Capillary electrophoresis-mass spectrometry based metabolomics approaches for volume-restricted applications

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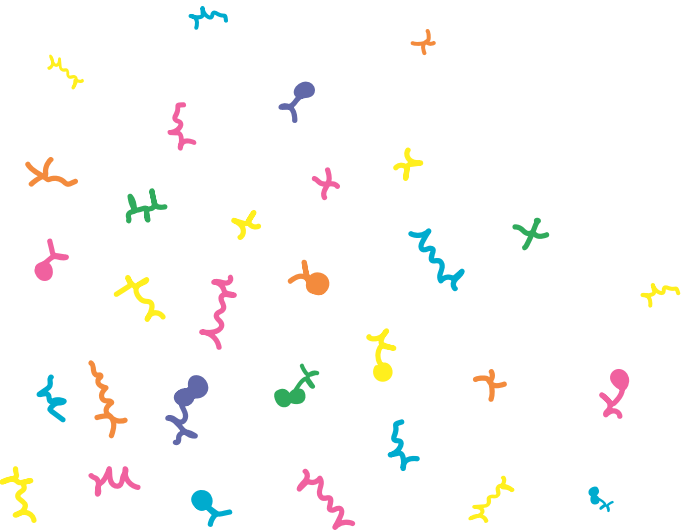

Chapter 6

Probing the effects of cortisol and specific receptor involvement in zebrafish larvae using a CE-MS metabolomics workflow

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Abstract

The zebrafish (Danio rerio) is increasingly used as a model system in biomedical research. Zebrafish embryos and larvae can fill the gap between cultured cells and mammalian animal models, because they can be obtained in large numbers, are small in size and can easily be manipulated genetically. Given that zebrafish larvae are intrinsically biomass-limited samples, we have developed a capillary electrophoresis-mass spectrometry (CE-MS) workflow for the profiling of (endogenous) metabolites in extracts from individual zebrafish larvae and pools of small numbers of larvae. The developed CE-MS workflow was used to analyse the metabolite levels in extracts from pools of 1, 2, 4, 8, 12, 16, 20 and 40 zebrafish larvae, and for six selected endogenous metabolites a linear response ($R2>0.98$) was obtained in extracts from these pools. The repeatability was satisfactory, with inter-day RSD values for peak area of 9.4-17.7% for biological replicates (n=3 over three days). Furthermore, the method allowed analysis of over 70 endogenous metabolites in a pool of 12 zebrafish larvae, and 29 endogenous metabolites in only an extract from one zebrafish larva. The CE-MS workflow was used to study the role of the mineralocorticoid receptor in mediating the effects of cortisol in wild-type (WT) and ubiquitous MR-knockout (MRKO) zebrafish larvae model systems.

Introduction

Stress can trigger a cascade of stress hormones that produce physiological changes via a multitude of metabolic pathways. The exact biological response to a stressor is highly individualized, as it depends on factors such as genetic background, biological age and personality [293]. In this context, metabolomics, i.e., the analysis of (endogenous) small molecules within a biological system, could be considered a valuable approach to gain a deeper understanding of the molecular responses occurring after exposure to a stressor. The primary stress hormone in humans is cortisol, which is mediated by two nuclear receptors, the glucocorticoid receptor (GR) and the mineralocorticoid receptor (MR). These receptors have different affinities for cortisol, with GR being activated at high cortisol levels, and MR being already activated at basal cortisol levels. While the role of cortisol-GR signaling during stress is well characterized [294], the role of MR on mediating the effects of cortisol is less known [295, 296].

Zebrafish (*Danio rerio*) are increasingly used as a model system in biomedical research, mainly because of their genetic and cellular homology with mammals [297]. Other advantages of this species include their rapid development (zebrafish larvae have functioning organs and a complex behavioral repertoire a few days post-fertilization) and high production of offspring (100-200 eggs), allowing for high throughput studies [298, 299]. Furthermore, zebrafish allow to fill the gap between *in vitro* studies and rodent models in predictive animal model studies of human physiology [300]. Within the last decade, zebrafish have proven to be a very effective stress-model [301, 302].

To study metabolism in zebrafish larvae, a number of analytical techniques have been considered, with chromatographic based techniques coupled to MS being the most popular, including RPLC-MS [303], HILIC-MS [304] and GC-MS [305]. Additionally, direct infusion MS [298] and NMR spectroscopy [306] have been used. Given that zebrafish larvae are intrinsically material-limited samples, capillary electrophoresis-(CE)-MS is a very suitable technique for zebrafish larvae analysis, mainly due to its microscale character, low sample consumption and complementary separation mechanism towards chromatographic techniques. A few years ago, the potential of CE-MS was already demonstrated for metabolic profiling of zebrafish larvae by our group [307], using a flow-through micro vial CE-MS interface for the analysis of an extract from a single zebrafish larva (48 h post fertilization). However, only a few metabolites could be detected with that approach and the coupling of CE to MS via this interface was quite complicated; moreover, the reproducibility of this method for metabolomics studies has not been demonstrated so far. In comparison, the use of a conventional co-axial sheath-liquid interface for coupling

CE to MS has proven to be robust and reproducible for metabolomics studies, as shown in a recent Metabo-ring study [271]. Shanmuganathan and co-workers have developed a robust sheath-liquid CE-MS-based workflow for metabolic profiling of more than 1000 serum samples from a multi-ethnic cohort of pregnant women with an acceptable precision [308]. Harada and co-workers used sheath-liquid CE–MS for the comprehensive profiling of charged and polar metabolites in more than 8000 human plasma samples from the Tsuruoka Metabolomics Cohort Study over a 52-month period [140]. In our group we have recently demonstrated that sheath-liquid CE-MS is well-suited for the efficient and sensitive profiling of polar ionogenic metabolites in various volume-restricted biological samples, demonstrating the value of this method for probing metabolic changes in biomass-restricted model systems [68, 111].

In this study, a CE-MS-based workflow has been developed for the profiling of polar ionogenic metabolites in extracts from zebrafish larvae in order to study the involvement of MR in mediating the effects of cortisol in zebrafish larvae. For this purpose, wild-type (WT) and ubiquitous MR-knockout (MRKO) zebrafish larvae were used and exposed to exogenous cortisol treatment. So far, it has hardly been investigated what the specific role of MR is during stress on metabolite levels, and which mechanisms might be important in this association.

Experimental section

Chemicals and reagents

All chemicals used were of analytical grade or higher purity. Acetic acid (99-100% m/m), methanol and isopropanol were acquired from Biosolve (Valkenswaard, The Netherlands). Ammonium hydroxide (28–30%) was purchased from Acros Organics (Amsterdam, the Netherlands). Sodium hydroxide (98% m/m) and hydrochloric acid (37% m/m) were from Merck (Darmstadt, Germany), and Thermo Fisher Scientific (Waltham, MA, USA), respectively. Amino acids standards were from Sigma-Aldrich (Steinheim, Germany). ^{13}C and/or ^{15}N stable-isotope labelled (SIL) internal standards were purchased from Cambridge Isotope Laboratories (Apeldoorn, the Netherlands). A Milli-Q Advantage A10 water purification system (Merck, Darmstadt, Germany) was used to obtain pure water. Ascorbic acid was from Sigma-Aldrich (Steinheim, Germany).

Ascorbic acid stock solution was prepared as a 1 mg/mL stock solution in water, and through dilution with water a working solution with a final concentration of 125 µM was prepared. A fresh solution of ascorbic acid was prepared daily.

Background electrolyte (BGE) solution was prepared by diluting acetic acid in water (10% *v*/*v*). As sheath-liquid, a mixture of water and isopropanol (50:50, *v*/*v*), containing 0.03% (*v*/*v*) acetic acid was used. Prior to first use, the sheath liquid was degassed for 10 minutes.

Sample solutions

Stable-isotope-labeled (SIL) metabolite standards (1 mg/mL) were dissolved in a Milli-Q water. SIL metabolite mixtures of 8 compounds (**Table S-1**) were prepared in water as 100 µM stock solutions, aliquoted and stored at -20°C. Migration time markers procaine, paracetamol and methionine sulfone were prepared in water as 100 µM mixed solutions, aliquoted and stored at -20°C. The final concentration of migration time markers was 50 µM.

Zebrafish larvae maintenance and cortisol treatment

Adult zebrafish (*Danio rerio*; AB/Tupfel long fin (ABTL) strain) were maintained in compliance with the directives of the local animal welfare committee of Leiden University. Specifically adults were housed on a recirculating system (Fleuren & Nooijen, The Netherlands) on a 14:10 light:dark cycle. Water was maintained at 28.5 °C, pH 7.4, 300 µS conductivity, and 10% of the water was exchanged daily. Animals were fed twice daily with Gemma micro 500 diet (Skretting, Norway) in the morning and live Artemia (Great Salt Lake Brine Shrimp Cooperative, USA) in the afternoon.

A zebrafish strain (*Danio rerio*) wild type (WT) and zebrafish mutants lacking nr3c2 ($^{inr11/inr11}$; hereafter MRKO). WT and MRKO zebrafish larvae were maintained at 28.5 ºC in 20 x 100 mm plates with 1x embryo medium (E3; 5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl2, 0.33 mM MgSO4 + 0.1 ppm methylene blue antifungal agent), with daily water changes until 3 days post-fertilization (dpf), according to guidelines of the Zebrafish Model Organism Database (zfin.org). At 3 dpf, larvae were transferred to 6-well plates (20 fish/well) and treated with either cortisol (5 µg/mL) or a vehicle control (0.05% ethanol). The larvae were then raised to 5 dpf, and all treatments were refreshed daily. Larvae were euthanized with an overdose of MS-222 (0.4 mg/L, Sigma). A pool of 1, 2, 4, 8, 12, 16, 20 and 40 larvae were collected, and each pool was considered a sample.

Experimental design

To study the role of MR in the metabolic response to cortisol in zebrafish larvae, a workflow using CE-MS has been developed and the following experimental design has been designed (**Figure 1**). First, homogenization and metabolite extraction were optimized, then the number of larvae per sample was set. Finally, four groups

have been used to survey the involvement of MR. For this purpose, WT zebrafish larvae and MRKO zebrafish larvae were used, both groups were stimulated with 5 µg/mL cortisol (F) (WT-F and MRKO-F) and by 0.05 % ethanol as a control-vehicle (V) (WT-V and MRKO-V). Ten samples per group divided into two batches were used, however, due to a pressure error during sample analysis, the second batch was eliminated.

Zebrafish larvae homogenization and metabolite extraction

To determine the most optimal metabolite extraction protocol, three homogenization strategies were compared for zebrafish larval samples, and were carried out using homogenization based on probe homogenization (strategy 1), homogenization using a Bullet Blender (strategy 2), and homogenization based on tip sonication (strategy 3). The conditions for each of the strategies are shown in **Table 1** and each was repeated three times. Three steps were applied in each experiment, for which groups of zebrafish larvae containing 8 zebrafish and 50 µl of methanol in a 1.5 ml Eppendorf vial were used. First, the extraction solvent was added. The second step was homogenization of the samples, and finally in the third step the samples were evaporated and reconstituted for analysis.

After homogenization, the homogenate was centrifuged (16,100 × *g*., 5 min, 4 °C), and 450 µL (strategy 1) or 90 µL (strategies 2 and 3) supernatant was pipetted into a 0.5 µL Eppendorf vial. The samples were evaporated in a SpeedVac Vacuum concentrator (Thermo Savant SC210A, Waltham, Massachusetts, United States), and reconstituted in 20 µL BGE prior to injection.

To determine the recovery of each strategy, SIL internal standards were spiked in the zebrafish larvae samples before homogenization and after sample preparation. Recovery was calculated based on **equation 1**:

$$\% \text{ Recovery} = \text{Area IS spiked before} / \text{Area IS spiked after} * 100\% \quad (1)$$

Table 1. Conditions used for each strategy to determine the best method of metabolite extraction from zebrafish larvae.

Strategy	Extraction Solvent	Sample homogenization
1	350 µL cold methanol 80 µL cold water 10 µL ascorbic acid (125 µM) 10 µL water with IS (100 µM)	Homogenizer (1000 × g, 30 s)
2	30 µL cold methanol 10 µL ascorbic acid (125 µM) 10 µL water with IS (100 µM)	Bullet blender (500 × g, 5 min)
3	30 µL cold methanol 10 µL ascorbic acid (125 µM) 10 µL water with IS (100 µM)	Tip sonication (1 x 3 s pulse, 30% amplitude)

(IS) internal standards (stable-isotope-labeled (SIL) metabolite standards and procaine, paracetamol and methionine sulfone)

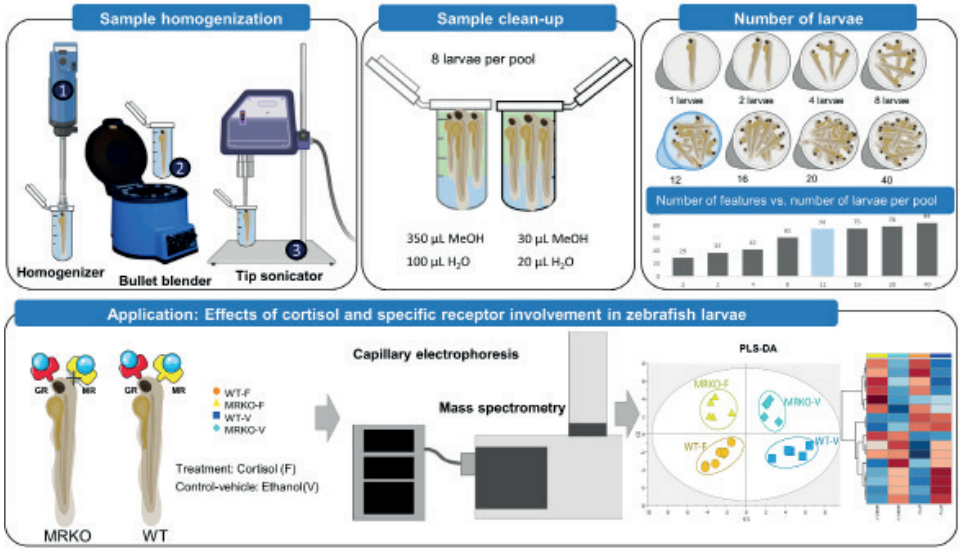

Figure 1. Workflow developed to study the effects of cortisol and involvement of specific receptors in zebrafish larvae using CE-MS.

CE-MS-based metabolic profiling of Zebrafish larvae extracts

Metabolic profiling was carried out with the 7100 CE system from Agilent Technologies (Waldbronn, Germany) which was coupled to a 6230 TOF-MS instrument from Agilent (Santa Clara, CA, USA) utilizing a co-axial sheath-liquid ESI interface equipped with a triple-tube sprayer. Sheath-liquid was delivered at a flow rate of 3 µL/min by an Agilent 1260 Infinity Isocratic Pump (Agilent Technologies) with a flow splitter that splits the sheath liquid in the ratio 1:100. Fused-silica capillaries with an internal diameter of 50 µm were purchased from BGB Analytik (Harderwijk, The Netherlands), and cut to a total length of 90 cm. New capillaries were conditioned by subsequently rinsing, at 5 bar for 1 minute, with methanol, water, sodium hydroxide 1M, water, hydrochloric acid 1M, water and background electrolyte (BGE). Injections were performed hydrodynamically for 54 s at 50 mbar (35.3 nL), and injected volumes were calculated with Zeecalc v1.0b (https://epgl.unige.ch/labs/fanal/zeecalc). For metabolite separation, a voltage of 30 kV was applied. The current observed during the experiment under these conditions was 12.9 µA. MS experiments were acquired in positive mode between 50 and m/z 1000 with an acquisition rate of 1.5 spectra/s. The nebulizer gas was set to 0 psi, and the sheath gas flow rate and temperature were set at 11 L/min and 100 °C, respectively and both were nitrogen. The ESI capillary voltage was set to 5500 V. Fragmentor and skimmer voltages were set at 100 V and 50 V, respectively. In order to obtain information about fragments, in-source fragmentation was used, applying 200 V at the fragmentor. MassHunter version B.06.00 (Agilent, Santa Clara, CA, USA) was used for data acquisition, instrument

control and data treatment. From the sheath-liquid, isopropanol ($C_3H_8¬OH^+$) and its clusters ($[(C_3H_8¬O)_2 +H]^+$ and $([(C_3H_8¬O)_3 +H]^+$) with corresponding m/z values of 61.06479, 121.12231 and 181.17982, respectively, were used as lock masses.

Data processing

Peak extraction was performed using MassHunter Quantitative Analysis (B.10.00, Agilent Technologies) with a mass error of 20 ppm and peak integrations were visually inspected to ensure correct integration. The response function of the measured peak area for 6 endogenous metabolites of which stable-isotope-labeled (SIL) internal standards were available for each was examined using 3 biological replicates for at least 8 different numbers of zebrafish larvae pools (from 40 larvae down to 1 larva). As internal standard, accompanying SIL standards (50 µM) were used. Repeatability (expressed as percentage relative standard deviation, % RSD) for peak area was determined by analyzing SIL standards spiked into a zebrafish sample (n=3). The last experiment has been repeated on three consecutive days to determine interday precision.

The data deconvolution process for the untargeted metabolomics was processed using MassHunter Profinder (B.10.00, Agilent Technologies), to obtain a data matrix in an appropriate format. Two consecutive algorithms were used in the software to perform the deconvolution. The first, performs the Molecular Features Extraction (MFE), reduces the data by removing non-specified information. The second algorithm, Find by Ion (FbI) was used for targeted feature extraction and to obtain a better accuracy of the data. Finally, abundance of the molecule, mass accuracy and migration time for each feature in all samples were obtained as matrix data [309]. Data quality was ensured by excluding background noise and unrelated ions, molecular features present in 50% of the quality control (QC) injections with a relative standard deviation (RSD) less than 30%, and present in 75% of the study samples, were maintained. Missing values were estimated using the k-nearest neighbours (kNN) algorithm.

Subsequently, the matrix was normalized by the total useful signal (TUS), then an unsupervised principal component analysis (PCA-X) model was generated in order to observe the trend of the QCs, detect outliers and observe natural clustering trends.

Statistical analysis

Multivariate data analysis (MVDA) was used to investigate the differences between the four groups. supervised models such as the partial least square discriminant analysis (PLS-DA) and orthogonal partial least square discriminant analysis (OPLS-

DA) were performed to discriminate the variation between groups. The quality of the models was evaluated by explained variance (R^2) evaluating the model by maximizing the variance given by the variables and predicted variance (Q^2) evaluating the predictive ability of the model. These parameters were provided by SIMCA-P (Version 17, Umetrics, Sartorius Stedim Biotech) software. Finally, the percentage change was calculated by comparing cases with controls. Following biomarker selection, a receiver operating characteristic (ROC) curve analysis was applied to the identified potential biomarker using the biomarker analysis module in the MetaboAnalyst 5.0 webserver. In addition, univariante ROC curve analysis was applied to calculate the area under the curve (AUC) for the characteristics identified from the comparison of MRKO-F vs WT-F, this was followed by a multivariate ROC curve analysis that was used to calculate AUCs for the combination of more than two characteristic metabolites. The ROC curves were generated by Monte-Carlo cross validation (MCCV) using balanced sub-sampling. For each MCCV at least two-thirds of samples were used to select significant features that were used to build the classification model. To validate the classification model, the remaining one-third of the samples were used to validate the classification model. The support vector machines (SVM), method was selected as the feature classification and ranking method and used the top six metabolites to generated a classification model.

Metabolite identification

Compounds were tentatively annotated based on their exact mass (maximum error of 20 ppm), isotopic distribution and adduct formation using the CEU Mass Mediator search tool [310]. Furthermore, to enhance confidence in metabolite identification, relative migration times (RMT), effective mobility values (μ_{eff}) and in-source fragments were compared with respect to standards analysed in a previous experiment [271, 311].

Results and discussion

CE-MS method development

In metabolomics studies, typically, the efficient homogenization of tissues and extraction of metabolites from tissue homogenates are the steps that are most time-consuming and error-prone [312]. Therefore, as a first step, the homogenization and metabolite extraction were evaluated for zebrafish larvae samples. For metabolite extraction, a single-phase extraction method based on protein precipitation my methanol was selected due to its effective capability to precipitate proteins [313, 314], speed and simplicity [315]. As the focus of the current study was solely on the polar metabolome, a single-phase extraction method is sufficient to extract

all the metabolites of interest. However, when the goal is to perform a global metabolomics study on zebrafish samples with interest on both polar and nonpolar metabolites of the same sample, a two-phase extraction protocol might be more beneficial (i.e., Bligh&Dyer extraction), as such an extraction protocol allows the analysis of both polar (aqueous layer) and nonpolar metabolites (chloroform layer) within the same sample, using different analytical platforms [316].

After selection of the extraction strategy, three different homogenization strategies were tested, i.e., homogenization based on standard homogenization (strategy 1), homogenization using a Bullet Blender (strategy 2) [11, 20], and homogenization based on tip sonication (strategy 3) [21]. All three strategies were applied to small pools of zebrafish larvae (8 larvae per pool) of 5 dpf. A selection of SIL standards (50 µM) (**Figure S-1**) was spiked into the zebrafish larvae sample before and after sample preparation to determine the recovery and repeatability of the different strategies. After complete homogenization, homogenates were collected. After metabolite extraction, the metabolite content of the homogenates was analyzed using CE-MS, whereafter recoveries of SIL standards were calculated. As shown in Table S-2, satisfactory recoveries were observed for all three homogenization strategies: between 84-98% for strategy 1, between 88-107% for strategy 2 and between 100-104% for strategy 3. The repeatability of peak areas of SIL standards was determined for SIL standards spiked at the beginning of the workflow for biological replicates as the relative standard deviation (%RSD), and was between 6.3-17.3% for strategy 1, between 4.5-15.0% for strategy 2 and between 3.1-8.4% for strategy 3.

Another important aspect during tissue homogenization is metabolite stability, as degradation could occur due to time duration, (high) temperatures and the applied frequency. As is it expected that catecholamines such as epinephrine and dopamine play an important role in mediating the stress response in zebrafish larvae [317], precautions were taken by adding an antioxidant at the beginning of the workflow in order to stabilize metabolites throughout the sample preparation. As antioxidant, ascorbic acid (12.5 µM) was selected due to its compatibility with MS detection [187]. Additionally, at pH 2.2, ascorbic acid will have no net charge, causing it to migrate with the EOF and thereby not disturbing the detection of cationic metabolites of interest. To evaluate the metabolite stability during sample preparation, two catecholamines epinephrine and dopamine, which are well-known metabolites that are prone to degradation, were included in the SIL standard mixture. As is shown in **Table S-2**, good recoveries for epinephrine and dopamine were observed for all homogenization strategies, with 96% and 98% for strategy 1, 88% and 96% for strategy 2 and 101% and 101% for strategy 3, which indicates there is no significant loss during sample preparation.

Furthermore, when selecting an appropriate sample preparation strategy, the time duration per sample is an important factor. Due to the size of the homogenizer tip for strategy 1, a minimal total volume of 500 µL needs to be present in the Eppendorf vial in order to perform proper homogenization, while in case of the Bullet Blender and tip homogenizer, 100 µL of total volume showed to be sufficient. After homogenization, either 450 µL (strategy 1) or 90 µL (strategies 2 and 3) of supernatant was transferred into another Eppendorf vial and dried in a Speedvac. Because the increased volume of strategy 1, the time for evaporation was more than tripled (2+ hours instead of 30 minutes). Additionally, because zebrafish larvae at 5 dpf encompass approximately 300 nL volume, it would be most beneficial to have as little dilution as possible during sample preparation. When comparing the time duration per sample for strategies 2 and 3, homogenization by the Bullet Blender takes 5 minutes per 24 samples (~13 seconds per sample), while tip sonication takes 3 seconds per sample. Taking into account the recovery and repeatability as well as the duration of the sample preparation strategy, strategy 3 (homogenization by tip sonication) was selected for further experiments.

The analytical performance of the CE-MS workflow was further evaluated for pools containing a small number of zebrafish larvae (8 fish per pool). Precision of the CE-MS workflow for profiling of endogenous metabolites in zebrafish larvae was assessed based on the repeated analyses of zebrafish samples spiked with glutamine-^{13}C as SIL internal standard (50 µM). Intraday RSD values (n=3) for corrected peak areas of all endogenous analytes for biological replicates were between 0.9-20.3%, while interday RSDs (n=3 over 3 days) were below 28.2% for 20 detected endogenous metabolites (**Table S-3**). Migration time repeatability was assessed by converting to effective mobilities (μ_{eff}), the repeatability was below 1.3% for interday analysis.

Subsequently, the response function of endogenous metabolites in decreasing numbers of zebrafish larvae per pool were investigated using SIL standards. Zebrafish pools of 40, 20, 16, 12, 8, 4, 2 and 1 zebrafish were spiked with SIL epinephrine-D_3, gamma-aminobutyric acid-D_6, glutamine-^{13}C, glutamic acid-^{13}C,^{15}N, asparagine-^{13}C,^{15}N, lysine-^{13}C and aspartic acid-^{13}C,^{15}N (50 µM), and calibration curves for endogenous metabolites corrected by their corresponding SIL analogue were prepared (in case the endogenous metabolites were detected). A linear response (and with R^2>0.98) for selected metabolites was obtained (**Figure 2A**). Thereafter, it was investigated how many features could be extracted from the data resulting from the different zebrafish pools. As can be seen in **Figure 2B**, 84 features were found in a pool of 40 zebrafish larvae, whereas only 29 features could be extracted from a single zebrafish larva. For this study, the zebrafish pools containing 12 larvae were selected for further experiments, as a lower amount of larvae showed

to result in more efficient metabolite homogenization when employing low solvent volumes during sample preparation. Furthermore, for our application it was not necessary to show inter-individual differences between the fish, therefore making it unnecessary to further scale down the amount of fish per pool.

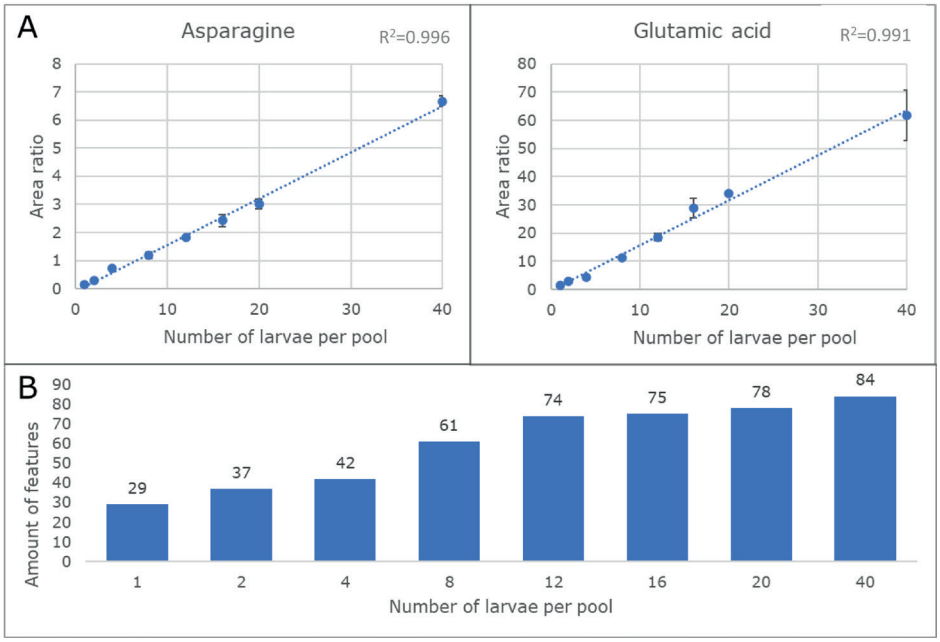

Figure 2. A) Response function for lysine, asparagine, glutamic acid, and aspartic acid in different zebrafish larvae pools as determined using CE-MS, and B) Number of features that could be extracted from CE-MS data obtained after analysis of zebrafish larvae pools.

Application of CE-MS to a zebrafish stress model

With the developed CE-MS workflow the aim was to study the role of polar ionogenic metabolites in zebrafish under a stress scenario and thus to potentially reveal the role of MR in mediating the effects of cortisol. After sample analysis, data processing was performed, which revealed 139 metabolic features. The signals were then filtered for presence in blanks, in clusters and in QC samples with a % RSD less than 30%. Then the signals belonging to adducts and fragments were removed. The final data set was reduced to 61 compounds (Table S-4). The analytical performance of CE-MS was evaluated using the unsupervised PCA-X (R2= 0.448), resulting in a clustering of quality control samples (QC's) distributed along the working list, showing high sensitivity and reproducibility throughout the analysis, indicating adequate data quality performance (**Figure 3A**).

Additionally, a supervised clustering PLS-DA model was constructed to obtain a better separation. The PLS-DA analysis score plot showed a clear separation between the four groups of cortisol-stimulated zebrafish larvae and controls.

The PLS-DA model had an R2 value of 0.611 and Q2 value of 0.487 (**Figure 3B**). After this evaluation, OPLS-DA analysis was performed to compare two groups at a time. Two comparisons were performed. First, we compared the group of Wild type zebrafish larvae stimulated with cortisol (WT-F) versus the group of Wild type zebrafish larvae stimulated with ethanol as control (WT-V), the model presented an R2 value of 0.987 and Q2 value of 0.841 (**Figure 3C**). Then, the groups of MR- Knockout zebrafish larvae stimulated with cortisol (MRKO-F) versus the group groups of MR- Knockout zebrafish larvae stimulated with ethanol (MRKO-V) as a control were compared, the model presented an R2 value of 0.998 and Q2 value of 0.736 (**Figure 3D**).

To select the metabolites responsible for the clustering and separation of the groups from the OPLS-DA models, Jack-Knife confidence intervals were calculated, variables importance in projection (VIP >1) and p(corr) >|0.5| were considered. In addition, for all OPLS-DA models, the validated cross-sectional scores (CV-scores) plots were shown, which revealed that 99% of the samples were correctly classified in their corresponding group. The results for the WT-F to WT-V and MRKO-F to MRKO-V comparisons of multivariate statistics are tabulated in Table 2.

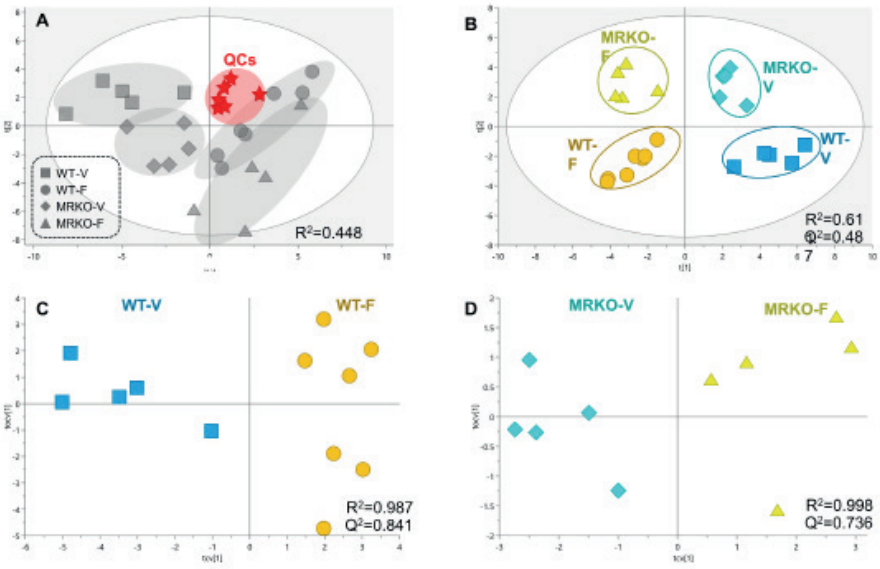

Figure 3. A) Principal component analysis (PCA-X) score plot with an explained variance R2 = 0.448, quality control (QC, red starts), B) PLS-DA model, C) CV-OPLS-DA model for WT-F (n=7) vs. WT-V (n=5) comparison, D) CV-OPLS-DA model for MRKO-F (n = 5) vs. MRKO-V (n=5) comparison.

Table 2. Differentially identified metabolite contents in the comparison of cortisol-stimulated zebrafish larval groups and control groups.

Compound	Mass (Da)	RSD (%) QC	p(corr)	VIP	JK	change (%)	p(corr)	VIP	JK	change (%)
Sarcosine	89.0475	8.0	0.9	1.6	Yes	58.8	0.9	1.8	Yes	47.5
Alanine	89.0476	2.4	0.9	1.4	Yes	23.2	0.8	1.7	Yes	12.8
Serine	105.0420	4.3	-1	1.6	Yes	-37.6	-0.8	1.7	Yes	-23.4
Cytosine	111.0427	27.6	0.7	1.3	Yes	83.9	0.5	1.1	-	47.2
Asparagine	132.0527	3.8	-0.8	1.4	Yes	-23.2	-0.6	1.1	-	-9.5
Ornithine	132.0890	7.2	-0.9	1.6	Yes	-66.6	-0.9	1.7	Yes	-49
Aspartic acid	133.0368	4.6	-0.5	1.0	-	-15.5	-0.7	1.6	Yes	-37.7
Acetylcholine	145.1091	6.0	NS	<1	-	NS	0.6	1.3	Yes	36.1
N_1-Acetylspermidine	187.1670	10.2	0.7	1.2	-	50.2	0.6	1.4	Yes	56.5
Cystathionine	222.0692	9.1	-0.6	1.2	Yes	-26.8	NS	<1	-	NS
Cytidine	243.0845	12.3	0.7	1.3	Yes	47.6	0.6	1.2	-	45
Aspartyllysine*	261.1314	20.5	-0.7	1.2	Yes	-49.9	-0.6	1.3	-	-40.6
Glutathione disulfide	612.1478	27.4	-0.8	1.4	Yes	-54.6	NS	<1	-	NS

(NS) Not significant, and (*) Contains multiple annotation options

To evaluate the regulation of MR and GR in response to cortisol stress in zebrafish larvae. The WT-F group was compared to WT-V, as a result 12 metabolites appeared to be modulated in response to stress. Increases in sarcosine, alanine, cytosine, N-acetylspermidine and cytidine levels were observed. In contrast, levels of serine, asparagine, ornithine, aspartic acid, cystathione, gutation disulfide and a dipeptide (Asp-Lys) were decreased (**Table 2**). On the other hand, MRKO-F larvae were compared with MRKO-V larvae. As a result, 11 metabolites were modulated. Increases in sarcosine, alanine, cytosine, acetylcholine, N-acetylspermidine and cytidine levels were observed.

However, decreased levels of serine, ornithine, aspartic acid, glutathione disulfide and a dipeptide (Asp-Lys) were observed (**Table 2**). Those metabolites that are altered in WT but not in MRKO were observed to highlight MR-dependent metabolites.

Interpretation of the main findings

Currently, zebrafish are used as a complement to rodent models to study neuropsychiatric disorders [31], because of their similarity to the manifestations of neuroendocrine mechanisms. So far, numerous behavioral and transcriptomic studies on zebrafish larvae in response to different stimuli have shown interesting results [32-34]. Cortisol is the main stress hormone and binds with different affinities to both GR and MR. High levels of cortisol bind to GR, whereas at basal levels of cortisol it binds to MR [35]. Even though the role of cortisol-GR signaling is well characterized, the role of MR is still less known. The metabolomic profile could help in understanding the role of both receptors under stress. Therefore, we present here an untargeted study based on CE-MS to determine the role of polar metabolites in mediating the GR and MR response to stress.

To evaluate the involvement of GR and MR in response to stress, we studied the effect of cortisol on the metabolome of both WT and MRKO zebrafish larvae. Metabolites

that were found to be commonly modulated following cortisol stimulation in both WT and MRKO zebrafish larvae compared to their respective controls were: alanine, sarcosine, serine, cytosine, asparagine, ornithine, aspartate, N-acetyl-spermidine, cytidine, and aspartyl-lysine. In MRKO, the independent role of GR in response to cortisol has been observed. However, it was observed that the involvement of the MR receptor in WT fish larvae cooperates in the modulation of these metabolites, showing slight increases in the percentage changes in the case of sarcosine, alanine, cytosine, N-acetylspermidine, and cytidine, as well as slight decreases in the levels of serine, asparagine, ornithine, and aspartyl-lysine (**Table 2**). These findings support the essential functions described for GR in energy homeostasis, stress response, and inflammation. Thus, it has also been observed that the cooperation between GR and MR is important for homeostasis.

Cortisol-generated stress has been associated with increased reactive oxygen species (ROS) and nitrogen species (RNS) leading to oxidative and nitrosative stress [36]. Ornithine is a substrate for putrescine production and complements polyamine metabolism, which may explain the decrease in ornithine levels during both GR activation and GR:MR cooperation in response to stress. Polyamines such as putrescine and spermidine are essential for decreasing reactive oxygen spice (ROS) damage in animals [37] and for protecting from stress, allowing the flow of K1 current, controlling brain glutamate receptors implicated in learning and memory [38]. On other hand, we found higher increased levels of N1-acetylspermidine when GR is activated, however, we did not find alterations in putrescine and spermidine levels. Spermidine is a substrate of spermidine/spermine N^1-acetyltransferas (SSAT), which is responsible for promoting acetylation at N1 in the presence of acetyl-CoA resulting in N1-acetylspermidine which in turn is converted to putrescine. These results suggested that GR promotes the acetylation of spermidine by altering the regulatory metabolism of polyamines [39]. In addition, ornithine is a substrate for proline production and contributes to the formation of glutamate. Glutamate is the main excitatory neurotransmitter in the central nervous system, and its regulation is important for maintaining the balance of the glutamate cycle. It has been reported that glutamatergic dysfunction causes neuropsychiatric conditions such as stress, anxiety and depression [40].

As previously mentioned, cortisol exposure causes increased oxidative stress resulting from the generation of ROS contributing to neuronal cell death. Glutathione (GSH) acts as an antioxidant defense and is responsible for the direct scavenging of ROS. During ROS scavenging, glutathione interacts with radicals such as superoxide radical anion, nitric oxide, or hydroxyl radical. On the other hand, GSH is an electron donor for the reduction of peroxides in the GPx reaction with the final oxidation product being glutathione disulfide (GSSH). However, GSH is

regenerated from GSSH through the reaction catalyzed by glutathione reductase [44]. In our study, decreased levels of GSSH were found in response against stimulation in WT fish larvae, whereas in MRKO the percentage change was not significant. This suggests the importance of the role of MR related to the activation of the antioxidant protection system.

Another metabolite that was modulated by the effects of stress was an N-methylated derivative of glycine. Sarcosine is produced from glycine via Glycine N-methyltransferase which utilizes a methyl group from S-adenosylmethionine (SAM) [45]. Sarcosine has been described to be an inhibitor of glycine transporter-1, increasing the levels of glycine, an inhibitor of rapid neurotransmission. Sarcosine is also responsible for enhancing the function of the N-methyl-D-aspartate receptor [45]. Previous studies have shown that sarcosine has a neuroprotective effect as it is effective in reducing oxidative stress [46]. These findings could justify the increase in sarcosine levels in response to stress during GR and MR activation and their cooperation with each other.

Moreover, decreases in asparagine and aspartate levels have been observed in response to cortisol stimulation in both GR activation and GR:MR involvement. Aspartate is converted to asparagine through asparagine synthetase using glutamine in response to cellular stress [41]. Glutamate and aspartate are both excitatory neurotransmitters, with aspartate as the most abundant in the central nervous system. Additionally, aspartate is converted to alanine via aspartate 4-decarboxylase [42, 43].

On the other hand, acetylcholine levels were found to be increased under stress conditions in MRKO-F compared to MRKO-V, whereas in WT-F compared to WT-V acetylcholine was not significant. This could suggest the involvement of GR in mediating acetylcholine levels. Acetylcholine is the main neurotransmitter reported to be released under stress conditions. These findings are consistent with previous work in which elevated levels of acetylcholine were observed in depressed individualS.

Interestingly, serine levels were found to be significantly decreased in WT-F and MRKO compared to their respective controls. Serine is a precursor of phospholipids, ATPs, and nucleic acids; serine is also used to produce SAM, which is responsible for donating ubiquitinated methyl groups to produce NADPH, which maintains cellular redox homeostasis [48].

These results suggest that the regulation of the balance between redox reactions within a cell depends on GR and MR and their cooperation together.

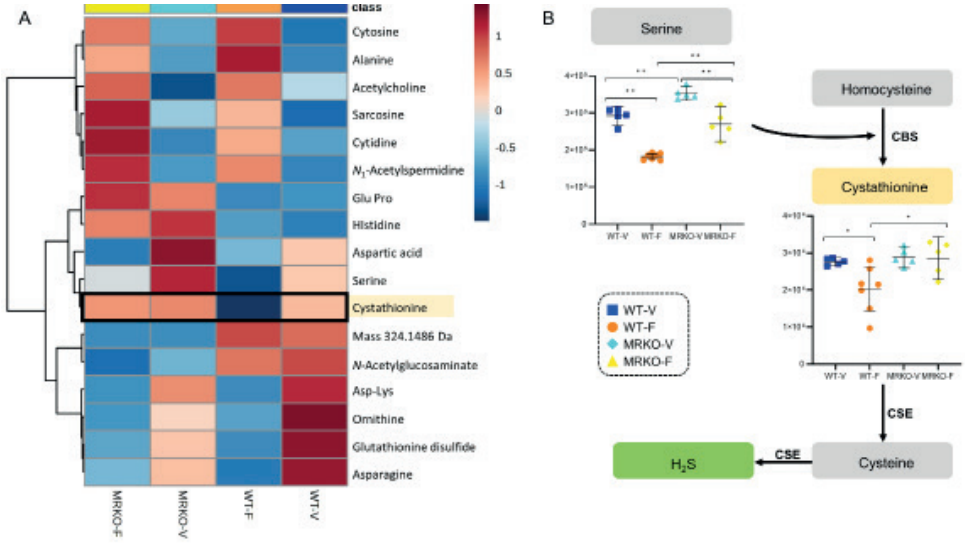

Figure 4. A) Heatmap of the 16 differentially modulated metabolites in metabolomic analysis of cortisol-stimulated WT or MRKO versus their respective controls. In each column the average concentrations of the samples, and each row represents a metabolite. The color coding within the heatmap represents the relative fold change of each metabolite between groups. Metrics used for the analysis were the Euclidean distance measure and Ward's clustering algorithm using MetaboAnalyst 5.0 software. B) Biogenesis of H2S by CSE or CBS.

In contrast to all metabolites, cystathionine regulation was observed to be MR-dependent only. Cystathionine is produced by cystathionine beta synthase (CBS) an enzyme that catalyses the first step of the transsulfuration pathway, leading to the production of cystathionine from serine and homocysteine. It has been described that under stress cystathionine levels are decreased consistent with what was observed in cortisol treated WT larvae in this study. Here we show that this decrease is likely due to MR as the effect of cortisol on cystathionine was abolished when MR was absent. Cystathionine can then be converted to cysteine and by extension H2S by the enzyme cystathionine gamma lyase (CSE) to produce H2S from cysteine and homocysteine (**Figure 4**) [49]. H2S is associated with many physiological processes, such as inflammation, neuromodulation and apoptosis and abnormal levels are associated with Alzheimer's diseases, liver cirrhosis and diabetes [50]. The data here suggests that MR may be a key regulator of enzymes involved in cystathionine production and subsequently impact key physiological processes.

Finally, in this study we have shown the role of MR in the response to cortisol at the metabolic level, which is consistent with a metabolic role previously described for MR in zebrafish larvae [47].

Conclusions and perspectives

In this work, a CE-MS-based analytical workflow has been developed for the profiling of polar ionogenic metabolites in extracts from zebrafish larvae with acceptable performance metrics. The workflow has been used successfully used for the profiling of metabolites in extracts from wild-type and zebrafish with a ubiquitous MR-knockout, providing insights into the role of MR and GR in mediating the effects of cortisol.

Through the present study, besides the ability of GR to modulate metabolism independently of MR, we observed the cooperation of both receptors in maintaining the balance of the metabolome, and that part of this profile is regulated by MR. Indeed, differential regulation of cystathionine by MR has been identified, and although this metabolite is known to be modulated during stress. Taken together, this technique has the potential to identify novel pathways and mechanisms of action in zebrafish larvae.

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Supplementary information

The supplementary files are available upon request.