value < 0.05 are identified in the figure. Bubble area is proportional to the impact of each pathway, with color denoting the significance from highest in red to lowest in white.

Ethical statement

Fish trials were conducted according to legal regulations (EU Directive 2010/63) and approved by the Ethical Committee of the Faculty of Sciences of the Lisbon University, overseen by the Portuguese National Competent Authority (Direção-Geral de Alimentação e Veterinária, DGAV). All researchers and technicians involved in the maintenance, handling and sampling of live animals were certified in Laboratory Animal Sciences by the Federation of European Laboratory Animal Science Associations (FELASA).

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References

Best, C., Melnyk-Lamont, N., Gesto, M., Vijayan, M.M., 2014. Environmental levels of the antidepressant venlafaxine impact the metabolic capacity of rainbow trout. Aquat. Toxicol. 155, 190-198. http://doi.org/10.1016/j.aquatox.2014.06.014

Bisesi Jr, J.H., Bridges, W., Klaine, S.J., 2014. Effects of the antidepressant venlafaxine on fish brain serotonin and predation behavior. Aquat. Toxicol. 148, 130-138. http://doi.org/10.1016/j.aquatox.2013.12.033

Bodin, N., Tapie, N., Le Ménach, K., Chassot, E., Elie, P., Rochard, E., Budzinski, H., 2014. PCB contamination in fish community from the Gironde Estuary (France): Blast from the past. Chemosphere 98, 66-72. https://doi.org/10.1016/j.chemosphere.2013.10.003

Bonnefille, B., Gomez, E., Alali, M., Rosain, D., Fenet, H., Courant, F., 2018. Metabolomics assessment of the effects of diclofenac exposure on *Mytilus galloprovincialis*: Potential effects on osmoregulation and reproduction. Sci. Total Environ. 613-614, 611-618. https://doi.org/10.1016/j.scitotenv.2017.09.146

Boulard, L., Dierkes, G., Schlüsener, M.P., Wick, A., Koschorreck, J., Ternes, T.A., 2020. Spatial distribution and temporal trends of pharmaceuticals sorbed to suspended particulate matter of German rivers. Water Res. 171, 115366. https://doi.org/10.1016/j.watres.2019.115366

Brew, D.W., Black, M.C., Santos, M., Rodgers, J., Henderson, W.M., 2020. Metabolomic Investigations of the Temporal Effects of Exposure to Pharmaceuticals and Personal Care Products and Their Mixture in the Eastern Oyster (*Crassostrea virginica*). Environ. Toxicol. Chem. 39, 419-436. 10.1002/etc.4627

Čelić, M., Gros, M., Farré, M., Barceló, D., Petrović, M., 2019. Pharmaceuticals as chemical markers of wastewater contamination in the vulnerable area of the Ebro Delta (Spain). Sci. Total Environ. 652, 952-963. https://doi.org/10.1016/j.scitotenv.2018.10.290

Costa, C., Semedo, M., Machado, S.P., Cunha, V., Ferreira, M., Urbatzka, R., 2021. Transcriptional analyses reveal different mechanism of toxicity for a chronic exposure to fluoxetine and venlafaxine on the brain of the marine fish *Dicentrarchrus labrax*. Comp. Biochem. Physiol. Part - C: Toxicol. Pharmacol. 250, 109170. https://doi.org/10.1016/j.cbpc.2021.109170

Courant, F., Antignac, J.-P., Dervilly-Pinel, G., Le Bizec, B., 2014. Basics of mass spectrometry based metabolomics. Proteomics 14, 2369-2388. 10.1002/pmic.201400255

David, A., Lange, A., Tyler, C.R., Hill, E.M., 2018. Concentrating mixtures of neuroactive pharmaceuticals and altered neurotransmitter levels in the brain of fish exposed to a wastewater effluent. Sci. Total Environ. 621, 782-790. https://doi.org/10.1016/j.scitotenv.2017.11.265

Dringen, R., 2000. Metabolism and functions of glutathione in brain. Progr. Neurobiol. 62, 649-671. https://doi.org/10.1016/S0301-0082(99)00060-X

Duarte, I.A., Pais, M.P., Reis-Santos, P., Cabral, H.N., Fonseca, V.F., 2019. Biomarker and behavioural responses of an estuarine fish following acute exposure to fluoxetine. Mar. Environ. Res. 147, 24-31. https://doi.org/10.1016/j.marenvres.2019.04.002

FAO, 2022. Cultured Aquatic Species Information Programme: *Argyrosomus regius* (Asso, 1801). Available in: https://www.fao.org/fishery/en/culturedspecies/argyrosomus_regius/en (accessed in 06/12/2022).

Fernández-Rubio, J., Rodríguez-Gil, J.L., Postigo, C., Mastroianni, N., López de Alda, M., Barceló, D., Valcárcel, Y., 2019. Psychoactive pharmaceuticals and illicit drugs in coastal waters

549 of North-Western Spain: Environmental exposure and risk assessment. Chemosphere 224, 379-
550 389. https://doi.org/10.1016/j.chemosphere.2019.02.041
551 Galus, M., Kirischian, N., Higgins, S., Purdy, J., Chow, J., Rangaranjan, S., Li, H., Metcalfe, C.,
552 Wilson, J.Y., 2013. Chronic, low concentration exposure to pharmaceuticals impacts multiple
553 organ systems in zebrafish. Aquat. Toxicol. 132, 200-211. 10.1016/j.aquatox.2012.12.021
554 Gaworecki, K.M., Klaine, S.J., 2008. Behavioral and biochemical responses of hybrid striped bass
555 during and after fluoxetine exposure. Aquat. Toxicol. 88, 207-213.
556 https://doi.org/10.1016/j.aquatox.2008.04.011
557 Golovko, O., Örn, S., Sörengård, M., Frieberg, K., Nassazzi, W., Lai, F.Y., Ahrens, L., 2021.
558 Occurrence and removal of chemicals of emerging concern in wastewater treatment plants and
559 their impact on receiving water systems. Sci. Total Environ. 754, 142122.
560 https://doi.org/10.1016/j.scitotenv.2020.142122
561 Gould, S.L., Winter, M.J., Norton, W.H.J., Tyler, C.R., 2021. The potential for adverse effects in
562 fish exposed to antidepressants in the aquatic environment. Environ. Sci. Technol.
563 10.1021/acs.est.1c04724
564 Grabicova, K., Grabic, R., Fedorova, G., Fick, J., Cerveny, D., Kolarova, J., Turek, J., Zlabek, V.,
565 Randak, T., 2017. Bioaccumulation of psychoactive pharmaceuticals in fish in an effluent
566 dominated stream. Water Res. 124, 654-662. https://doi.org/10.1016/j.watres.2017.08.018
567 Huerta, B., Rodriguez-Mozaz, S., Lazorchak, J., Barcelo, D., Batt, A., Wathen, J., Stahl, L., 2018.
568 Presence of pharmaceuticals in fish collected from urban rivers in the U.S. EPA 2008–2009
569 National Rivers and Streams Assessment. Sci. Total Environ. 634, 542-549.
570 https://doi.org/10.1016/j.scitotenv.2018.03.387

571 Ings, J.S., George, N., Peter, M.C.S., Servos, M.R., Vijayan, M.M., 2012. Venlafaxine and atenolol
572 disrupt epinephrine-stimulated glucose production in rainbow trout hepatocytes. Aquat. Toxicol.
573 106-107, 48-55. https://doi.org/10.1016/j.aquatox.2011.10.006
574 Lopez, F.J., Pitarch, E., Botero-Coy, A.M., Fabregat-Safont, D., Ibáñez, M., Marin, J.M., Peruga,
575 A., Ontañón, N., Martínez-Morcillo, S., Olalla, A., Valcárcel, Y., Varó, I., Hernández, F., 2022.
576 Removal efficiency for emerging contaminants in a WWTP from Madrid (Spain) after secondary
577 and tertiary treatment and environmental impact on the Manzanares River. Sci. Total Environ. 812,
578 152567. https://doi.org/10.1016/j.scitotenv.2021.152567
579 Magalhães, P., Alves, G., Llerena, A., Falcão, A., 2014. Venlafaxine pharmacokinetics focused on
580 drug metabolism and potential biomarkers. Drug Metabol. Drug Interact. 29, 129-141. DOI
581 10.1515/dmdi-2013-0053
582 Maulvault, A.L., Camacho, C., Barbosa, V., Alves, R., Anacleto, P., Pousão-Ferreira, P., Rosa, R.,
583 Marques, A., Diniz, M.S., 2019. Living in a multi-stressors environment: An integrated biomarker
584 approach to assess the ecotoxicological response of meagre (*Argyrosomus regius*) to venlafaxine,
585 warming and acidification. Environ. Res. 169, 7-25. https://doi.org/10.1016/j.envres.2018.10.021
586 Maulvault, A.L., Santos, L.H.M.L.M., Paula, J.R., Camacho, C., Pissarra, V., Fogaça, F., Barbosa,
587 V., Alves, R., Ferreira, P.P., Barceló, D., Rodriguez-Mozaz, S., Marques, A., Diniz, M., Rosa, R.,
588 2018a. Differential behavioural responses to venlafaxine exposure route, warming and
589 acidification in juvenile fish (*Argyrosomus regius*). Sci. Total Environ. 634, 1136-1147.
590 https://doi.org/10.1016/j.scitotenv.2018.04.015
591 Maulvault, A.L., Santos, L.H.M.L.M., Camacho, C., Anacleto, P., Barbosa, V., Alves, R., Pousão
592 Ferreira, P., Serra-Compte, A., Barceló, D., Rodriguez-Mozaz, S., Rosa, R., Diniz, M., Marques,
593 A., 2018b. Antidepressants in a changing ocean: Venlafaxine uptake and elimination in juvenile

fish (*Argyrosomus regius*) exposed to warming and acidification conditions. Chemosphere 209, 286-297. https://doi.org/10.1016/j.chemosphere.2018.06.004

Mishra, P., Gong, Z., Kelly, B.C., 2017. Assessing biological effects of fluoxetine in developing zebrafish embryos using gas chromatography-mass spectrometry based metabolomics. Chemosphere 188, 157-167. https://doi.org/10.1016/j.chemosphere.2017.08.149

Moreno-González, R., Rodríguez-Mozaz, S., Huerta, B., Barceló, D., León, V.M., 2016. Do pharmaceuticals bioaccumulate in marine molluscs and fish from a coastal lagoon? Environ. Res. 146, 282-298. http://doi.org/10.1016/j.envres.2016.01.001

Painter, M.M., Buerkley, M.A., Julius, M.L., Vajda, A.M., Norris, D.O., Barber, L.B., Furlong, E.T., Schultz, M.M., Schoenfuss, H.L., 2009. Antidepressants at Environmentally Relevant Concentrations Affect Predator Avoidance Behavior of Larval Fathead Minnows (*Pimephales Promelas*). Environ. Toxicol. Chem. 28, 2677-2684. 10.1897/08-556.1

Parrott, J.L., Metcalfe, C.D., 2017. Assessing the effects of the antidepressant venlafaxine to fathead minnows exposed to environmentally relevant concentrations over a full life cycle. Environ. Pollut. 229, 403-411. https://doi.org/10.1016/j.envpol.2017.06.009

Ramirez, G., Gomez, E., Dumas, T., Rosain, D., Mathieu, O., Fenet, H., Courant, F., 2022. Early Biological Modulations Resulting from 1-Week Venlafaxine Exposure of Marine Mussels *Mytilus galloprovincialis* Determined by a Metabolomic Approach. Metabolites 12, 197. 10.3390/metabo12030197

Rodrigues, P., Cunha, V., Oliva-Teles, L., Ferreira, M., Guimarães, L., 2020. Norfluoxetine and venlafaxine in zebrafish larvae: Single and combined toxicity of two pharmaceutical products relevant for risk assessment. J. Hazard. Mat. 400, 123171. https://doi.org/10.1016/j.jhazmat.2020.123171

Rojo, M., Álvarez-Muñoz, D., Dománico, A., Foti, R., Rodriguez-Mozaz, S., Barceló, D., Carriquiriborde, P., 2019. Human pharmaceuticals in three major fish species from the Uruguay River (South America) with different feeding habits. Environ. Pollut. 252, 146-154. https://doi.org/10.1016/j.envpol.2019.05.099

Salahinejad, A., Attaran, A., Meuthen, D., Chivers, D.P., Niyogi, S., 2022. Proximate causes and ultimate effects of common antidepressants, fluoxetine and venlafaxine, on fish behavior. Sci. Total Environ. 807, 150846. https://doi.org/10.1016/j.scitotenv.2021.150846

Santos, L.H.M.L.M., Araujo, A.N., Fachini, A., Pena, A., Delerue-Matos, C., Montenegro, M.C.B.S.M., 2010. Ecotoxicological aspects related to the presence of pharmaceuticals in the aquatic environment. J. Hazard. Mat. 175, 45-95. 10.1016/j.jhazmat.2009.10.100

Santos, L.H.M.L.M., Maulvault, A.L., Jaén-Gil, A., Marques, A., Barceló, D., Rodríguez-Mozaz, S., 2020. Insights on the metabolization of the antidepressant venlafaxine by meagre (*Argyrosomus regius*) using a combined target and suspect screening approach. Sci. Total Environ. 737, 140226. https://doi.org/10.1016/j.scitotenv.2020.140226

Schymanski, E.L., Jeon, J., Gulde, R., Fenner, K., Ruff, M., Singer, H.P., Hollender, J., 2014. Identifying Small Molecules via High Resolution Mass Spectrometry: Communicating Confidence. Environ. Sci. Technol. 48, 2097-2098. 10.1021/es5002105

Serra-Compte, A., Álvarez-Muñoz, D., Solé, M., Cáceres, N., Barceló, D., Rodríguez-Mozaz, S., 2019. Comprehensive study of sulfamethoxazole effects in marine mussels: Bioconcentration, enzymatic activities and metabolomics. Environ. Res. 173, 12-22. https://doi.org/10.1016/j.envres.2019.03.021

Tang, Y., Fan, Z., Yang, M., Zhang, S., Li, M., Fang, Y., Li, J., Feng, X., 2022. Low concentrations of the antidepressant venlafaxine affect courtship behaviour and alter serotonin and dopamine

systems in zebrafish (*Danio rerio*). Aquat. Toxicol. 244, 106082. https://doi.org/10.1016/j.aquatox.2022.106082

Thompson, W.A., Vijayan, M.M., 2020. Zygotic Venlafaxine Exposure Impacts Behavioral Programming by Disrupting Brain Serotonin in Zebrafish. Environ. Sci. Technol. 54, 14578-14588. 10.1021/acs.est.0c06032

Voloshenko-Rossin, A., Gasser, G., Cohen, K., Gun, J., Cumbal-Flores, L., Parra-Morales, W., Sarabia, F., Ojeda, F., Lev, O., 2015. Emerging pollutants in the Esmeraldas watershed in Ecuador: discharge and attenuation of emerging organic pollutants along the San Pedro–Guayllabamba–Esmeraldas rivers. Environ Sci Process Impacts 17, 41-53. 10.1039/c4em00394b

Webhofer, C., Gormanns, P., Tolstikov, V., Zieglgänsberger, W., Sillaber, I., Holsboer, F., Turck, C.W., 2011. Metabolite profiling of antidepressant drug action reveals novel drug targets beyond monoamine elevation. Transl. Psychiatry 1, e58-e58. 10.1038/tp.2011.56

Wigner, P., Synowiec, E., Czarny, P., Bijak, M., Jóźwiak, P., Szemraj, J., Gruca, P., Papp, M., Śliwiński, T., 2020. Effects of venlafaxine on the expression level and methylation status of genes involved in oxidative stress in rats exposed to a chronic mild stress. J. Cell. Mol. Med. 24, 5675-5694. https://doi.org/10.1111/jcmm.15231

Wilkinson, J.L., Boxall, A.B.A., Kolpin, D.W., Leung, K.M.Y., Lai, R.W.S., Galbán-Malagón, C., Adell, A.D., Mondon, J., Metian, M., Marchant, R.A., Bouzas-Monroy, A., Cuni-Sanchez, A., Coors, A., Carriquiriborde, P., Rojo, M., Gordon, C., Cara, M., Moermond, M., Luarte, T., Petrosyan, V., Perikhanyan, Y., Mahon, C.S., McGurk, C.J., Hofmann, T., Kormoker, T., Iniguez, V., Guzman-Otazo, J., Tavares, J.L., Gildasio De Figueiredo, F., Razzolini, M.T.P., Dougnon, V., Gbaguidi, G., Traoré, O., Blais, J.M., Kimpe, L.E., Wong, M., Wong, D., Ntchantcho, R., Pizarro, J., Ying, G.-G., Chen, C.-E., Páez, M., Martínez-Lara, J., Otamonga, J.-P., Poté, J., Ifo, S.A.,

Wilson, P., Echeverría-Sáenz, S., Udikovic-Kolic, N., Milakovic, M., Fatta-Kassinos, D., Ioannou-Ttofa, L., Belušová, V., Vymazal, J., Cárdenas-Bustamante, M., Kassa, B.A., Garric, J., Chaumot, A., Gibba, P., Kunchulia, I., Seidensticker, S., Lyberatos, G., Halldórsson, H.P., Melling, M., Shashidhar, T., Lamba, M., Nastiti, A., Supriatin, A., Pourang, N., Abedini, A., Abdullah, O., Gharbia, S.S., Pilla, F., Chefetz, B., Topaz, T., Yao, K.M., Aubakirova, B., Beisenova, R., Olaka, L., Mulu, J.K., Chatanga, P., Ntuli, V., Blama, N.T., Sherif, S., Aris, A.Z., Looi, L.J., Niang, M., Traore, S.T., Oldenkamp, R., Ogunbanwo, O., Ashfaq, M., Iqbal, M., Abdeen, Z., O'Dea, A., Morales-Saldaña, J.M., Custodio, M., de la Cruz, H., Navarrete, I., Carvalho, F., Gogra, A.B., Koroma, B.M., Cerkvenik-Flajs, V., Gombač, M., Thwala, M., Choi, K., Kang, H., Ladu, J.L.C., Rico, A., Amerasinghe, P., Sobek, A., Horlitz, G., Zenker, A.K., King, A.C., Jiang, J.-J., Kariuki, R., Tumbo, M., Tezel, U., Onay, T.T., Lejju, J.B., Vystavna, Y., Vergeles, Y., Heinzen, H., Pérez-Parada, A., Sims, D.B., Figy, M., Good, D., Teta, C., 2022. Pharmaceutical pollution of the world's rivers. PNAS 119, e2113947119. 10.1073/pnas.2113947119

Xie, Z., Lu, G., Li, S., Nie, Y., Ma, B., Liu, J., 2015. Behavioral and biochemical responses in freshwater fish *Carassius auratus* exposed to sertraline. Chemosphere 135, 146-155. https://doi.org/10.1016/j.chemosphere.2015.04.031

Ziarrusta, H., Ribbenstedt, A., Mijangos, L., Picart-Armada, S., Perera-Lluna, A., Prieto, A., Izagirre, U., Benskin, J.P., Olivares, M., Zuloaga, O., Etxebarria, N., 2019. Amitriptyline at an Environmentally Relevant Concentration Alters the Profile of Metabolites Beyond Monoamines in Gilt-Head Bream. Environ. Toxicol. Chem. 38, 965-977. 10.1002/etc.4381

Ziegler, M., Eckstein, H., Ottmann, S., Reinelt, L., Stepinski, S., Köhler, H.-R., Triebskorn, R., 2020. Biochemical and cellular biomarkers in brown trout (*Salmo trutta* f. *fario*) in response to the

antidepressants citalopram and venlafaxine. Environ. Sci. Eur. 32, 158. 10.1186/s12302-020-00437-z

Figure 1

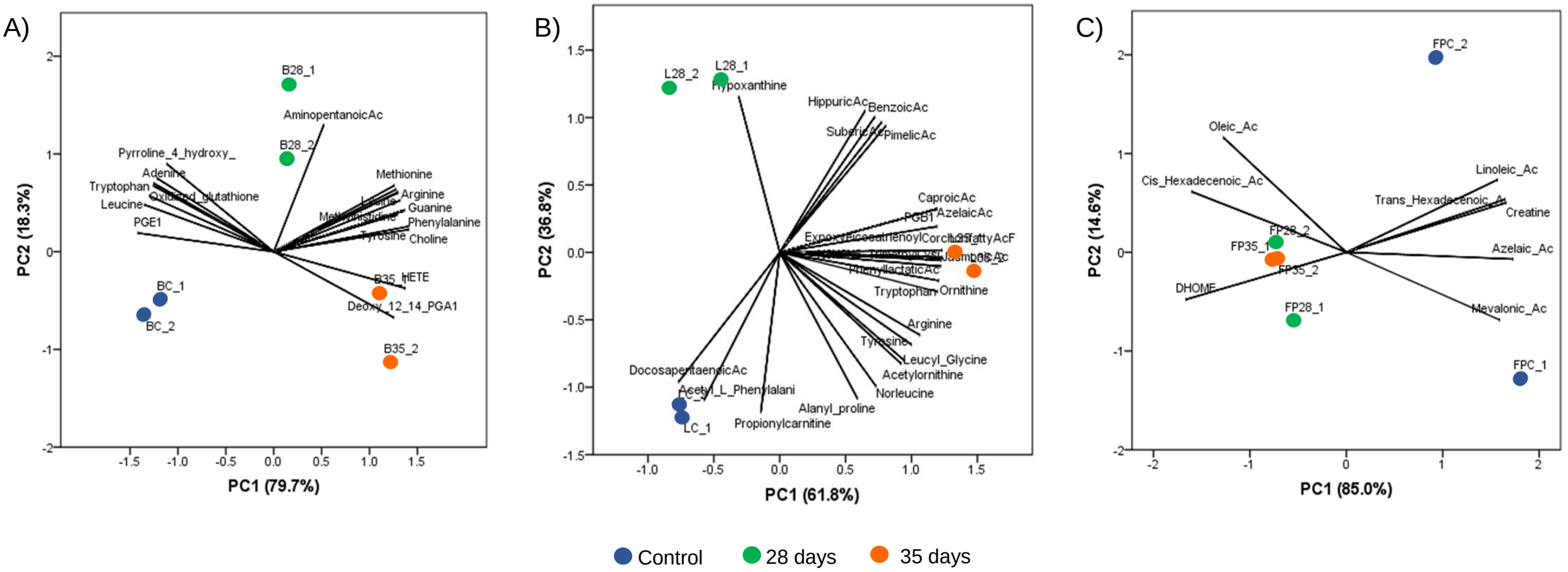

A)
B28_1
AminopentanoicAc
B28_2
Pyrroline_4_hydroxy_
Adenine
Tryptophan
Leucine
PGE1
Methionine
Arginine
Guanine
Phenylalanine
Choline
BC_1
BC_2
B35_HETE
Deoxy_12_14_PGA1
B35_2
PC2 (18.3%)
PC1 (79.7%)
B)
L28_2
L28_1
Hypoxanthine
HippuricAc
BenzoicAc
SubericAc
PimelicAc
CaproicAc
AzelaicAc
PGB1
Phenyllactic Ac
Tryptophan
Ornithine
Arginine
Tyrosine
Leucyl_Glycine
Acetylornithine
Norleucine
Alanyl_proline
Propionylcarnitine
DocosapentaenoicAc
Acetyl_L_Phenylalani
LC_2
LC_1
PC2 (36.8%)
PC1 (61.8%)
C)
FPC_2
Oleic_Ac
Cis_Hexadecenoic_Ac
Linoleic_Ac
Trans_Hexadecenoic_A
Creatine
FP28_2
FP35_1
FP35_2
Azelaic_Ac
DHOME
FP28_1
Mevalonic_Ac
FPC_1
PC2 (14.6%)
PC1 (85.0%)
Control
28 days
35 days

Figure 2

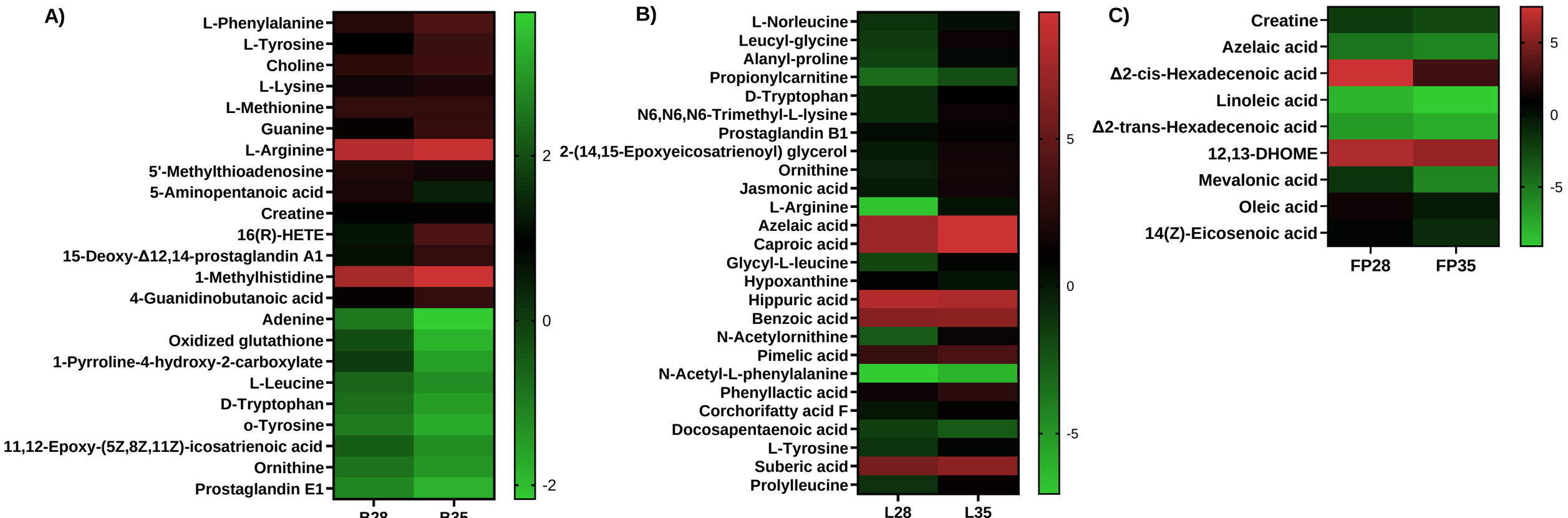
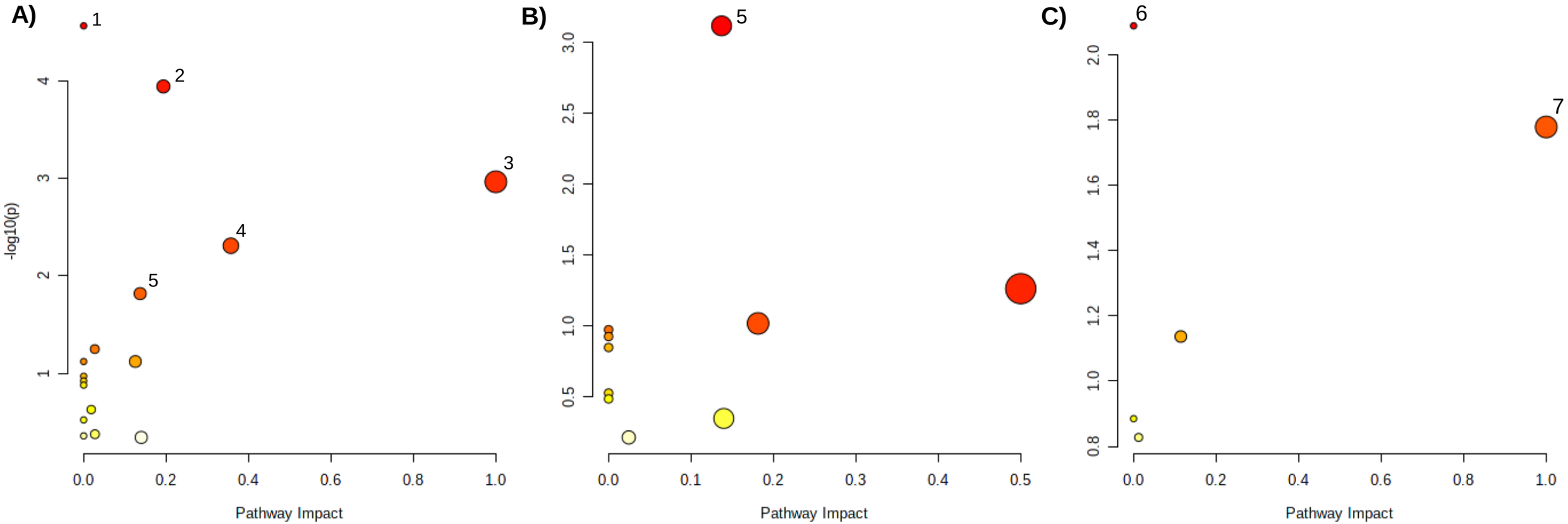

A)
L-Phenylalanine
L-Tyrosine
Choline
L-Lysine
L-Methionine
Guanine
L-Arginine
5'-Methylthioadenosine
5-Aminopentanoic acid
Creatine
16(R)-HETE
15-Deoxy-Δ12,14-prostaglandin A1
1-Methylhistidine
4-Guanidinobutanoic acid
Adenine
Oxidized glutathione
1-Pyrroline-4-hydroxy-2-carboxylate
L-Leucine
D-Tryptophan
o-Tyrosine
11,12-Epoxy-(5Z,8Z,11Z)-icosatrienoic acid
Ornithine
Prostaglandin E1
2
0
-2
B28
B35
B)
L-Norleucine
Leucyl-glycine
Alanyl-proline
Propionylcarnitine
D-Tryptophan
N6,N6,N6-Trimethyl-L-lysine
Prostaglandin B1
2-(14,15-Epoxyeicosatrienoyl) glycerol
Ornithine
Jasmonic acid
L-Arginine
Azelaic acid
Caproic acid
Glycyl-L-leucine
Hypoxanthine
Hippuric acid
Benzoic acid
N-Acetylornithine
Pimelic acid
N-Acetyl-L-phenylalanine
Phenyllactic acid
Corchorifatty acid F
Docosapentaenoic acid
L-Tyrosine
Suberic acid
Prolylleucine
5
0
-5
L28
L35
C)
Creatine
Azelaic acid
Δ2-cis-Hexadecenoic acid
Linoleic acid
Δ2-trans-Hexadecenoic acid
12,13-DHOME
Mevalonic acid
Oleic acid
14(Z)-Eicosenoic acid
FP28
FP35
5
0
-5

Figure 3


A)
B)
C)
1
2
3
4
5
6
7
-log10(p)
Pathway Impact
Pathway Impact
Pathway Impact