Effect of Acute Stresses on Zebra Fish (*Danio rerio*) Metabolome Measured by NMR-Based Metabolomics

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Key words
- zebra fish
- NMR
- metabolomics
- stress

Abstract

We applied an acute stress model to zebra fish in order to measure the changes in the metabolome due to biological stress. This was done by submitting the fish to fifteen minutes of acute confinement (netting) stress, and then five minutes for the open field and light/dark field tests. A polar extract of the zebra fish was then subjected to ^{1}H nuclear magnetic spectroscopy. Multivariate data analysis of the spectra showed a clear separation associated to a wide range of metabolites between zebra fish that were submitted to open field and light/dark field tests. Alanine, taurine, adenosine, creatine, lactate, and histidine were high in zebra fish to which the light/dark field test was applied, regardless of stress, while acetate and isoleucine/lipids appeared to be higher in zebra fish exposed to the open field test. These results show that any change in the environment, even for a small period of time, has a noticeable physiological impact. This research provides an insight of how different mechanisms are activated under different environments to maintain the homeostasis of the body. It should also contribute to establish zebra fish as a model for metabolomics studies.

Supporting information available online at http://www.thieme-connect.de/products

Introduction

Stress can be defined as a condition in which the dynamic equilibrium or homeostasis of an organism is disturbed by an internal or external factor, called stressor [1]. A stressor can act in two ways either by disturbing the internal equilibrium or homeostasis of the organism and/or forcing a set of behavioral and physiological responses that work as adaptive mechanisms and help the organism to cope with the changes [2, 3]. In their natural environment, fish are continuously exposed to different kinds and intensities of stressors, such as changes in water flow, toxicity or pH, and exposure to pathological microorganisms. When they are bred under controlled conditions, either as food or for research purposes, they additionally suffer other kinds of stress derived from handling, overcrowding, and transport. In general, the unique response of fight or flight helps fish to cope with the stress but the reaction to specific stressors will ultimately depend upon the type, duration, and intensity of the stressor. The teleost fish stress response has been grouped as primary, secondary, and tertiary. The primary response is the release of catecholamines or corticosteroids [2]. This phenomenon occurs by the stimulation of two endocrine routes, the hypothalamic autonomic nervous system-adrenal medulla axis, and the hypothalamic-pituitary-adrenal axis [4]. Catecholamines increase branchial blood flow, branchial oxygen diffusing capacity, oxygen transport, and the oxygen uptake rate of gills [5]. Also, elicitation of beta-adrenergic receptors causes stimulation of glucogenolysis and gluconeogenesis during stress [3]. The secondary response is a consequence of the effect of these hormones on blood and tissue levels, and includes an increase in cardiac output, oxygen uptake, disturbance of the hydro-mineral balance, and elevatation of blood glucose levels [6]. The prolonged stress leading to a continued loss of natural body balance to which the fish cannot adapt results in immunity loss, defective reproduction, and growth, all of which characterize the tertiary response [5].

Most researchers use blood plasma cortisol or glucose levels as indicators of stress levels [5]. However, there is always a risk that this type of analysis might not wholly represent the overall response of the stressed organism [7]. Thus, a ho-

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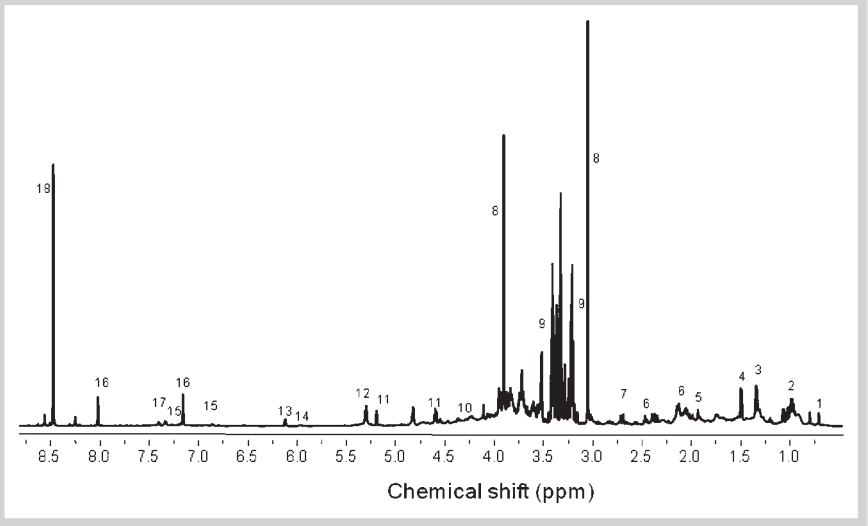

Fig. 1 600 MHz ^{1}H NMR of adult zebra fish. 1, bile acid conjugates; 2, isoleucine/leucine; 3, lactate/threonine; 4, alanine; 5, acetate; 6, glutamate/glutamine; 7, aspartic acid; 8, creatine; 9, taurine; 10, ribose; 11, glucose; 12, lipids; 13, ATP; 14, uridine; 15, tyrosine; 16, histidine; 17, phenylalanine; 18, formate.

listic approach-based method, such as metabolomics, should be able to assess the overall physiological response of an organism more accurately, since its ultimate goal is to measure qualitatively and quantitatively of all the metabolites in an organism [8]. The zebra fish has proved to be adequate as a model for the investigation of stress response [9]. As a model, it is quite similar in terms of brain system, neurochemical mechanism, and behavior response to the rodent model [10, 11], providing, thus, a good and cost-effective alternative to this classical model. The response of the zebra fish after stress exposure can be measured by submitting them to tests, such as the novel diving tank, light/dark field, or open field [10, 12]. Each of these tests provides different types of stress as they consist in the exposure of the fish to different novel environments and this very likely results in different kinds of chemical perturbations. In this study, we attempted to understand the physiological effects of these novel environments when combined with acute stress.

Results

Representative 600 MHz ^{1}H NMR spectra are displayed in **Fig. 1**. The NMR spectra showed hundreds of overlapping signals that were resolved using 2D NMR spectroscopy. The comparison of chemical shifts and splitting patterns with our in-house library of more than 600 common metabolites and with available literature [13–15] allowed us to identify most of the compounds as shown in **Table 1**.

To differentiate among the stressed and non-stressed zebra fish, ^{1}H NMR data was first subjected to unbiased multivariate data analysis (MvDA), principal component analysis, or PCA. It is an unbiased approach, which helps to correlate observations with variables and represent the data in three-dimensional spaces. The scatter plot of the PCA model obtained with data of 76 samples of adult zebra fish can be observed in **Fig. 2**. For PCA, the cross validation of the predicted model showed a PC1 value of 22% for the first component and a PC2 value of 14% for the second component. These values indicate a weak model for such kind of data. Visual inspection of PCA also revealed no significant trend between stressed and non-stressed zebra fish.

The reason for the minimal separation and low score was considered to be more likely due to high biological variation than the stress and non-stress variables or the presence of non-related variables.

To improve our model, data was further tailored and subjected to partial least square discriminatory analysis (PLS DA). After applying PLS DA, no improvement in the discrimination of stressed and non-stressed zebra fish samples was observed (**Fig. 1S**, Supporting Information). Therefore, spectral values were subjected to an orthogonal signal correction filter in order to remove systematic

Table 1 ^{1}H NMR chemical shifts and coupling constants of the metabolites identified from the polar extract of whole adult zebra fish (600 MHz).

Metabolites	Chemical shifts (δ) and coupling constants (J)
Acetate	δ 1.97 (s)
Alanine	δ 1.48 (d, J = 7.3), δ 3.74 (q, J = 7.2)
Asparagine	δ 2.83 (m), δ 2.96 (m), δ 3.94 (m)
Aspartate	δ 2.64 (dd, J = 17.5, 8.6), δ 2.80 (dd, J = 17.5, 3.5), δ 3.84 (dd, J = 8.9, 3.5)
ATP	δ 6.13 (d, J = 6.4), δ 8.26 (s), 8.56 (s)
Betaine	δ 3.29 (s), δ 3.87(s)
Phosphocholine	δ 3.22 (s)
Choline	δ 3.24 (s)
Citrate	δ 2.70 (d, J = 16.9), δ 2.35 (d, J = 16.9)
Creatine	δ 3.06 (s), δ 3.91 (s)
Formate	δ 8.48 (s)
GABA	δ 1.90 (m), δ 2.31 (t, J = 7.6), δ 3.03 (m)
Glucose	δ 4.60 (d, J = 7.9), δ 5.20 (d, J = 4.0)
Glutamate	δ 2.05 (m), δ 2.40 (m), δ 3.71 (m)
Glutamine	δ 2.14 (m), δ 2.46 (m), δ 3.71 (m)
Glycine	δ 3.52 (s)
Histidine	δ 7.07 (s), δ 3.13 (dd, J = 15.3, 7.2), δ 3.94 (dd, J = 8.0, 4.5)
Isoleucine	δ 0.92 (t, J = 7.3), δ 1.70 (d, J = 7.1)
Lactate	δ 1.32 (d, J = 7.0), δ 4.06 (q, J = 6.9)
Leucine	δ 0.99 (d, J = 7.0), δ 1.0 (d, J = 7.0), δ 1.68 (m), δ 3.68 (m)
Methionine	δ 2.14 (s)
Phenylalanine	δ 7.33–7.42 (m), δ 3.10 (dd, J = 14.5, 8.5)
Proline	δ 2.02 (m), δ 2.34 (m), δ 4.11 (dd, J = 8.6, 6.3)
Threonine	δ 1.33 (d, J = 7.1), δ 4.23 (m)
Tryptophan	δ 7.13 (t, J = 7.1), δ 7.21 (t, J = 7.06), δ 7.30 (s), δ 7.48 (d, J = 8.1), δ 7.73 (d, J = 7.8)
Tyrosine	δ 6.89 (d, J = 8.5), δ 7.20 (d, J = 8.5), δ 3.18
Taurine	δ 3.21 (t, J = 6.4), δ 3.41 (t, J = 6.4)

variations or uncorrelated parts since this technique appeared to be effective in previous studies [16,17]. The orthogonal signal correction (OSC) filter removes and subtracts the uncorrelated or orthogonal variation with regards to the Y variable from the spectral data. The remaining spectral data, which is correlated to the Y variable, is then subjected to PLS DA modeling. By applying this filter to the PLS DA model, we were able to get a well-explained model. The OSC filter was created by the use of two variables, stress and novel environment (open field and light/dark field), as Y variables. The resulting model was quite promising as compared to PCA or PLS DA. The first component of the OSC-PLS DA score plot was able to explain 41% of the variation while the second component explained 16% (**Fig. 3**).

Inspection of the score plot showed a separation based on the field test variable, while the stress-based variable was insignificant (**Fig. 3**). On the basis of these results, we decided to exclude one variable at a time and create two different models. Score and loading plots are shown in **Fig. 4A–C**. As it can be clearly observed in **Fig. 4A**, our model failed to reveal any significant difference between the stressed and control groups. In the next model, we omitted the Y variable for stress and used novel environment exposure as a Y variable. We then got a different picture (**Fig. 4B**) that clearly showed the difference between the fish exposed to the two different fields, regardless of being stressed or non-stressed. The separation was obtained along PLS1 for both groups exposed to the two different environments. Also, both models were validated by a permutation test with 100 permutations (**Fig. 2S**, Supporting Information). The low Q2 value (0.35) for the first model with stress as a Y variable revealed it to be a weak model, while the Q2 value (0.85) obtained for the second model with the novel field as a Y variable suggested a validated model with high predictive power.

A representative S-plot (weight of variables vs. Pearson's correlation) of the second model (**Fig. 4C**) shows the variable responsible for the separation. In this case, taurine and acetate seemed to be the most affecting variables. The S-plot also revealed a negative correlation between acetates, isoleucine/lipids, and the rest of the metabolites.

Once the variables responding to stress were identified, they were subjected to statistical analysis using relative quantification values. Relative quantification depends upon the values of the mean peak area obtained from the bucket table. These values represent the area of a specific peak in that bucket. In order to confirm the results from MvDA, we used the values (mean peak area from the bucket table) of some metabolites selected after examining the S-plot. These values were subjected to ANOVA ($p < 0.05$) followed by Bonferroni's multiple comparison tests, in which all groups were compared with each other to check significance. The ANOVA confirmed the participation of the relative metabolites shown in **Fig. 5**. The bar graphs represented in **Fig. 5** clearly show that the most significant differences observed between the groups were related to the exposure to two different environmental conditions rather than netting stress exposure.

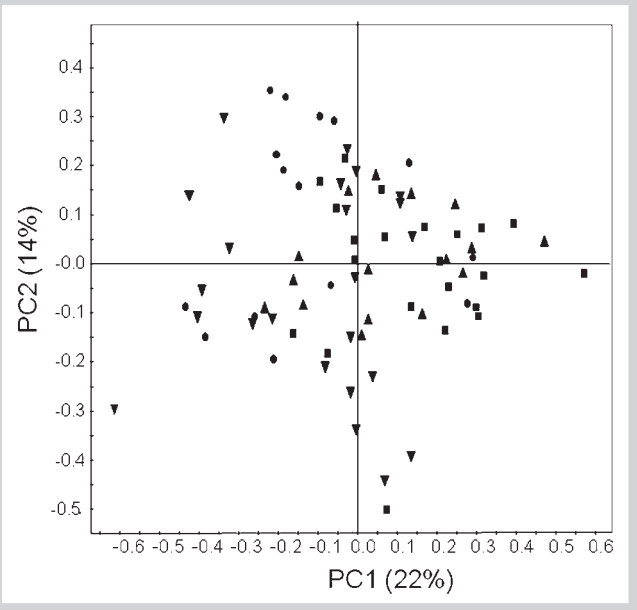

Fig. 2 Principal component analysis score scatter plot of ^{1}H NMR spectra of 76 zebra fish samples. (▲) Control group submitted to the light/dark field; (•) control group submitted to the open field; (■) stress group submitted to the light/dark field; (▼) stress group submitted to the open field.

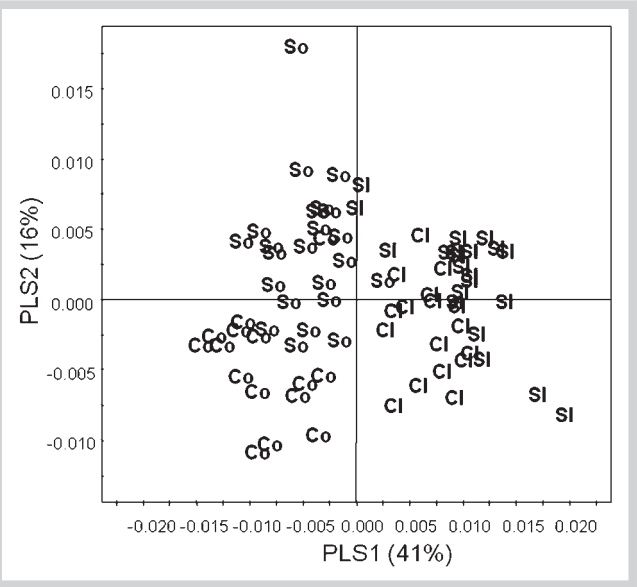

Fig. 3 Score plot of partial least square modeling-discriminant analysis after orthogonal signal correction using two variables of stress and novel environment. Co, control group exposed to the open field; So, stress group exposed to the open field; Cl, control group exposed to the light/dark field; Sl, stress group exposed to the light/dark field.

Discussion

We used NMR as our main tool for metabolite detection. Sensitivity of NMR can be an issue as it limits the number of metabolites detected as compared to other detection tools like mass spectrometer or gas chromatography. This disadvantage can easily be outweighed with other advantages offered by NMR like easy sample preparation, low technical variability, nondestructive nature of sample analysis, and easy quantification of detected metabolites. Here, in this study, we demonstrate the power of a ^{1}H NMR-based metabolomic technique as a tool to assess abrupt changes in the metabolome of zebra fish. We demonstrated how their acute exposure to different environments induces changes

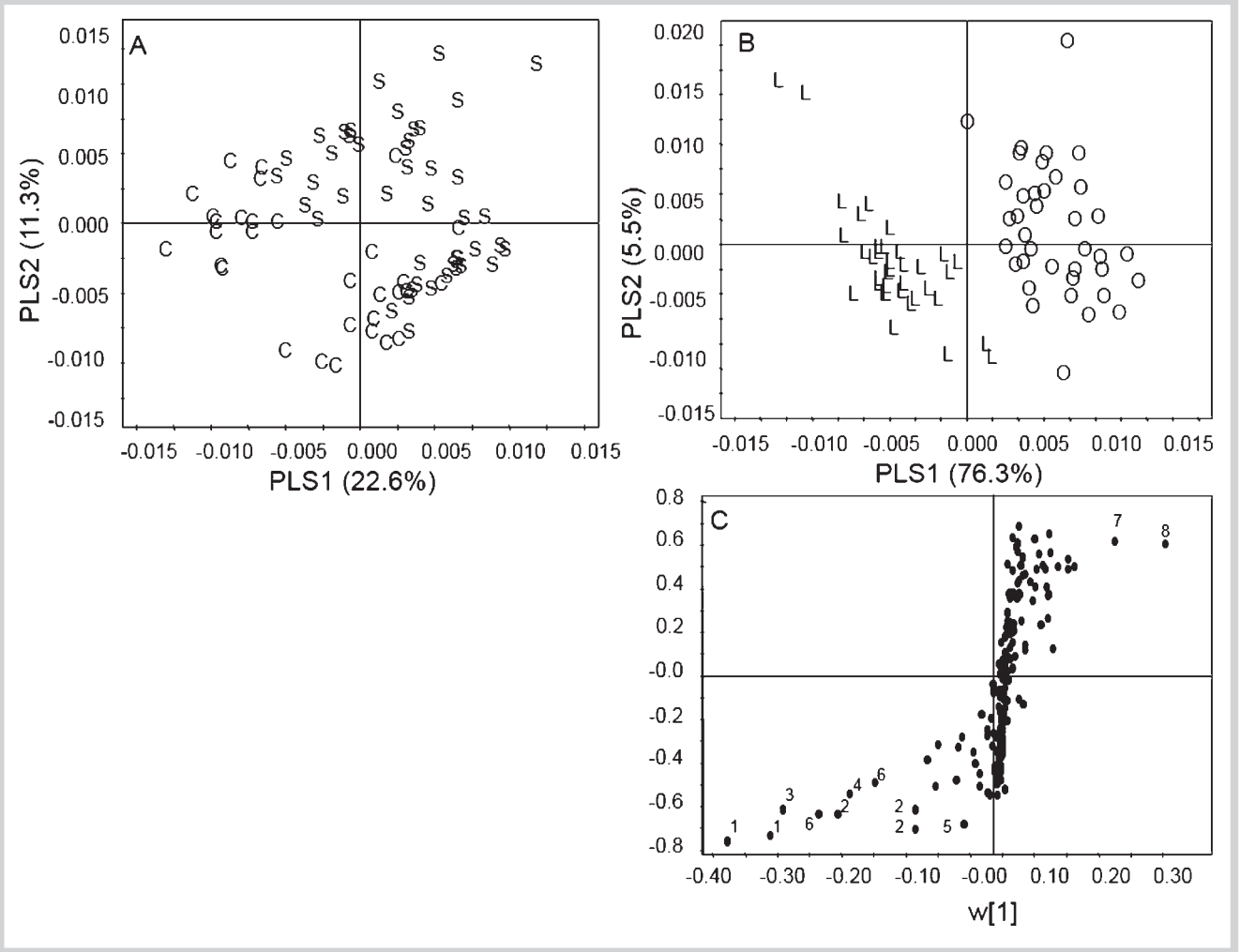

Fig. 4 Score plot of partial least square modeling-discriminant analysis after orthogonal signal correction with stress as a Y variable (**A**), novel environment (light/dark and open field) as a Y variable (**B**), and S-plot indicating markers responsible for the separation in the score plot (**C**). C, control group of zebra fish without netting strain; S, stress group exposed to netting strain; L, group exposed to the light/dark field; O, group exposed to the open field. 1, taurine; 2, histidine; 3, lactate; 4, alanine; 5, adenosine; 6, creatine; 7, isoleucine; 8, acetate.

in the metabolic flux in different ways. The use of NMR together with multivariate data analysis is clearly useful to assess the overall metabolic effect of stress or anxiety in zebra fish in different environments.

Open field and light/dark field tests have been used for many years to measure the behavior or anxiolytic/anxiogenic response of organisms to different stressors [18, 19]. These tests are based on the hypothesis that an animal will spontaneously explore novel environments. They are usually performed for quantitative and behavioral analyses of animals treated with drugs but have never been applied to study the effect of environmental changes at a metabolomic level. Classically, they have been used for rodent behavior studies, but have now also been validated for zebra fish [12]. Here we have used a chemometric technique (MvDA) to show that even a little exposure to these environments has an effect on zebra fish metabolome that can be revealed using NMR and multivariate data analysis. Many studies have been conducted to measure the effect of different types of stress on aquatic animals, but, to date, there is no report of a study such as this one.

Alanine accumulation is now universally accepted to be the first signal of stress in a variety of organisms [20]. In this case, we also detected a significant difference among the fish exposed to the two different environments, showing the evidence of stress. Alanine has been found to modulate the peripheral nervous system and affect the cardiac response to circulating catecholamines [21]. In our study, we found that alanine tended to be relatively increased in fish exposed to a light/dark field, as compared to fish exposed to an open field, irrespective of stress. Alanine is also a major raw material for gluconeogenesis and is produced by skeletal muscles and erythrocytes [1]. Any change in the levels of free alanine in the body can be an indicator of its usage in the gluconeogenesis pathway under stress conditions. The injection of alanine has been reported to produce sedative effects in chicks and this has been interpreted to occur through the activation of glycine receptors, as glycine is an inhibitory neurotransmitter [22]. It is thus possible that under stress, the production of alanine in the body helps to overcome the anxiety and facilitate the movement of muscles. The exact function of alanine during stress is not clearly understood but it has been speculated that it could stimulate the gene encoding for the synthesis of stress protein.

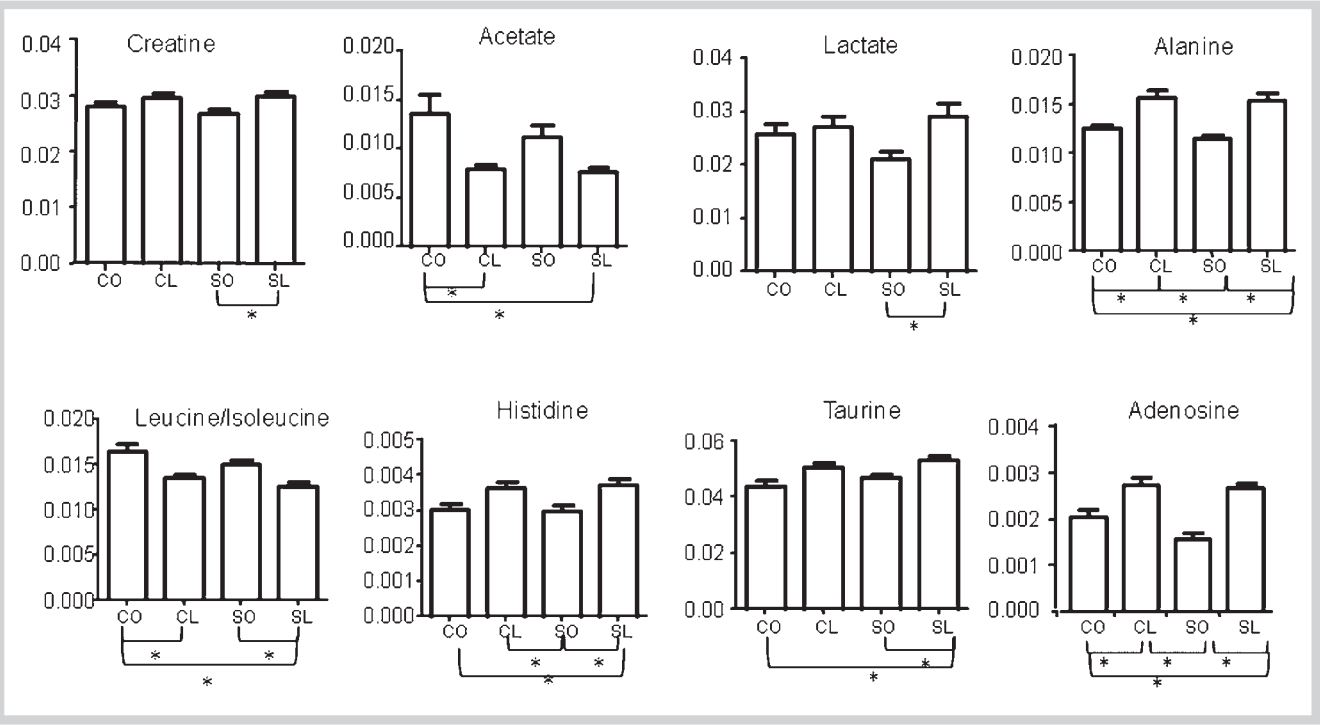

Fig. 5 Relative quantification graphs of the contributing metabolites based on ^{1}H NMR intensity. Line joining on the base shows the significant differences (< 0.05 %) among groups. CO, control group exposed to the open field; CL, control group exposed to the light/dark field; SO, stress group exposed to the open field; SL, stress group exposed to the light/dark field. * Statistically significant.

Alanine was also found to be increased in fish after two hours of stress. This may be attributed to the fact that alanine is the preferred amino acid for glucogenolysis under aerobic conditions in fish [22, 23].

In the case of lactate and creatine, the only difference we detected was among the stressed fish exposed to the light/dark and open fields. Lactate is generally a product of the glucogenolysis pathway and is produced under anaerobic conditions. It is also a major raw material for the gluconeogenesis pathway, so a decrease in its level can be an indicator of its utilization in this pathway [24]. Thus, any change in the quantity of lactate can be attributed to decreased aerobic conditions or to the utilization of lactate in the formation of glucose, as both glucose and lactate levels alter under stress or struggle [25, 26]. The OSC-PLS DA model, however, did not show glucose to be one of the main variables affecting the zebra fish. On the other hand, creatine is also an energy related compound, which is responsible for the supply of energy to all parts of the body, especially muscles. Creatine, in the form of phosphocreatine, usually helps to replenish the adenosine tri-phosphate (ATP) pool. Here we also found adenosine to be increased in the light/dark field-exposed group, thus supporting the existence of a direct correlation between creatine production and ATP synthesis.

Our study also revealed a significant difference in taurine levels in the stress-affected fish exposed to the light/dark field as compared to the control fish exposed to the open field. Taurine can also be a stress indicator, as taurine levels have been reported to increase during stress periods [27]. Taurine is a sulphur-containing amino acid that does not participate in peptide formation but still plays a very vital role. Taurine biosynthesis involves the formation of cysteine sulfinate catalyzed by the enzyme cysteine dioxygenase and the decarboxylation of cysteine sulfinate to hypotaurine by sulfinate decarboxylase [28]. It is known to be abundant in the heart, kidneys, and muscles of animals [29], and is involved in many physiological functions, mainly as a neurotransmitter, conjugated with bile acids, in the cardiovascular system, and in the prevention of oxidative stress or other toxicity-related stress [29]. Taurine has also been recognized as a metabolite that is affected by stress [30].

The adenosine moiety seemed to be highly significant in different groups as seen in **Fig. 5**. The level of adenosine was found to be higher in groups exposed to the light/dark field as compared to the open field. The main function of adenosine in the body is to act as an energy carrier in the form of ATP. It is also known for its activity as a homeostatic modulator, a function common to all cell types [31]. Along with these functions, adenosine also acts as an inhibitory neurotransmitter, confers neuronal protection, and is a learning facilitator [32]. The higher level in the light/dark field-exposed zebra fish group might be due to many factors, such as neuronal activity under different environments or the metabolic demand. It has been established that adenosine can produce a sleep-like effect [32], but at this stage in our study, it is difficult to establish the precise reason for this shift in zebra fish.

In the case of histidine, we found a significant difference among the control and stressed groups exposed to different novel environments and also among the stressed zebra fish of both groups (**Fig. 5**). Histidine is an alpha amino acid that is synthesized from adenosine triphosphate and phosphoribosyl pyrophosphate. It was found in high levels in the light/dark field-exposed group then in the open field-exposed group. As discussed earlier, the same pattern was found in the adenosine moiety, so it seems quite likely that an increase in adenosine could also result in an increase in histidine levels. Histidine is also a precursor of the

neurohormone and neurotransmitter histamine, which in turns regulates the histaminergic system involved in the alertness of animals [33]. It is thus quite possible that the metabolism of zebra fish reacts accordingly when exposed to a novel environment. A negative correlation between histidine production and anxiety-related behavior similar to that induced by novel environment exposure in HDC (histidine decarboxylase)-lacking female mice was observed [34]. It thus seems possible that during the period of exposure to novel environments applied in our experiment, this system affected the exploration and learning behavior of zebra fish in different ways [35].

In this study, we applied the powerful approach provided by metabolomics to understand the effect of exposing adult zebra fish to different novel environments. The study showed how minor changes in the environment could regulate the physiological functions of an organism, proving that it is crucial to take even minimum variables into consideration. Furthermore, this kind of study may be able to help in developing new strategies that include metabolomics along with the traditional approach in the screening of novel compounds.

Materials and Methods

Animal husbandry

Male and female adult zebra fish (*Danio rerio* F. Hamilton, 1882) of AB wild-type were purchased from Selecta Aquarium Speciaalzaak (Leiden, The Netherlands), which obtains stock from Europet Bernina International BV (Gemert-Bakel). Fish were kept at a maximum density of 100 individuals in glass recirculation aquariums (L 80 cm; H 50 cm, W 46 cm) on a 14-h light:10-h dark cycle (lights on at 8 : 00 in the morning). Water and air temperature were maintained at 24.5–27 °C and 23 °C, respectively. Newly purchased fish were allowed to adapt to our facility for at least four months before experimental testing began. The fish were fed twice daily with "Spirulina" brand flake food (O. S. l. Marine Lab., Inc.) and twice a week with frozen food (Dutch Select Food, Aquadistri BV). All experimental procedures were approved by the local committee on animal bioethics and welfare of Leiden University (app. no. 08 229, app. date: March 2009).

Stress procedure

The procedure for stress exposure was adapted from [12]. A total of 76 male zebra fish, 12 months of age, were used in this experiment and divided into control (n = 31) and stress (n = 45) groups. Briefly, the procedure consisted of exposing the zebra fish to 15 min netting strain, after which the stress group was released into two different environments: light/dark field (n = 22) and open field (n = 23). Controls were exposed directly to the light/dark field (n = 16) and open field (n = 15) for 5 min without netting stress. After 5 min, the zebra fish were collected and flash frozen in liquid nitrogen for further analysis.

NMR sample preparation

A simple sample preparation method that is already in use by our group was applied [36–40]. Zebra fish were ground to total homogenization in liquid nitrogen, freeze-dried, and transferred to a micro tube (2 mL) to which 1.5 mL 50% CD_3OD and 50% D_2O (KH_2PO_4 buffer, pH 6.0) containing 0.01% TMSP (w/w) were added. The mixture was vortexed for 1 min, ultrasonicated for a further 20 min, and centrifuged at 13 000 rpm at room temperature for 10 min. An aliquot of 750 µL was then transferred to a 5-mm NMR tube.

NMR measurements

NMR spectra were recorded at 25 °C on a 600 MHz Bruker DMX-600 spectrometer (Bruker) operating at a proton NMR frequency of 600.13 MHz. CD_3OD was used as the internal lock. Each ^{1}H-NMR spectrum consisted of 128 scans complying with the parameters used by our group [36].

Data preprocessing and statistical analysis

All of the spectra were referenced, base-lined, and phase-corrected using Topspin v. 2.1 (Bruker). Visual inspection was done by superimposing and stacking the spectra using MestReNova v.6.0.2 (Mestrelab Research S. L.).

^{1}H NMR spectra (from zebra fish) were automatically reduced to ASCII files. Spectral intensities of ^{1}H-NMR spectra were scaled to total intensity and reduced to integrated regions of equal width (0.04 ppm) corresponding to the region of δ 0.4–δ 10.0 using Amix software (Bruker). The regions of δ 4.8–δ 4.9 and δ 3.30–δ 3.34 were excluded from the analysis because of the residual signal of the deuterated solvents. PCA and OSC combined with the PLS DA test were performed with SIMCA-P software (v. 12.0, Umetrics) based on a Pareto scaling method. Statistical analyses were performed using GraphPad Prism (v. 5.03 for Windows, GraphPad Software). The region of the spectra between δ 0.8 and δ 4.0 corresponds to aliphatic compounds including amino acids and organic acids, from δ 4.0 to δ 5.5 to carbohydrates, while the remaining region δ 5.5 to δ 8.6 corresponds to phenolic compounds.

Supporting information

Score plot of PLS-DA and validation of model by permutation test can be found as Supporting Information.

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Conflict of Interest

No conflict of interest exists.

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