**Goal:** Analysis the concentration of betaine, choline, TMA, TMAO and amino acids in plasma in *Megalobrama amblycephala*.

**Experimental design:**

1. Healthy juvenile fish (*Megalobrama amblycephala*: average weight, 5.47±0.085g) were obtained and acclimated. Fish were randomly distributed into 12 plastic tanks (25 fish/tank). These tanks were randomly assigned to four groups (CD, HCD, HCB and HC4B), ensuring three replicates per group (4×3=12).
2. Control diet (CD group) was formulated to contain 27.11±0.38% carbohydrates. The high-carbohydrate diet (HCD group) was formulated to contain over 36.75±0.92% of easily digestible carbohydrates (mostly from flour). The betaine treatment diet (HCB) to contain a high level of carbohydrates (35.64±0.43%) with 1% betaine supplemented for 16 weeks. HC4B group was fed a combination of HCD (first twelve weeks) and HCB diets (last four weeks).
3. After 16 weeks of the feeding trial, approximately 24h after the last feeding, fish in all tanks were anaesthetized with MS-222 (Sigma Aldrich, USA) at the concentration of 100 mg L-1. Fifteen specimens from each tank were selected randomly to obtain the blood. Blood was obtained from the caudal vein using sterile syringes with pre-added anticoagulant solution and then centrifuged (3000×g) at 4°C for 10 min to obtain the serum, which was quickly frozen in liquid nitrogenand stored at -80°C for biochemical assays.
4. betaine, choline, TMA, TMAO and amino acids in plasma were determined using high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) on the Ultimate3000 (Dionex, Sunnyvale，USA)-API 3200Q TRAP (AB Sciex, Framingham, MA, USA). The analyses were performed by the Beijing Mass Spectrometry Medical Research Co.,Ltd. (Beijing, China).
5. HPLC-MS/MS detail: Fifty μl of protein precipitant (containing NVL) was added to 50 μl of serum solution of each sample, mixed thoroughly, and centrifuged at 13200 rpm for 4 min. The supernatant (10 μl) was mixed with 50 μl methanol borate buffer (0.1 M, pH=8.8) thoroughly. For amino acids, the above solution was mixed with 20 μl of diethyl ethoxymethylene-malonate at 55°C for 15 min. Fifty μl of the solution was analysed by the HPLC-MS/MS system consisting of a SRD-3600 Solvent Rack with analytical 6-channel vacuum degasser, a DGP-3600A pump, WPS-3000TSL analytical autosampler, and a tcc-3200 column compartment. Chromatographic separations were performed on an MSLab C18 column (150×4.6 mm, 5 μm). The mobile phase A was 0.1% formic acid in water (v/v), and the organic mobile phase B was 0.1% ammonium formate in acetonitrile (v/v) with pH=5.8. The solvent was delivered to the column at a flow rate of 1 ml min−1, and retention times are shown in **Table 1**. The conditions for mass spectrometry detection, optimized to obtain the highest signal intensity, were as follows: mode=positive-ion mode; ion spray voltage=5500 V; nebulizer gas pressure=55 psi; curtain gas pressure=20 psi; collision gas pressure=medium; turbo gas temperature=500°C; entrance potential=10 V; collision cell exit potential=2 V. Nitrogen gas was used as the collision gas in the multiple reaction monitoring mode (**Table 2**). Calibration solution (**Table 3**) was used for calibration of the system and quantification of metabolites (**Tables 4 and 5**). The data were processed using Analyst software version 1.5.1 (Applied Biosystems).

**Samples names**

CD group (included sample named 1, 2, 3, 4, 5, 6); HCD group (included sample named 7, 8, 9, 10, 11, 12); HCB group (included sample named 13, 14, 15, 16, 17, 18); HC4B group (included sample named 19, 20, 21, 22, 23, 24) (**Table 6**). Abbreviation and full name showed in **Table 7**.

**Table 1** Flow retention time and gradient (v/v) of chromatographic separations

|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Retention time (min) | 0.0 | 1 | 1.1 | 5 | 5.1 | 8 | 8.1 | 12 |
| A (%) | 90 | 90 | 30 | 30 | 0 | 0 | 90 | 90 |
| B (%) | 10 | 10 | 70 | 70 | 100 | 100 | 10 | 10 |

**Table 2** Parameters of multiple reaction monitoring (MRM) mode

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Chemical Name | MRM (precursor ion/fragment ion) | Internal Standard | Declustering Potential (v) | Impact Energy (v) |
| TMA | 60.080/44.000 | TMAO-d9 (85.050/66.050) | 20 | 15 |
| TMAO | 76.080/58.050 | TMAO-d9 (85.050/66.050) | 20 | 15 |
| Betaine | 118.070/59.100 | TMAO-d9 (85.050/66.050) | 20 | 15 |
| Choline | 104.300/60.200 | TMAO-d9 (85.050/66.050) | 20 | 15 |
| Ala | 260.100/171.010 | NVL(288.150/171.010) | 30 | 20 |
| Pro | 286.150/171.010 | NVL(288.150/171.010) | 30 | 20 |
| Val | 288.150/171.010 | NVL(288.150/171.010) | 30 | 20 |
| Ile | 302.150/171.010 | NVL(288.150/171.010) | 30 | 20 |
| Gln | 317.150/171.010 | NVL(288.150/171.010) | 30 | 20 |
| Glu | 318.100/171.010 | NVL(288.150/171.010) | 30 | 20 |
| Phe | 336.200/171.010 | NVL(288.150/171.010) | 30 | 20 |
| Arg | 345.210/171.010 | NVL(288.150/171.010) | 30 | 20 |
| Tyr | 352.200/171.010 | NVL(288.150/171.010) | 30 | 20 |
| Trp | 375.200/171.010 | NVL(288.150/171.010) | 30 | 20 |
| Lys | 487.200/171.010 | NVL(288.150/171.010) | 30 | 20 |
| Leu | 302.150/171.010 | NVL(288.150/171.010) | 30 | 20 |

TMAO: trimethylamine-N-oxide, NVL: norvaline

**Table 3** Metabolite standards origins

|  |  |  |
| --- | --- | --- |
| Chemical Name | Origin | Product Number |
| TMA | Aladdin, Shanghai, China | [T108428](http://www.aladdin-e.com/zh_cn/t108428.html) |
| TMAO | Aladdin, Shanghai, China | [T162063](http://www.aladdin-e.com/zh_cn/t162063.html) |
| Betaine | Aladdin, Shanghai, China | [B105555](http://www.aladdin-e.com/zh_cn/b105555.html) |
| Choline | Aladdin, Shanghai, China | [C108898](http://www.aladdin-e.com/zh_cn/c108898.html) |
| Amino acids | Mslab, Beijing, China | MSLAB-50AA |

**Table 4** Accuracy of calibration solution in HPLC-MS/MS system

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| Analyte Peak Name | Sample Type | Analyte Peak Area (counts) | Analyte Peak Height (cps) | Analyte Concentration (ng/mL) | Calculated Concentration (ng/mL) | Accuracy (%) |
| TMA | Standard | 0.00E+00 | 0.00E+00 | / | No Peak | / |
| TMA | Standard | 7.87E+02 | 2.03E+02 | 25 | 15.4 | 61.6 |
| TMA | Standard | 3.80E+03 | 9.61E+02 | 125 | 165 | 132 |
| TMA | Standard | 1.43E+04 | 2.91E+03 | 625 | 683 | 109.28 |
| TMA | Standard | 2.62E+04 | 5.72E+03 | 1250 | 1270 | 101.6 |
| TMA | Standard | 4.87E+04 | 1.04E+04 | 2500 | 2390 | 95.6 |
| TMAO | Standard | 4.09E+03 | 7.86E+02 | 6.25 | 6.78 | 108.48 |
| TMAO | Standard | 2.22E+04 | 3.51E+03 | 31.3 | 34 | 108.6261981 |
| TMAO | Standard | 3.38E+04 | 5.76E+03 | 62.5 | 51.5 | 82.4 |
| TMAO | Standard | 8.26E+04 | 1.20E+04 | 125 | 125 | 100 |
| TMAO | Standard | 1.63E+05 | 2.39E+04 | 250 | 245 | 98 |
| TMAO | Standard | 3.41E+05 | 4.93E+04 | 500 | 513 | 102.6 |
| Betaine | Standard | 0.00E+00 | 0.00E+00 | / | No Peak | / |
| Betaine | Standard | 1.23E+03 | 2.99E+02 | 25 | < 0 | / |
| Betaine | Standard | 4.01E+03 | 1.00E+03 | 125 | 119 | 95.2 |
| Betaine | Standard | 1.42E+04 | 3.30E+03 | 625 | 681 | 109.0 |
| Betaine | Standard | 2.53E+04 | 5.94E+03 | 1250 | 1300 | 104.0 |
| Betaine | Standard | 4.63E+04 | 1.12E+04 | 2500 | 2460 | 98.4 |
| Choline | Standard | 2.27E+01 | 1.33E+01 | / | No Peak | / |
| Choline | Standard | 6.70E+02 | 1.50E+02 | 25 | 24.1 | 96.4 |
| Choline | Standard | 2.62E+03 | 5.89E+02 | 125 | 152 | 121.6 |
| Choline | Standard | 1.02E+04 | 2.15E+03 | 625 | 651 | 104.2 |
| Choline | Standard | 1.79E+04 | 3.79E+03 | 1250 | 1160 | 92.8 |
| Choline | Standard | 3.88E+04 | 6.80E+03 | 2500 | 2540 | 101.6 |
| Ala | Standard | 0.00E+00 | 0.00E+00 | / | No Peak | / |
| Ala | Standard | 2.53E+05 | 2.00E+06 | 50 | 53.3 | 106.6 |
| Ala | Standard | 4.39E+05 | 1.93E+06 | 100 | 103 | 103 |
| Ala | Standard | 6.44E+05 | 1.66E+06 | 200 | 183 | 91.5 |
| Ala | Standard | 1.44E+06 | 1.84E+06 | 400 | 378 | 94.5 |
| Ala | Standard | 2.96E+06 | 1.75E+06 | 800 | 832 | 104 |
| Pro | Standard | 0.00E+00 | / | / | No Peak | / |
| Pro | Standard | 2.54E+05 | 2.00E+06 | 25 | 23.7 | 94.8 |
| Pro | Standard | 5.22E+05 | 1.93E+06 | 50 | 56.9 | 113.8 |
| Pro | Standard | 6.72E+05 | 1.66E+06 | 100 | 88.2 | 88.2 |
| Pro | Standard | 1.68E+06 | 1.84E+06 | 200 | 206 | 103 |
| Pro | Standard | 3.05E+06 | 1.75E+06 | 400 | 400 | 100 |
| Val | Standard | 0.00E+00 | / | / | No Peak | / |
| Val | Standard | 2.60E+05 | 2.00E+06 | 25 | 22.6 | 90.4 |
| Val | Standard | 4.55E+05 | 1.93E+06 | 50 | 50.1 | 100.2 |
| Val | Standard | 7.70E+05 | 1.66E+06 | 100 | 110 | 110 |
| Val | Standard | 1.52E+06 | 1.84E+06 | 200 | 205 | 102.5 |
| Val | Standard | 2.65E+06 | 1.75E+06 | 400 | 387 | 96.75 |
| Ile | Standard | 0.00E+00 | / | / | No Peak | / |
| Ile | Standard | 8.90E+04 | 2.00E+06 | 6.25 | 5.96 | 95.36 |
| Ile | Standard | 1.69E+05 | 1.93E+06 | 12.5 | 12.8 | 102.4 |
| Ile | Standard | 2.76E+05 | 1.66E+06 | 25 | 25.4 | 101.6 |
| Ile | Standard | 6.06E+05 | 1.84E+06 | 50 | 51.3 | 102.6 |
| Ile | Standard | 1.09E+06 | 1.75E+06 | 100 | 98.3 | 98.3 |
| Gln | Standard | 0.00E+00 | / | / | No Peak | / |
| Gln | Standard | 1.56E+05 | 2.00E+06 | 50 | 46.9 | 93.8 |
| Gln | Standard | 3.29E+05 | 1.93E+06 | 100 | 101 | 101 |
| Gln | Standard | 5.65E+05 | 1.66E+06 | 200 | 203 | 101.5 |
| Gln | Standard | 1.32E+06 | 1.84E+06 | 400 | 426 | 106.5 |
| Gln | Standard | 2.26E+06 | 1.75E+06 | 800 | 772 | 96.5 |
| Glu | Standard | 0.00E+00 | / | / | No Peak | / |
| Glu | Standard | 2.10E+04 | 2.00E+06 | 6.25 | 5.67 | 90.72 |
| Glu | Standard | 4.55E+04 | 1.93E+06 | 12.5 | 13.2 | 105.6 |
| Glu | Standard | 7.81E+04 | 1.66E+06 | 25 | 26.7 | 106.8 |
| Glu | Standard | 1.58E+05 | 1.84E+06 | 50 | 49.1 | 98.2 |
| Glu | Standard | 3.02E+05 | 1.75E+06 | 100 | 99.1 | 99.1 |
| Phe | Standard | 0.00E+00 | / | / | No Peak | / |
| Phe | Standard | 5.71E+04 | 2.00E+06 | 6.25 | 5.87 | 93.92 |
| Phe | Standard | 1.20E+05 | 1.93E+06 | 12.5 | 12.4 | 99.2 |
| Phe | Standard | 2.24E+05 | 1.66E+06 | 25 | 26.6 | 106.4 |
| Phe | Standard | 4.87E+05 | 1.84E+06 | 50 | 51.8 | 103.6 |
| Phe | Standard | 8.68E+05 | 1.75E+06 | 100 | 97.1 | 97.1 |
| Arg | Standard | 0.00E+00 | / | / | No Peak | / |
| Arg | Standard | 4.90E+03 | 2.00E+06 | 12.5 | 13.2 | 105.6 |
| Arg | Standard | 1.01E+04 | 1.93E+06 | 25 | 24 | 96 |
| Arg | Standard | 2.05E+04 | 1.66E+06 | 50 | 51.9 | 103.8 |
| Arg | Standard | 4.09E+04 | 1.84E+06 | 100 | 90.4 | 90.4 |
| Arg | Standard | 9.14E+04 | 1.75E+06 | 200 | 208 | 104 |
| Tyr | Standard | 0.00E+00 | / | / | No Peak | / |
| Tyr | Standard | 3.85E+04 | 2.00E+06 | 6.25 | 6.12 | 97.92 |
| Tyr | Standard | 6.59E+04 | 1.93E+06 | 12.5 | 12.2 | 97.6 |
| Tyr | Standard | 1.09E+05 | 1.66E+06 | 25 | 25.4 | 101.6 |
| Tyr | Standard | 2.42E+05 | 1.84E+06 | 50 | 52.6 | 105.2 |
| Tyr | Standard | 4.18E+05 | 1.75E+06 | 100 | 97.4 | 97.4 |
| Trp | Standard | 0.00E+00 | / | / | No Peak | / |
| Trp | Standard | 4.13E+04 | 2.00E+06 | 6.25 | 6 | 96 |
| Trp | Standard | 8.24E+04 | 1.93E+06 | 12.5 | 11.9 | 95.2 |
| Trp | Standard | 1.67E+05 | 1.66E+06 | 25 | 27.3 | 109.2 |
| Trp | Standard | 3.49E+05 | 1.84E+06 | 50 | 51.2 | 102.4 |
| Trp | Standard | 6.33E+05 | 1.75E+06 | 100 | 97.4 | 97.4 |
| Lys | Standard | 0.00E+00 | / | / | No Peak | / |
| Lys | Standard | 1.26E+04 | 2.00E+06 | 12.5 | 13.1 | 104.8 |
| Lys | Standard | 2.33E+04 | 1.93E+06 | 25 | 25.8 | 103.2 |
| Lys | Standard | 3.61E+04 | 1.66E+06 | 50 | 47.2 | 94.4 |
| Lys | Standard | 7.91E+04 | 1.84E+06 | 100 | 94.2 | 94.2 |
| Lys | Standard | 1.64E+05 | 1.75E+06 | 200 | 207 | 103.5 |
| Leu | Standard | 0.00E+00 | / | / | No Peak | / |
| Leu | Standard | 2.03E+05 | 2.00E+06 | 12.5 | 12.4 | 99.2 |
| Leu | Standard | 3.32E+05 | 1.93E+06 | 25 | 23 | 92 |
| Leu | Standard | 6.32E+05 | 1.66E+06 | 50 | 55 | 110 |
| Leu | Standard | 1.25E+06 | 1.84E+06 | 100 | 101 | 101 |
| Leu | Standard | 2.28E+06 | 1.75E+06 | 200 | 196 | 98 |

Accuracy= (Calculated concentration - Analyte Concentration)/Analyte Concentration.

**Table 5** Regression equations between ‘Analyte Concentration’ (x; ng/ml) and ‘Analyte Peak Area’ (y; cps) for each metabolite

|  |  |
| --- | --- |
| Metabolites | Analyte regression equation |
| TMA | y = 20.2 x + 476 (r = 0.9967) |
| TMAO | y = 665 x + -417 (r = 0.9984) |
| Betaine | y = 18.1 x + 1860 (r = 0.9987) |
| Choline | y = 15.2 x + 304 (r = 0.9987) |
| Ala | y = 0.00201 x + 0.0194 (r = 0.9977) |
| Pro | y = 0.00431 x + 0.0249 (r = 0.9971) |
| Val | y = 0.00381 x + 0.0441 (r = 0.9980) |
| Ile | y = 0.00628 x + 0.00711 (r = 0.9996) |
| Gln | y = 0.00168 x + -0.000377 (r = 0.9984) |
| Glu | y = 0.00174 x + 0.000641 (r = 0.9990) |
| Phe | y = 0.00514 x + -0.00156 (r = 0.9988) |
| Arg | y = 0.000256 x + -0.000938 (r = 0.9969) |
| Tyr | y = 0.00241 x + 0.0045 (r = 0.9990) |
| Trp | y = 0.00374 x + -0.00181 (r = 0.9984) |
| Lys | y = 0.000452 x + 0.000402 (r = 0.9982) |
| Leu | y = 0.00655 x + 0.0206 (r = 0.9984) |

**Table 6** Samples names

|  |  |
| --- | --- |
|  |  |
| Sample names | Experimental variables |
| 1 | Control group |
| 2 | Control group |
| 3 | Control group |
| 4 | Control group |
| 5 | Control group |
| 6 | Control group |
| 7 | High carbohydrates group |
| 8 | High carbohydrates group |
| 9 | High carbohydrates group |
| 10 | High carbohydrates group |
| 11 | High carbohydrates group |
| 12 | High carbohydrates group |
| 13 | Betaine supplementary for 16 weeks |
| 14 | Betaine supplementary for 16 weeks |
| 15 | Betaine supplementary for 16 weeks |
| 16 | Betaine supplementary for 16 weeks |
| 17 | Betaine supplementary for 16 weeks |
| 18 | Betaine supplementary for 16 weeks |
| 19 | Betaine supplementary for 4 weeks |
| 20 | Betaine supplementary for 4 weeks |
| 21 | Betaine supplementary for 4 weeks |
| 22 | Betaine supplementary for 4 weeks |
| 23 | Betaine supplementary for 4 weeks |
| 24 | Betaine supplementary for 4 weeks |

**Table 7**. Abbreviation and full name in experiment

|  |  |
| --- | --- |
| Abbreviation | Full name |
| S0 | Standard solution=0 ng/ml |
| S5 | Standard solution=25 ng/ml |
| S25 | Standard solution=125 ng/ml |
| S125 | Standard solution=625 ng/ml |
| S250 | Standard solution=1250 ng/ml |
| S500 | Standard solution=2500 ng/ml |
| TCJ | betaine |
| DJ | choline |