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The Investigation of Early Metabolic Level Perturbation of Northern Quahog (*Mercenaria mercenaria*) in Response to Brevetoxin

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Abstract

Brevetoxins are a type of neurotoxin produced in red tide blooms. Northern quahogs (*M. mercenaria*) are extensively used in commercial aquaculture farming, and early-stage metabolomics studies can provide early warnings of brevetoxins for farmers. In this study, NMR-based metabolomics was performed to investigate the response of clam gills and digestive glands under a series of sublethal doses of brevetoxins. Our study showed that the brevetoxin PbTx-2 had minimal influence on the physical activities of *M. mercenaria* for a short exposure time (24 hours). However, major metabolic level perturbations were observed in the clam gill extracts from the 1 ppb treatment. In addition, in the low concentration (0.1ppb) study, clam gills showed combinational metabolite perturbations, as observed by an OPLS-DA study. The highly disturbed metabolites in the gill samples were the upregulated serine, glucose, hypotaurine, and glycine and the downregulated lactate, leucine, isoleucine, threonine, biotin, taurine, and valine. The results indicated that the brevetoxin PbTx-2 potentially affects glycolysis, glycine, serine, and threonine metabolism, taurine and hypotaurine metabolism, and biotin metabolism. While the digestive

gland had less significantly changed metabolites, the potential combinational metabolite changes from PCA were observed from the 5-ppb treatment. Glucose and glycine are the primary metabolites that showed high contributions to the OPLS-DA model, which indicates the potential influence of digestive activities. The study indicated that metabolomic analysis of the gills and digestive glands of *M. mercenaria* is a feasible method to monitor the toxicity of brevetoxins, especially under sublethal doses in marine water.

1 Introduction

Harmful algal blooms (HAB), also called red tide, are seriously damaging to the coastal aquatic environment(1). The toxic dinoflagellate *Karenia brevis* (*K. brevis*) is the main algae of the red tides, naturally occurring blooms along Florida's Gulf of Mexico coast(2). Brevetoxins are a family of neurotoxins produced by *K. brevis*(3–5) that bind to voltage-gated sodium ion channels in marine organisms that ingest them, disrupting neurological function in a manner that often proves fatal. The adverse effects of brevetoxins are not limited to only the aquatic environment but also to humans living or engaging in activities around these aquatic environments(6). Brevetoxins are broadly classified as A-type and B-type, based on their structures(7). A three-year-long study examined the lingering effects of brevetoxin on hard clams and was still able to detect persistent B-type brevetoxin in shellfish months after the blooms had dispersed(8). Even though both toxin types are of concern, major studies have focused on B-type brevetoxins, which are naturally abundant in nature(9). Therefore, our work chose the B-type brevetoxin, PbTx-2 for further studies. In 2014, Rolton and associates discovered higher mortality rates in *M. mercenaria* larvae exposed to brevetoxins and a higher percentage of larvae with physical abnormalities(10). *Mercenaria mercenaria* (Linnaeus 1758), also known as the northern quahog, is one of the two species of hard clam that are prolific in the state of Florida. Clam larvae also had a lower shell length on average, which may be particularly concerning when considering the implications for shell length at harvest (11). In 2018, Rolton and associates also found that sexually mature *M. mercenaria* demonstrate an increase in undetermined gender and a reduced rate of fertilization in clam gametes (12). During red tide, the average concentration of brevetoxins is 5–30 ppb(13) with aerosol diameters around 6-8 μm(14) At these low concentrations, significant respiratory challenges have been reported.. Brevetoxin-contaminated shellfish also pose a significant risk to human health, and cause a disease known as Neurotoxic Shellfish Poisoning (NSP) (15). In order

to test for and prevent NSP, three metabolites are used as biomarkers in hard clams: S-desoxy BTX-B2, BTX-B2, and BTX-B1(16). Red tide threatens public health, tourism, economic activity, and aquatic organisms (17–19). As a result, there is a growing need for methods of early detection of brevetoxins that are cheaper, quicker, and more cost-efficient.

M. mercenaria and other marine bivalves are often used as bioindicators due to their ability to accumulate environmental pollutants through the filter-feeding process, their low mobility, and their ease of access(20). In addition, the metabolic enzymes of bivalves are less active than the other marine organisms, causing them to retain pollutants for a longer time(7). The *M. mercenaria* is a highly regarded species and an excellent source of omega-3 fatty acids(21,22). They are therefore harvested year-round, making them economically significant(23). The production of *M. mercenaria* has been reported to be over 8 million pounds in the U.S.(24)

Metabolomics is one approach that can simultaneously study the changes of a large number of metabolites using platforms such as nuclear magnetic resonance (NMR) after various treatments(25–27). It has been applied to the study of ocean acidification(28) and coastal herbicide pollutants(29), and the siphonal autotomy of clams(30). Environmental stressors such as high heat exposure, salinity, and pharmaceutical pollutants have also been studied using metabolomics(31–33). An NMR metabolomics analysis of zebrafish embryos exposed to sublethal doses of PbTx-2 discovered that PbTx-2 is neuronal excitotoxicity, and the carbohydrate and energy metabolism pathways are seriously interrupted (34). Therefore, studying *M. mercenaria* using metabolomics can provide early markers before the critical damage in the red tide season. In this study, we applied PbTx-2 to *M. mercenaria* in a 24-hour exposure using different concentrations ranging from 0.1 ppb to 10 ppb, which represents the early-stage exposure during red tides(35). NMR-based metabolomics was applied to study the metabolic level changes in clam gills and digestive glands, which can provide early warning and treatment to minimize the damage of clams in the red tide season.

2 Methodology

2.1 Clam exposure experiment

M. mercenaria samples were acquired from Bay Shellfish Co. in Terra Cierra, Florida. The clams acquired ranged from 16 to 18 months old. *M. mercenaria* were acquired from Bay Shellfish Co. in Terra Cierra, Florida, with clams ranging from 16 to 18 months of age. Upon

acquisition, the clams were transferred to a cooler for transport to the laboratory. A drip acclimation method was employed, allowing water from the tank to flow into the cooler over a period of 4 hours to facilitate gradual acclimatization to the new water conditions. Following acclimation, the clams were introduced into a designated tank. After an initial 12-hour period, they were fed 15 mL of a shellfish diet composed of marine microalgae species, including Isochrysis, Pavlova, and Tetraselmis. Water quality parameters were monitored daily, maintaining salinity at 35 ppt, pH at 7.5, dissolved oxygen at 7 mg/L, and temperature at 24 °C. Clams were fed every other day and maintained in the tank for two weeks to ensure adequate acclimation. Any dead clams were promptly removed to prevent adverse effects on water quality. Aerators with sponge filters were applied to provide dissolved oxygen for the clams and promote the growth of algae and plankton that the clams could eat.

PbTx-2 was freshly prepared and added to each tank with different concentrations, and seven different tanks were used, including a control tank. The Brevetoxin PbTx-2 concentrations were 0, 0.1, 0.5, 1, 2,5, and 10 ppb. Ten clams were placed in each of the tanks and all of them were used for the study without exclusion. Clams were left in various concentrations of brevetoxins for 24 hours before harvesting, and the samples were immediately frozen in liquid nitrogen and transferred into a Thermo Scientific freezer at -80℃ for further studies.

2.2 Metabolites extraction

The clam gill and digestive glands were collected after the clams were thawed on ice. The polar metabolites were extracted using a chloroform, methanol, and water approach with slight changes (36). Briefly, the tissue was broken down using a BEADBUG™3 Microtube homogenizer with beads in the 0.4 mL of methanol and 0.085 mL of deionized water solution mixture for 180 seconds at 3500 RMP. Samples in each tube were vortexed and mixed with 0.4 mL of chloroform and 0.2 mL of deionized water. It was then centrifuged under 14,000 RPM at 4 ℃. The polar phase was collected and dried using a Savant Speed Vacuum Concentrator SPD120 and stored in a -80℃ freezer for further analysis.

2.3 NMR metabolomics

The dried sample was resuspended in 0.1 M phosphate buffer in D_2O for the NMR study, and the final sample included 0.5 mM TSP and 0.05% NaN_3 (650 µL). The samples were centrifuged

under 14,000 RPM at 4 ℃, and a solution of 600 μL was transferred in a 5mm NMR tube for each sample before further study.

A JEOL 400 MHz NMR was used for this experiment. All 70 samples were placed in the NMR and run using a presaturation experiment on Delta 5.3.3 (JEOL Ltd.) with 64 scans and 4s relaxation delay. Peak shapes were monitored with the TSP half peak width (smaller than 1.5 Hz). NMR spectra were zero filled 1 time and windows function single exponential 0.25 Hz, baseline was corrected using automatic baseline correction by Delta. The data was transferred to Matlab after NMR data collection. NMR peaks were extracted using the average peak width based on a published work(37). After analysis through Matlab, the specific metabolites were analyzed using Chenomx 8.6 manually. The principal component analysis (PCA) and orthogonal partial least squares-discriminant analysis (OPLS-DA) were carried out using PLS-Toolbox (Eigenvector Research, Inc). The NMR peaks were aligned using the internal standard TSP. Samples were normalized to total intensity. The mean center and pareto scaling were applied for PCA and auto scaling was applied for OPLS-DA.

3 Results

3.1 *M. mercenaria* observation

During the brevetoxin test, all clams survived with no physical changes observed. The clams started to show minor signs of impact by burying themselves in the sand. This was found after the treatment, when the concentrations were higher than 2 ppb, indicating signs of burrowing behavior(38). The behavior is a sign of various biotic and abiotic factors and an indication of the clam responses to brevetoxins in this study. Previous studies by Sokolova et al. have revealed that abiotic factors such as an increase in salinity have an intracellular imbalance in clams leading to burrowing(39). In their work, they postulated other factors such as oxygen concentration, pH levels, and other contaminants could lead to similar observations. The investigation of behavioral changes in metabolic alteration is well established. Bailey, Lauren A., et al. have discovered that the abiotic variable (temperature) is linked to protein phosphorylation leading to high mortality in *Mytilus galloprovincialis*(40). Therefore, the weakened activity observed here (burrowing) provides an opportunity to study the metabolic level response before physical damage can be observed.

3.2 Metabolic response to gill extracts

In the gill extracts, 36 metabolites were identified (Table S1), and various types of metabolites showed significant differences from the control ($p < 0.05$) (Table 1). Specifically, hypotaurine was significantly upregulated in all groups, while lactate and threonine were significantly downregulated. In the PCA study, the score plot showed a distinct difference from the 1 ppb treatment to the 10 ppb treatment (Figure S1 C-F), but 0.1 and 0.5 ppb treatment has mixed score values (Figure S1 A and B). The OPLS-DA results showed that the 0.1 and 0.5 ppb treatment can be distinguished from the control with relatively reliable models (Figure 1) with a cross-validation error rate of 0.35 for 0.1 ppb and five LVs for 0.5 ppb.

3.3 Metabolic response to digestive gland extracts

In the digestive gland, 35 metabolites were identified (Table S2). The metabolite changes were less significant than the gill extract. Alanine, glucose, glutamate, and malate were the metabolites that significantly changed ($p < 0.05$) in at least one test group (Table 2). The PCA score showed that all the groups were mixed without a clear pattern and the example PCA score plot for the higher concentration groups (5 and 10 ppb) is shown in Figure S3. However, OPLS-DA results showed that 5 and 10 ppb treatment can be distinguished from the control with relatively reliable models (Figure 3), which indicates the combinational changes in the metabolic level.

4 Discussion

4.1 The gill metabolite responses to brevetoxin

While individual metabolite changes are important, metabolomics studies also pay high attention to the combinational contributions of the metabolites. Therefore, chemometric approaches such as PCA and OPLS-DA are applied in this study. PCA is an approach designed for description and is used to interpret information on combination metabolites. OPLS-DA was designed for classification, mainly applied to show the combinational effect only when PCA cannot clearly describe the data. The OPLS-DA was only applied when cross-validation had an error rate lower than 0.4 in this study. The cross-validation error rate was also tested with permutation tests ($n = 1000$) with average p values smaller than 0.3 (70% chance of difference). Since the gradual changes were observed in the PCA study (Figure 2), and similar error rates were obtained in different groups of data sets with consistent results, the overfitting risk of the OPLS-DA is relatively low. The Q2 values of the OPLS-DA in 0.1 and 0.5 ppb of the gill samples were around

0.3 (Figure S8). Since overfitting models are usually negative (41), the models were relatively good. In addition, the 10 ppb study has good PCA separation and also has a Q2 of around 0.3 (Figure S8. The relatively low Q2 may indicate that the whole metabolic profiling perturbance is not critical, but metabolic pathway perturbation was obtained, which can also be observed by the pathway analysis.

Major metabolites perturbation

The PCA score plot showed distinct separation in 1, 2, 5, and 10 ppb treatment, indicating that the major metabolites perturbation can be observed after 1 ppb brevetoxin treatment. The 1 ppb concentration of brevetoxin is likely to cause major damage to the health of clams even when no physical damage can be observed.

The PCA score and loading biplot show high similarity in the separation and the metabolites that highly contributed to the separation (Figure S4). The PCA loadings are the metabolites contribution to the PCA model, and the loadings values (metabolites) contribute more to the score values (sample) on the same separation directions. Taurine, lactate, biotin, threonine, valine, leucine, isoleucine, and tartrate were downregulated, whereas maltose, glycine, glucose, and hypotaurine were upregulated in all the treatment groups from 1 ppb to 10 ppb. Though the p-values of some metabolites are higher than 0.05, the PCA results showed confidence in the high metabolic perturbation (Figure 5).

The PCA plots in all the tested groups revealed a notable pattern with increased brevetoxin concentration even at the lower concentrations where no distinctive difference was observed. (Figure 2) The overall PCA score plot indicates further evidence of the low-concentration metabolic perturbation. The major metabolites contributing to the separation trend are the upregulated serine, glucose, hypotaurine, and glycine, and the downregulated lactate, leucine, isoleucine, threonine, biotin, taurine, and valine. (Figure S2). The metabolites are highly related to the metabolic pathways that brevetoxin affected, which are likely glycolysis, glycine, serine and threonine metabolism, taurine and hypotaurine metabolism, and biotin metabolism.

Low-concentration brevetoxin study

The metabolites' repose or perturbation to low concentration treatments is critical in studying the brevetoxin metabolism. The metabolite changes can be observed in lower concentrations even though no distinct metabolite changes were observed in the PCA study. Since the statistical t-test

could not represent the combined effect, an OPL-DA model was built to distinguish these metabolites at lower concentrations. In both the 0.1 and 0.5 ppb treatment studies, the OPLS-DA study showed relatively high reliable models built with cross-validation error rates lower than 0.4. The biplot showed that the major metabolites contributing to the models are glycine, hypotaurine (upregulated), valine, lactate, biotin, and threonine (downregulated) in both the 0.1 and 0.5 ppb treatment. In the lowest concentration 0.1 ppb, which is a very low level of brevetoxin, the metabolites biotin, lactate, threonine, and taurine were significantly downregulated, while hypotaurine and taurine were significantly upregulated ($p < 0.05$). In addition, the upregulation of glycine and downregulation of taurine, serine, and valine showed combinational effects in response to the brevetoxin at 0.1 ppb level while the single metabolite t-test was not significant. The metabolite changes in the PCA model overlap with the OPLS-DA study in the low concentration (0.1 ppb). In conclusion, the metabolites in glycolysis, glycine, serine and threonine metabolism, taurine and hypotaurine metabolism, and biotin metabolism showed higher perturbation in low concentration (0.1 ppb). In conclusion, the metabolites, glucose, glycine, serine, threonine, taurine hypotaurine, and biotin in their respective metabolism showed higher perturbation at the lowest brevetoxin concentration (0.1 ppb).

Overall metabolic pathway

The gill metabolites showed high perturbation in response to the brevetoxin PbTx-2 exposure within 24 hours of investigation. Due to the filter-feeding nature of the clams, the gills are one of the first organs that came in contact with the brevetoxin. Biotin was downregulated and could have effects on the citric acid cycle (TCA) since it involves as a cofactor with propionyl CoA[27]. The down-regulation of biotin is a sign of energy source consumption in response to brevetoxin. A similar trend can be observed on lactate. Lactate, a product of anaerobic glycolysis can also be used as an energy source. However, the sugars including glucose and maltose, were upregulated which indicates the clams tend to use other forms of energy than glucose. Similar phenomena can also be observed by the TCA closely related amino acids, including leucine, isoleucine, valine, and threonine. Acetate was also downregulated, which is likely related to the TCA cycle (Figure 4). Other amino acids, including serine and alanine were also downregulated in a low significant level and have less importance in the PCA model. Glycine and choline were reported as the source of sarcosine, which can act as an osmolyte(42),(43). Meanwhile, the hypotaurine and taurine are

also osmolytes which are associated with osmotic regulation(44). Those metabolites are also related to TCA via serine, but serine is not highly affected. Therefore, the upregulation of glycine, choline, and hypotaurine is a likely response to osmotic regulation. The downregulation of taurine was likely a result of its conversion into hypotaurine(44). Similar results have been discovered in a recent study of paralytic shellfish toxin contamination; high levels of shellfish toxin exposure to mussels lead to disturbances in osmoregulation and alterations to energy and amino acid metabolism (45).

4.2 The digestive glands metabolite responses to brevetoxins

The digestive gland of *M. mercenaria* produces digestive enzymes which help food absorption and is critical to the health of the clams. Unlike the gill, the digestive gland metabolic changes in response to brevetoxin were relatively weak. While the PCA score plot showed no clear patterns, OPLS-DA studies suggested that the metabolic changes can be observed in the combinational effect at 5 and 10 ppb cases, while single metabolite changes are not significant using the critical p-value as 0.05 (Figure 3B). In the 5 and 10 ppb groups, Q2 were both larger than 0 but smaller than the gill samples with a larger error rate (around 0.4), which indicated lower whole metabolic profiling perturbance. Judging by the plot with both loadings and scores, the major metabolites that highly contributed to the separation were the upregulation of glucose ($p < 0.1$), and the downregulation of glycine ($p < 0.1$). The upregulation of glucose is the same as in the gill samples, where it is likely caused by the energy conversion from other metabolites such as lactate (weak downregulation). Glycine plays an essential role in digestion, and the downregulation of glycine in the digestive gland is likely caused by the digestion activity, which has an opposite response than in the gill samples (46,47).

In summary, the digestive glands showed signs of similar metabolic pathway influence in the glycolysis but much weaker. The significant osmolytes-related metabolite changes were not observed, indicating less influence in the short time exposure. However, the downregulation of glycine indicates that digestive activities may be affected.

5 Conclusion

NMR metabolomics was performed to investigate the response of clam gills and digestive glands under a series of sublethal doses of brevetoxin PbTX-2. Our study showed that the brevetoxin PbTx-2 has very limited influence on the physical activities of the *M. mercenaria* for a short exposure time (24 hours) when the concentration is lower than 10 ppb. However, major metabolic

level perturbations can be observed in the clam gill extracts from the 1 ppb treatment. In addition, in the low concentration (0.1ppb) study, clam gills have combinational metabolite perturbations judging by an OPLS-DA study. The metabolites perturbation in the gill samples were the upregulated serine, glucose, hypotaurine, and glycine and the downregulated lactate, leucine, isoleucine, threonine, biotin, taurine, and valine. Therefore, the brevetoxin PbTx-2 potentially affected the glycolysis, glycine, serine, and threonine metabolism, taurine and hypotaurine metabolism, and biotin metabolism. The gill osmolytes-related metabolites were also highly influenced. The digestive glands had less perturbation but showed potential combinational metabolite changes from the 5 ppb level. Glucose and glycine are the main metabolites that showed high contributions to the OPLS-DA model, which indicates the potential digestive activities influences. The study indicated that the metabolomics of the gills and digestive glands of *M. mercenaria* is a potential approach that can study the early stage brevetoxins impact during red tide.

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7 Data Availability

Data for this paper, including the NMR raw data are available at Mendeley Data at https://data.mendeley.com/datasets/ysrx6322tc/1

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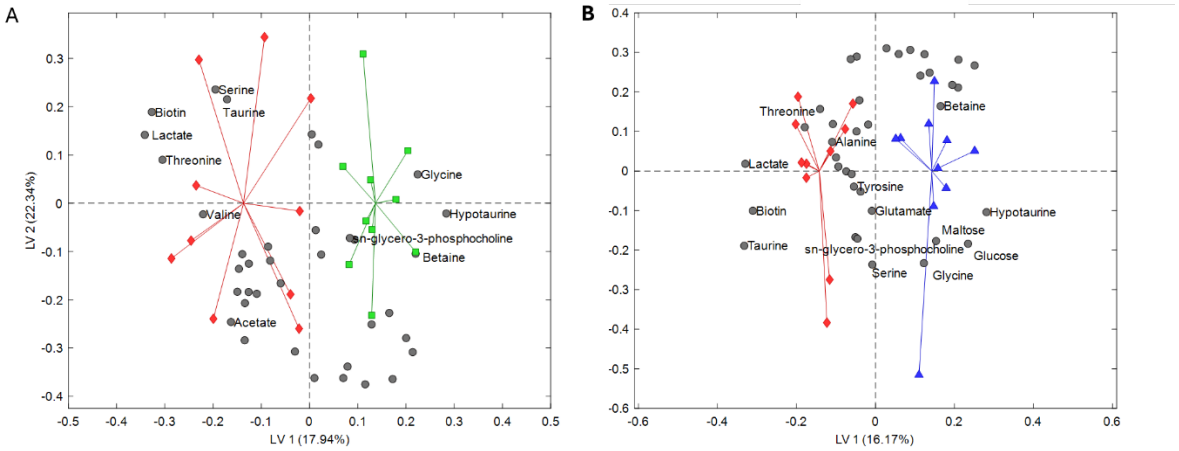

Figure 1. The OPLS-DA study for lower concentration treatment (0.1 and 0.5 ppb) for gill samples. PLS-DA Error rate (average of false positive rate and false negative rate for class) in cross validation is **A**, 0.35 and **B**, 0.35 A, R2 0.88 Q2 0.15 B, R2 0.60 Q2 0.31. The pertutation test with 1000 trials showed Cross-Validated Wilcoxon P values **A** 0.143 and **B** 0.344.

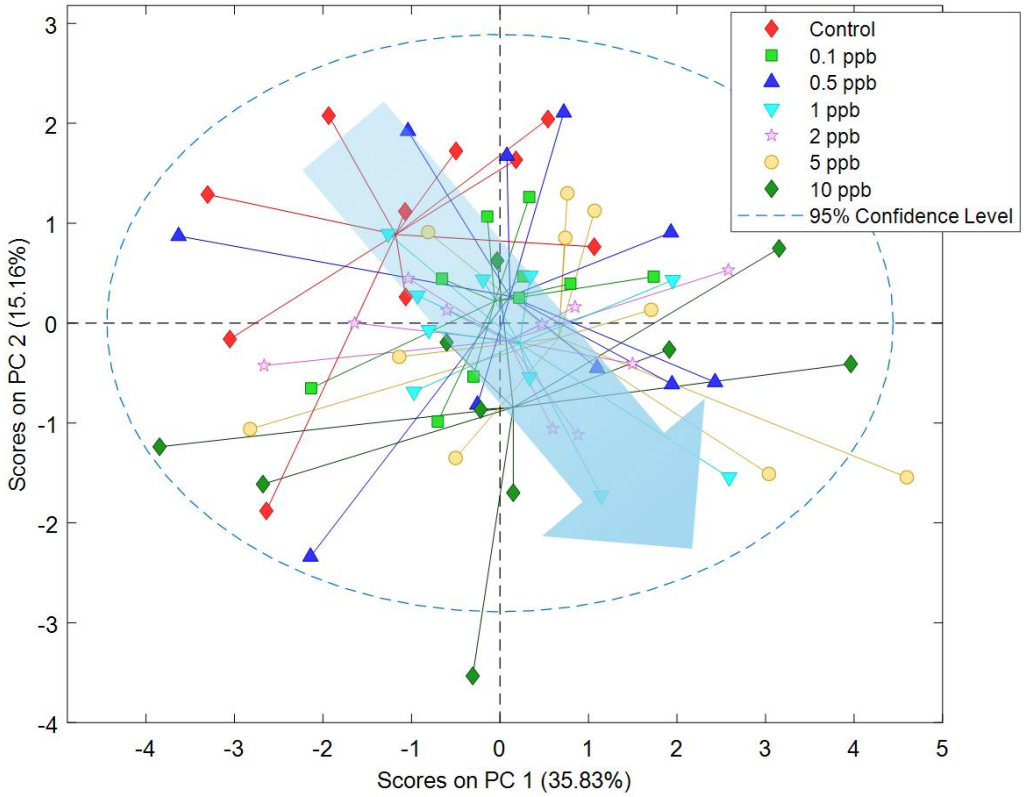

Figure 2. The PCA score for all the treatment groups in gill extracts which can show the trend of the concentration influence.

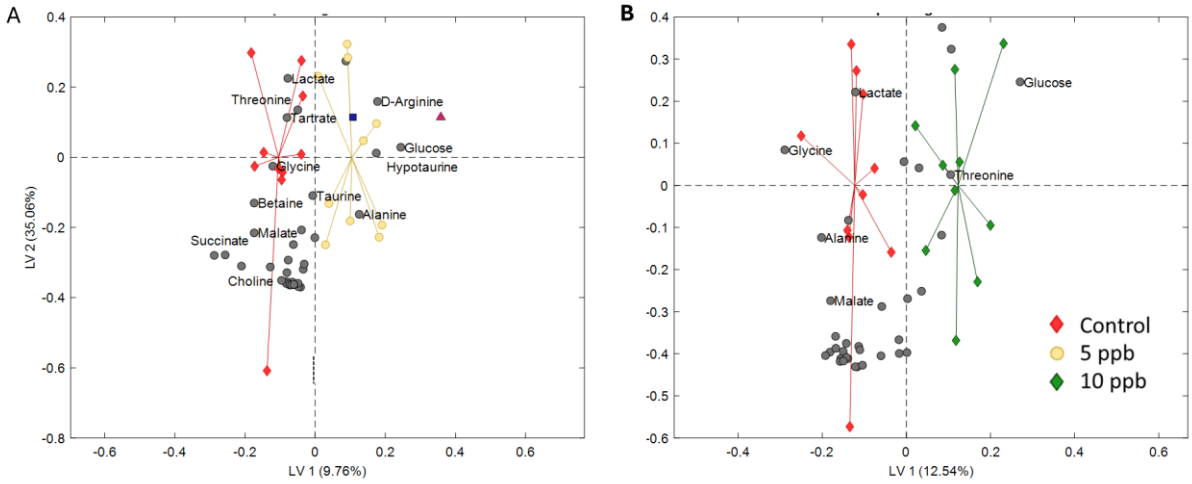

Figure 3. The digestive glands study. The OPLS-DA biplot of the control vs 5 and 10 ppb treatment, cross validation error rates for **A** and **B** are 0.4 (5 LVs) and 0.35 (3 LVs). A, R2 0.58 Q2 0.14 B, R2 0.67 Q2 0.03. The pertutation test with 1000 trials showed the cross-validated Wilcoxon p values A 0.424 and B 0.288.

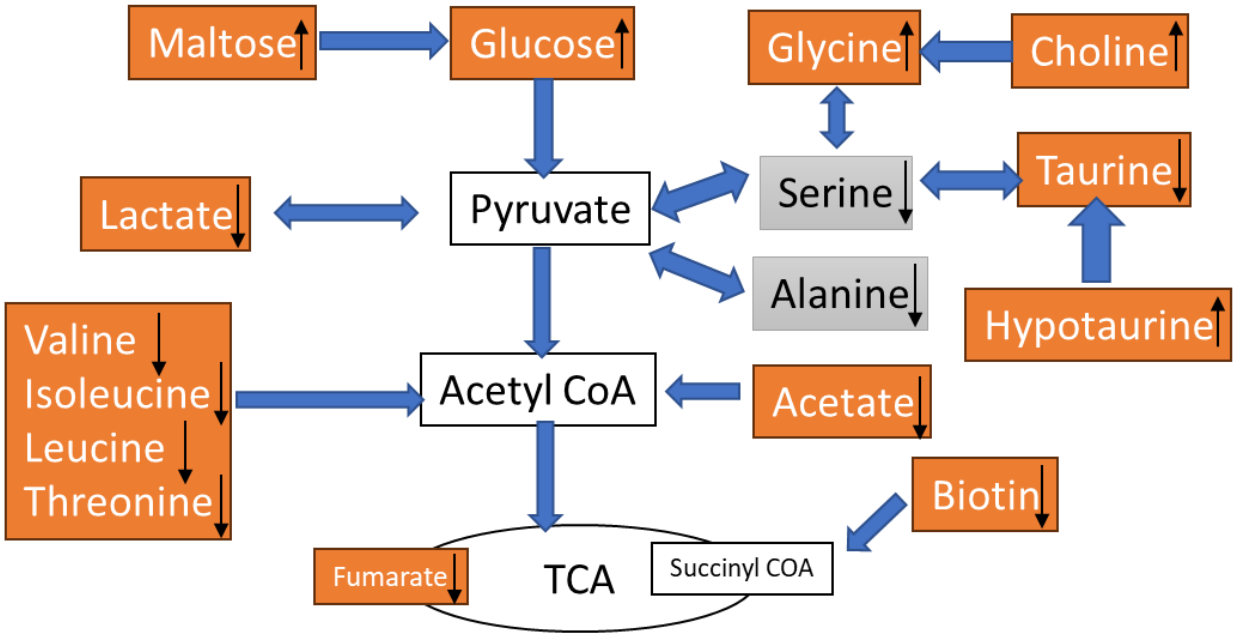

Figure 4. The major metabolic pathway observed in the gill extracts after brevetoxin treatment. The orange box indicates the metabolites showed high contribution in the PCA plot and the arrows indicate the metabolites change directions, upregulation and downregulation.

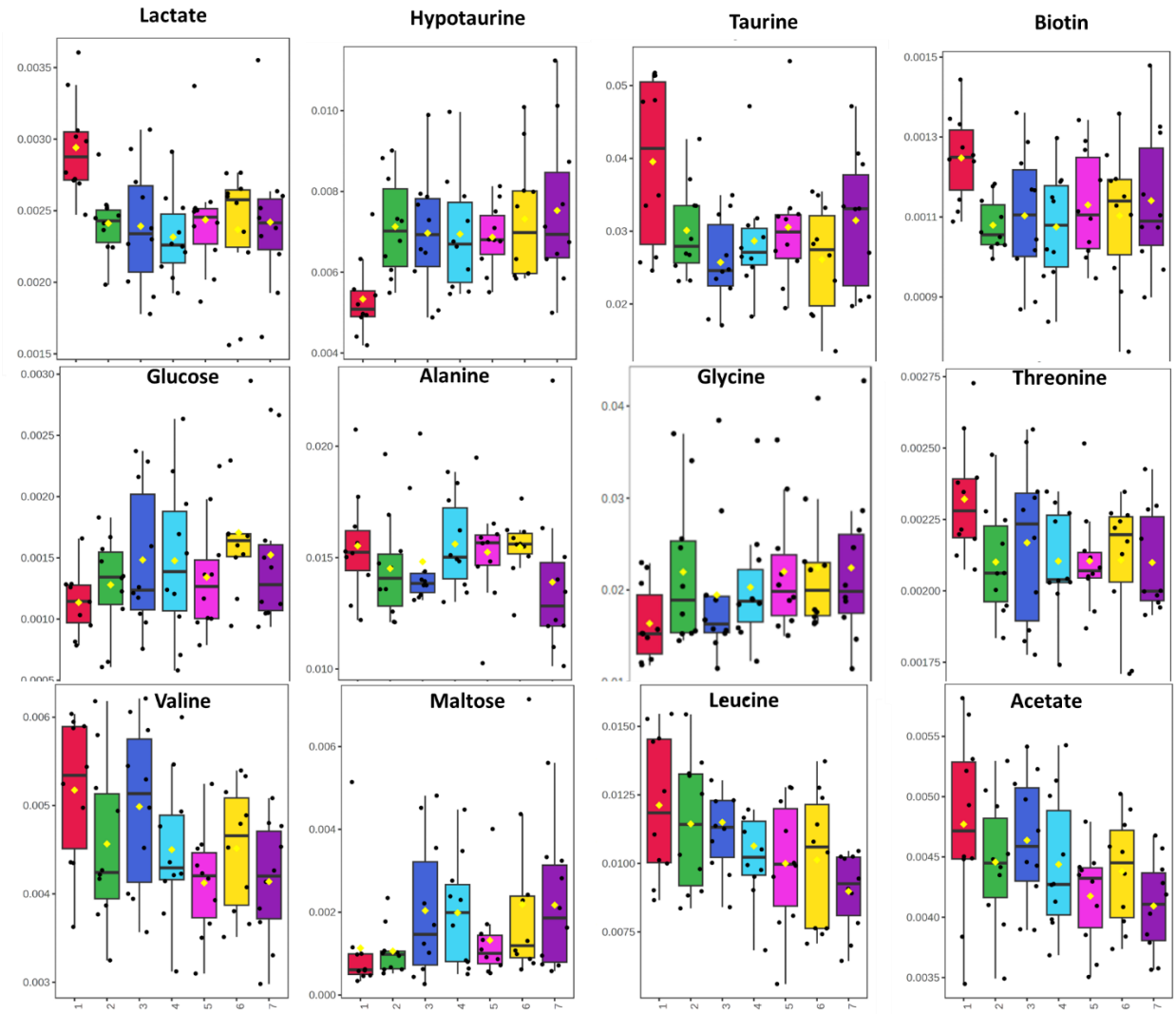

Figure 5. The box plots of selected major metabolites in gill extracts. The labels are 1, control (red); 2, 0.1 ppb (green); 3,0.5 ppb (blue); 4, 1 ppb (cyan); 5, 2 ppb (pink); 6, 5 ppb (yellow); 7, 10 ppb (purple).

Table 1 Selected gill metabolites extract changes after various concentrations of brevotoxin treatment. FC is folder change which is the average values of each study divided by the average of the control group. P values were calculated from student t-tests. The metabolites that showed significant regulations in several of the experimental groups were highlighted in bold.

		0.1 ppb		0.5 ppb		1 ppb		2 ppb		5 ppb		10 ppb	
Metabolites	ppm	p values	FC	p values	FC	p values	FC	p values	FC	p values	FC	p values	FC
Leucine	0.97	5.61E-01	0.94	5.68E-01	0.95	2.06E-01	0.9	7.47E-02	0.82	1.01E-01	0.84	4.18E-03	0.74
Hypotaurine	2.65	2.02E-03	1.34	9.40E-03	1.31	8.60E-03	1.3	1.23E-03	1.29	3.00E-03	1.37	5.20E-03	1.41
Valine	0.99	1.41E-01	0.88	6.44E-01	0.96	8.56E-02	0.9	4.69E-03	0.8	7.15E-02	0.87	7.05E-03	0.8
Lactate	4.12	8.36E-04	0.82	5.60E-03	0.81	3.64E-04	0.8	7.43E-03	0.83	4.92E-03	0.81	1.51E-02	0.82
4-hydroxyphenylacetate	7.19	2.61E-01	0.91	8.86E-01	0.99	1.30E-01	0.9	1.50E-01	0.89	5.98E-01	0.96	1.55E-02	0.81
Choline	3.2	2.89E-02	1.32	8.78E-02	1.26	5.79E-02	1.3	8.42E-02	1.27	8.56E-02	1.37	1.62E-02	1.43
Threonine	4.26	2.37E-02	0.91	1.90E-01	0.93	2.37E-02	0.9	2.18E-02	0.91	4.11E-02	0.91	2.00E-02	0.9
Acetate	1.92	3.04E-01	0.93	6.59E-01	0.97	2.86E-01	0.9	4.36E-02	0.88	1.82E-01	0.92	2.30E-02	0.86
Isoleucine	1.02	7.41E-01	0.97	6.33E-01	0.96	1.08E-01	0.9	1.00E-01	0.86	1.17E-01	0.87	4.35E-02	0.82
Phenylalanine	7.41	5.53E-01	0.96	8.35E-01	0.98	2.79E-01	0.9	3.02E-01	0.93	4.84E-01	0.95	4.85E-02	0.85
Glycine	3.56	7.02E-02	1.34	2.97E-01	1.19	1.32E-01	1.2	3.98E-02	1.34	4.37E-02	1.37	6.74E-02	1.37
Glucose	5.23	3.56E-01	1.13	1.04E-01	1.31	1.44E-01	1.3	2.39E-01	1.18	9.35E-03	1.5	9.77E-02	1.34
Taurine	3.29	3.74E-02	0.76	3.81E-03	0.65	2.30E-02	0.7	7.09E-02	0.77	6.62E-03	0.66	1.05E-01	0.8
Biotin	4.46	6.43E-04	0.86	3.43E-02	0.88	6.71E-03	0.9	4.81E-02	0.91	4.05E-02	0.88	1.26E-01	0.91
Asparagine	2.93	7.52E-02	1.28	1.07E-01	1.34	1.37E-01	1.3	2.42E-01	1.22	1.08E-01	1.34	3.35E-01	1.21
Butyrate	0.9	1.12E-01	1.11	5.42E-02	1.12	1.35E-01	1.1	3.90E-01	1.05	1.08E-01	1.12	4.53E-01	1.07
Betaine	3.27	8.23E-02	1.08	2.51E-01	1.05	3.79E-01	1	3.24E-02	1.1	1.30E-01	1.06	5.20E-01	1.04

Table 2 Selected metabolites change in the digestive glands compared to the control. FC is folder change which is the average values of each study divided by the average of the control group. P values were calculated from student t tests.

		0.1 ppb	0.5 ppb	1 ppb	2 ppb	5 ppb	10 ppb

	ppm	p values	FC	p values	FC	p values	FC	p values	FC	p values	FC	p values	FC
Glycine	3.56	9.47E-01	0.99	6.23E-02	0.8	3.14E-01	1.11	5.92E-01	0.94	5.05E-01	0.94	7.90E-02	0.82
Glucose	5.23	7.58E-01	0.96	8.53E-01	0.98	9.00E-01	0.99	2.28E-01	0.84	2.41E-01	1.12	8.20E-02	1.23
Alanine	1.5	2.32E-01	1.23	8.39E-01	1.03	2.76E-01	1.19	7.42E-02	1.24	5.04E-01	1.07	2.17E-01	0.86
Malate	4.29	3.83E-02	1.21	4.01E-01	1.07	6.03E-01	0.98	4.63E-01	0.97	3.36E-01	0.96	2.27E-01	0.95
Uracil	7.55	2.14E-01	1.32	7.86E-01	1.05	8.01E-01	0.96	1.87E-01	0.8	7.13E-01	0.94	2.42E-01	0.84
sn-Glycero-3-phosphocholine	3.23	4.94E-01	1.07	7.26E-01	0.96	9.04E-01	1.01	8.34E-01	1.03	1.82E-01	0.85	2.61E-01	0.88
Fumarate	6.53	1.83E-01	1.28	7.91E-01	1.05	7.84E-01	0.96	1.23E-01	0.81	5.93E-01	0.93	3.47E-01	0.89
Aspartate	2.7	2.87E-01	1.14	8.83E-01	1.02	7.81E-01	1.03	7.80E-01	0.97	8.09E-01	0.97	3.50E-01	0.89
Phenylalanine	7.42	2.87E-01	1.21	8.80E-01	1.02	7.41E-01	0.96	1.82E-01	0.84	7.83E-01	0.96	3.54E-01	0.89
Glutamine	2.43	2.98E-01	1.2	9.95E-01	1	9.71E-01	0.99	3.04E-01	0.85	7.87E-01	0.96	3.55E-01	0.87

Data Availability

Data for this paper, including the NMR raw data are available at Mendeley Data at https://data.mendeley.com/datasets/ysrx6322tc/1