Plasma metabolomic profiles reveal sex- and maturation-dependent metabolic strategies in sea lamprey (Petromyzon marinus)

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Abstract
Introduction Adult sea lamprey (Petromyzon marinus) cease feeding and migrate to spawning streams where males build nests, undergo final sexual maturation, and subsequently produce and release large quantities of bile acid pheromones that attract mature females. These animals are predicted to rearrange their metabolic pathways drastically to support their reproductive strategies, presenting advantageous opportunities to examine how sex and the maturation processes affect metabolism.

Objectives The objective is to investigate the metabolic differences between sexes and maturation states in sea lamprey that support changes in physiological functions.

Methods We compared plasma metabolomes of spawning and prespawning sea lamprey in both sexes using both non-targeted and targeted metabolomics approaches using UPLC/MS–MS with electrospray ionization in both positive and negative modes. The data were processed using Progenesis QI, Compound Discoverer and XCMS softwares for alignment, peak picking, and deconvolution of the peaks. Principle component analyses (PCA) and partial least squares discriminant analyses (PLS-DA) were performed using SIMCA and Metaboanalyst softwares to identify discriminating features, followed by fragmentation matching with extensive database search and pathway mapping.

Results The pheromonal bile acid biosynthesis was upregulated significantly in males compared to females. Spermiating males further upregulated bile acid biosynthesis by altering amino acid metabolisms, upregulating cofactors and nucleotide metabolisms, but downregulating carbohydrate and energy metabolisms.

Conclusion Plasma metabolomes are sex- and maturation-dependent and reflect the special metabolic demands at each life stage and reproductive strategy.

Keywords Petromyzon marinus · Bile acid biosynthesis · Targeted analyses · Untargeted analyses · Metabolic pathways · Sexual selection

1 Introduction

Adult sea lamprey (Petromyzon marinus) must rearrange their metabolomes drastically to support the demand for reproduction after they cease feeding. One of the pronounced physiological changes during their spawning migration is that they stop feeding and the lipid reserves function as the primary energy source during this period (Bird et al., 1993; Sheridan, 1988). It provides a unique opportunity to study how animals alter metabolic processes to mobilize energy reserves to fulfill biological functions.

In early spring adult sea lamprey cease feeding and migrate to spawning grounds (Applegate, 1951) where sexually mature spermiating males (SM) release large quantities of bile acid pheromones that attract ovulatory females (OF) to the nest for spawning (Buchinger et al., 2015; Chung-Davidson et al., 2010; Fissette et al., 2021; Johnson et al., 2006, 2009; Li et al., 2002; Scott et al., 2019). One SM can release up to 0.5 mg/h of a major component of the male sex pheromone, 3-keto petromyzonol sulfate (3kPZS; Li et al., 2002). On the contrary, females and prespermiating males (PSM) only release trace or non-detectable amounts of bile acids (Chung-Davidson et al., 2021). Since bile acid
pheromones are synthesized in the liver, transported via blood circulation to the gills, and released into the water (Brant et al., 2013; Fissette et al., 2021; Li et al., 2002; Siefkes et al., 2003), comparing sea lamprey plasma metabolomes between sexes and maturation states may help to infer metabolic strategies that sustain high pheromone production and release in SM.

The intricate networks of metabolic pathways are coordinated to meet the needs of the whole organism, i.e., to generate ATP for energy consumption and the building blocks for biosynthesis. ATP is generated by oxidation of fuel molecules such as glucose, fatty acids and amino acids, whereas the building blocks for biosynthesis are usually intermediates from various metabolic pathways (Stryer, 1995). We expect that SM employ effective metabolic strategies to generate ATP and building blocks for massive bile acid biosynthesis. Since blood plasma mediate biological functions between organs, plasma metabolomes provide a proxy for the metabolic status of an organism.

We hypothesize that adult sea lamprey alter metabolic pathways to facilitate different biological functions necessitated by sex and maturation status, resulting in dramatic differences in plasma metabolomes in preovulatory females (POF), PSM, OF and SM. Using both non-targeted and targeted approaches, we found that sea lamprey exhibited distinct plasma metabolomes in different sexes and maturation states, and SM maximized pheromone production by downregulating energy metabolisms but upregulating bile acid biosynthesis. There is an apparent trade-off between sexual signals (bile acid pheromones) and energy consumption in sea lamprey.

2 Methods

2.1 Animals

Migratory adult sea lampreys were collected from Ocqueoc River (Presque Isle, Michigan, USA) in the summer of 2019 by agents of the US Geological Survey at Hammond Bay Biological Station, Great Lakes Science Center, Millersburg, Michigan, USA. Pre-spawning lamprey were held in the lower Ocqueoc River (Presque Isle County, Michigan, USA) to induce sexual maturation, which was assessed daily by visual inspection of secondary sex characteristics and gentle expression of gametes (Brant et al., 2013). All animals were then transferred to Michigan State University (East Lansing, Michigan, USA) where samples were collected immediately upon arrival. Standard operating procedures for transporting, maintaining, handling, anesthetizing, and euthanizing sea lampreys were approved by the Institutional Committee on Animal Use and Care of Michigan State University (AUF # Li-02-17-030-99) and in compliance with standards defined by the National Institutes of Health Guide for the Care and Use of Laboratory Animals (Institute for Laboratory Animal Research, 2011). All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

2.2 Chemicals

HPLC grade methanol, acetonitrile, chloroform, and ammonium acetate, cholic acid (CA), deoxycholic acid (DCA), taurochenedeoxycholic acid (TCDDCA), and MS222 were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). Petromyzonol sulfate (PZS), 3-keto petromyzonol sulfate (3kPZS), petromyzonamine disulfate (PADS), petromyzonamine-24-monosulfate (PAMS-24), allocholic acid (ACA or 5α-cholic acid), 3-keto allocholic acid (3kACA) and deuterated 3-keto petromyzonol sulfate ([1H5]-3kPZS) were custom-synthesized from Bridge Organic, Inc. (Vicksburg, Michigan, USA).

2.3 Sample analyses

2.3.1 Sample preparation

Blood samples (n = 10 each for POF, PSM, OF, and SM) were collected by cardiac puncture with 10 mL heparinized vacutainers after animals were anesthetized with 0.02% MS222. Plasma samples (supernatant) were obtained by centrifugation at 1000×g, 4 °C for 20 min. Sample proteins were precipitated by adding 600 µl of ice-cold methanol to 300 µl of plasma (2:1, v/v), incubated at −20 °C overnight and centrifuged at 15,800×g at 4 °C for 15 min. The resulting supernatant was mixed with 600 µl of chloroform at 4 °C (on ice) at 100 rpm for 20 min, incubated at −20 °C for 30 min, and then centrifuged at 10,000×g at 4 °C for 30 min. The aqueous (top) layer was freeze dried and then reconstituted in 100 µl of 50% methanol in water. Quality control (QC) samples for male and female groups were prepared separately by pooling equal volumes of PSM and SM samples for male QC, and POF and OF for female QC.

2.3.2 Untargeted analyses

Samples were analyzed on a UPLC Q-Exactive Orbitrap system equipped with a heated electrospray ionization source (HESI). Chromatographic separations were optimized (detail see Supplementary Information) using an Acquity UPLC BEH C18 column (100 × 2.1 mm; 1.7 µm particle) with 10 mM ammonium acetate in water as solvent A and acetonitrile as solvent B. The injection volume was 10 µl. The mobile phase gradient was maintained as follows, 0 min: 5% B; 5 min: 38% B; 7 min: 55% B; 10 min: 70% B; 16 min: 95% B; 21 min: 100%
B; 21.5–25 min: 5% B. The flow rate was maintained at 0.3 ml/min and the column temperature at 55 °C throughout the analyses. Samples were analyzed in both positive and negative ionization modes using the full scan MS with data-dependent (dd)-MS2 acquisition mode. The Orbitrap parameters for the full scan acquisition were set as follows, resolution: 17,500; AGC target: 3e6; maximum injection time (IT): 50 ms; scan range: 80 to 1200 m/z. The parameters for dd-MS2 were set as follows, resolution: 17,500; AGC target: 1e6; IT: 50 ms; scan range: 200 to 2000 m/z; normalized collision energy: 10, 30, and 60. Source parameters were as follows, sheath gas flow rate: 48 AU; spray voltage: 3.5 kV; capillary temperature: 256 °C; auxiliary gas flow rate: 11; sweep gas flow rate: 2; auxiliary gas heater temperature: 413 °C.

### 2.3.3 Targeted analyses

Nine bile acids (including 6 sea lamprey-specific bile acids) were quantified in all samples. A Waters Acquity H-Class UPLC system connected to a Xevo TQ-S Triple Quadrupole mass spectrometer was used for the analyses. Separation was achieved using a Waters BEH C18 column (2.1 × 100 mm; 1.7 µm particle size) coupled to an Acquity UPLC BEH C18 VanGuard Pre-column (2.1 × 5 mm; 1.7 µm particle size). For sea lamprey-specific bile acids (3kPZS, PZS, PADS, PAMS-24, ACA, and 3kACA), 10 mM triethylamine (TEA) in water was used as mobile phase A and methanol was used as mobile phase B. The gradient was maintained as follows, 0 min: 40% B; 7–9 min: 99% B; 10–12 min: 40% B. The flow rate was maintained at 0.25 µl/min and the column temperature at 35 °C (Wang et al., 2015). For DCA, CA, and TCDDCA, water containing 7.5 mM ammonium acetate and 0.1% formic acid was used as solvent A, and acetonitrile/methanol (1:9; v/v) containing 7.5 mM ammonium acetate and 0.1% formic acid was used as solvent B. The gradient was maintained as follows, 0 min: 40% B; 7 min: 74% B; 7.01–9 min: 100% B; 9.01–12 min: 40% B. The flow rate was maintained at 0.25 µl/min and the column temperature at 30 °C. The injection volume was 10 µl in both cases (Li et al., 2015).

All bile acids were analyzed by electrospray ionization in the negative mode. The ESI–MS/MS parameters were set as follows, capillary voltage: 2.60 kV; extractor voltage: 5 V; source temperature: 150 °C; desolvation temperature: 500 °C; desolvation gas flow: 800 L/h (N2, 99.9% purity). Argon (99.9% purity) was introduced as the collision gas into the collision cell at a flow rate of 0.15 mL/min. The multiple reaction monitoring (MRM) and other parameters are provided in Supplementary Information (Table S1). MassLynx 4.2 software was used for data acquisition and data were processed using TargetLynx XS (Waters Corp., Milford, MA, USA).

### 2.4 Data processing

#### 2.4.1 Untargeted analyses

The workflow applied for data processing is illustrated in Fig. S1. Progenesis QI and Compound Discoverer 3.1 softwares were used to maximize the number of metabolite identification. Briefly, the spectra were selected from the raw data and then aligned with a retention time (RT) tolerance of 0.2 min and mass error of 5 ppm. The discriminating features were selected based on the ANOVA p value (<0.01) and fold change (FC > 1.2). Compound annotations of significant metabolites were based on the fragment matching with available databases, especially Human Metabolome Database (HMDB) (https://hmdb.ca/) and KEGG (https://www.genome.jp/kegg). Principal component analyses (PCA) and partial least squares discriminant analyses (PLS-DA) were performed by exporting the data to SIMCA 17 software (Umetrics, Sweden). Raw files were converted to.mzXML files using ProteoWizard (MS Converter; Chambers et al., 2012). Converted files were uploaded into an online platform XCMS (Smith et al., 2006) to produce a data matrix following the same discrimination criteria as described above (p value <0.01, FC > 1.2, and abundance >10,000).

#### 2.4.2 Targeted analyses

Data were processed using MassLynx 4.2 software (Waters Corp., Milford, MA, USA). The software produced a table with m/z, RT, and intensity (peak area) values for each variable in each sample. Linear relationship calculations between peak areas and concentrations were adopted by weighted least squares regression. For targeted analyses, FC between groups was calculated as the ratio of the mean concentrations, e.g., dividing the mean concentration of SM by the mean concentration of PSM.

#### 2.4.3 Statistical analyses

Multivariate data analyses were performed to find the similarities and differences between sample groups. PCA, an unsupervised dimensionality-reduction tool, was used for feature selection and classification, i.e., to identify patterns in the data and to check the trends and outliers, using SIMCA 17 software (Umetrics, Sweden) (Smilowitz et al., 2013). PLS-DA, a supervised machine learning dimensionality-reduction tool, was also performed as it is suited for metabolomics data with large number of features, noise and missing data, and fewer samples than features (Ruiz-Perez et al., 2020). The PCA and PLS-DA models were evaluated...
in terms of their goodness of fit (R2Xcum, R2Ycum) and goodness of prediction (Q2cum). R2Xcum is the cumulative modeled variation in X, R2Ycum is the cumulative variation in X correlated to Y, and Q2cum estimates the cumulative predictive ability of the model. A Q2cum value > 0.5 is considered a good model for metabolomics analyses (Dembélé et al., 2020; Wang et al., 2022).

2.4.4 Pathway analyses

Potential impacted pathways were assessed by pathway analyses using MetaboAnalyst 4.0 (http://www.metaboanalyst.ca/) and the Human Metabolome Database (HMDB; https://hmdb.ca/) (Chong et al., 2019). The platform iPath v.3 was used to map the impacted pathways whenever the annotated metabolites were listed in its database (Darzi et al., 2018).

3 Results

3.1 Metabolomic profiles showed sex differences and dramatic changes during sexual maturation

LC-HRMS analyses revealed 6554 significant features of metabolic fingerprints in ESI− mode and 7105 features in ESI+ mode. PCA analyses in both negative and
positive modes showed significant differences between PSM vs. SM, POF vs. OF, OF vs. SM, and POF vs. SM (Figs. 1, S2; Table S2). The goodness of fit for these models resulted in $R^2_X = 0.69$ for male sexual maturation (PSM vs. SM), $R^2_X = 0.715$ for female sexual maturation (POF vs. OF), and sex differences in mature adults (OF vs. SM: $R^2_X = 0.751$) and immature adults (POF vs. PSM: $R^2_X = 0.917$). The loadings plots for PSM vs. SM, POF vs. OF, OF vs. SM, and POF vs. SM in positive (Fig. S3) and negative (Fig. S4) modes are provided as supplementary information. In each comparison, the goodness of prediction $(Q^2)$ was greater than 0.5, indicating that the model was acceptable for metabolonomic analysis (Table S2). PLS-DA plots also confirmed significant differences between PSM vs. SM, POF vs. OF, OF vs. SM, and POF vs. SM (Figs. S5, S6). The goodness of fit for each comparison (PSM vs. SM; POF vs. OF; POF vs. PSM; OF vs. SM) resulted in $R^2_X > 0.5$, $R^2_Y > 0.8$, and $Q^2 > 0.5$ (Table S2). Quality control samples (QC) were injected to determine the intragroup variability and remove all features showing relative standard deviation (RSD) > 30%. PCA analysis detected high variation between two groups of QC samples in the female groups (POF, OF) in the first instance, and those samples were identified and replaced as QC.

Discriminant analyses revealed dramatic upregulations in sea lamprey-specific bile acids in the comparisons between sexes and male sexual maturation (Table 1). For example, PSM contained 244-fold (↑) PZS compared to POF, SM contained 155-fold (↑) PZ and 53-fold (↑) ACA compared to OF, and SM contained 314-fold (↑) ACA and 201-fold (↑) PZ compared to PSM. Other bile acids, including cholic acid, lithocholic acid and chenodeoxycholic acid-3-sulfate were also upregulated hundreds of folds in SM compared to PSM or OF. Changes in nucleotide and amino acid metabolisms were apparent in all comparisons. SM showed increases in adenine (↑ 77-fold) but decreases in creatine (↓ 81-fold) compared to OF. On the other hand, OF showed downregulations of fatty acid metabolism compared to POF (11-dehydrothromboxane: ↓ fourfold; palmitic acid: ↓ twofold).

### 3.2 Bile acid biosynthesis was increased drastically in conjunction with altered amino acid metabolite levels and reduced sugar and energy metabolite levels during male sexual maturation

Pathway analyses revealed that the most prominent changes between SM and PSM were the metabolites in primary and secondary bile acid biosynthesis (Table 1); e.g., lithocholic acid (↑ 153-fold) and cholic acid (↑ 134-fold). Interestingly, lamprey-specific bile acids were upregulated at least 2 times more than those common bile acids (Table 1); e.g., ACA (5α-cholic acid ↑ 314-fold), PZ (↑ 201-fold), and PZS (↑ 212-fold). Several amino acid metabolic pathways were affected during male sexual maturation (impact > 0.15, Table S5); e.g., tyrosine (↑ twofold), arginine (↓ twofold), glutamate (↑ twofold), and glutamine (↑ fourfold). Significantly changed metabolites are listed in Table 1, and their associated primary metabolic pathways are shown in Fig. S5. In general, metabolic pathway maps indicated that SM reduced TCA cycle-related activities and carbohydrate and energy metabolisms, and at the same time increased biosynthesis of bile acids, cofactors, and vitamins (Fig. S7).

On the contrary, most metabolic pathways were downregulated in OF compared to POF, including lipid metabolism (e.g., palmitate ↓ twofold; Tables 1, S5 and Fig. S8). Notably, two eicosanoid metabolites were downregulated (Table 1); i.e., prostaglandin C1 (↓ threefold) and 11-dehydrothromboxane B2 (↓ fourfold). Other notable changes were amino acid metabolisms (Table 1); e.g., methionine (↑ fourfold), tryptophan (↑ threefold), creatine (↑ fivefold), and pretyrosine (↑ 13-fold). Interestingly, D-aspartate but not L-aspartate were downregulated (↓ tenfold, Table 1).

Sex differences are most prominent in the pathways involved in biosynthesis of bile acids, cofactors, and vitamins (Tables 1 and S5, and Fig S9: OF vs. SM and Fig S10: POF vs. PSM), as males contained more bile acids than females (Tables S3, S4); e.g., PZS: ↑ 244-fold in PSM vs. POF; PZ: ↑ 155-fold in SM vs. OF (Tables 1, S5).

Since bile acid biosynthetic pathways showed the most dramatic changes between sexes and during male sexual maturation, we used targeted analyses to confirm the results from untargeted analyses. Indeed, SM plasma contained the highest levels of most bile acids, except TCDCA (Tables S3 and S4). PZS was the most abundant bile acid in SM and PSM, with concentrations of 19 µg/ml and 2 µg/ml, respectively (Table S3). Upregulations of bile acids were seen during sexual maturation (PSM vs. SM and POF vs. OF, Tables S3, S4), and the most dramatic changes were observed between sexes (males > females; OF vs. SM and POF vs. PSM; Tables S3, S4), consistent with the untargeted analysis results. A putative sea lamprey bile acid biosynthetic pathway is shown in Fig. 2. Higher levels of bile acids in SM vs. OF (Fig. 2 and Tables S3, S4) were exemplified by PZS (↑ 388-fold), ACA (↑ 138-fold), and 3kACA (↑ 83-fold). For pre-spawning adults, higher levels of bile acids in PSM vs POF (Tables S3, S4) were found in PZS (↑ 388-fold), 3kPZS (↑ 255-fold), and PAMS-24 (↑ 69-fold). ACA was probably important for sea lamprey sexual maturation since it was the most upregulated metabolite in PSM vs. SM (↑ 87-fold; Table S4) and POF vs. OF (↑ 15-fold; Table S4).
Table 1  List of metabolic pathways and the most discriminant metabolites in the comparison of different sea lamprey groups between sexes and maturation states

| Group                        | Metabolism pathway                          | Metabolite                  | Regulation | Fold change | p value     | q value     |
|------------------------------|---------------------------------------------|-----------------------------|------------|-------------|-------------|-------------|
| PSM vs SM                    | Secondary bile acid biosynthesis            | Lithocholic acid            | ↑          | 152.9       | 9.66E−11    | 2.55E−08    |
|                              | −                                           | Petromyzon sul fate         | ↑          | 212.3       | 1.38E−10    | 3.37E−08    |
|                              | −                                           | Chenodeoxycholic acid 3-sulfate | ↑          | 264.2       | 1.83E−09    | 3.68E−07    |
|                              | −                                           | Petromyzonol                | ↑          | 201.0       | 4.45E−09    | 6.49E−07    |
| Purine metabolism            |                                             | Adenine                     | ↑          | 46.8        | 2.21E−07    | 4.95E−05    |
| Amino acid metabolism        |                                             | 3-Hydroxypropenoate         | ↓          | 8.4         | 1.63E−06    | 2.11E−04    |
| Primary and Secondary bile   |                                             | Cholic acid                 | ↑          | 134.0       | 2.44E−04    | 2.72E−03    |
| acid biosynthesis            |                                             |                             |            |             |             |             |
| Secondary bile acid          | 5α-Cholic acid                              |                             | ↑          | 313.9       | 1.07E−04    | 4.26E−03    |
| biosynthesis                 | −                                           | D-Xylulosonic acid          | ↑          | 11.2        | 1.64E−04    | 6.07E−03    |
| Tyrosine metabolism          |                                             | Tyrosine                    | ↑          | 1.9         | 2.61E−02    | 7.76E−02    |
| Amino acid metabolism        |                                             | Arginine                    | ↓          | 2.2         | 5.64E−02    | 1.08E−01    |
| Lysine degradation           |                                             | Lysopine                    | ↓          | 1.5         | 6.98E−02    | 1.18E−01    |
| Amino acid metabolism—Gluthione metabolism | | Ornithine | ↑ | 1.5 | 1.04E−01 | 1.44E−01 |
| Arginine and proline         | Octopine                                    |                             | ↓          | 1.2         | 4.52E−01    | 3.02E−01    |
| metabolism                   | Biliverdin                                  |                             | ↑          | 1.4         | 5.14E−01    | 3.25E−01    |
| Purine metabolism            | Hypoxanthine                                |                             | ↑          | 1.2         | 7.15E−01    | 3.97E−01    |
| Histidine metabolism         | Formimino-γ-glutamic acid                   |                             | ↑          | 1.2         | 7.29E−01    | 4.01E−01    |
| Pyrimidine metabolism        | Cytosine                                    |                             | ↑          | 37.2        | 4.70E−05    | 1.10E−03    |
| Amino acid metabolism        | Glutamic acid                               |                             | ↑          | 1.7         | 4.50E−03    | 2.60E−02    |
| Histidine metabolism         | Methylhistidine                             |                             | ↑          | 2.8         | 1.00E−02    | 5.10E−02    |
| Amino acid metabolism        | Glutamine                                   |                             | ↑          | 4.4         | 7.40E−05    | 3.80E−03    |
| Amino acid metabolism        | Glutamic acid                               |                             | ↑          | 1.5         | 1.60E−03    | 3.30E−02    |
| −                            | N3,N4-Dimethyl-γ-arginine                   |                             | ↓          | 7.1         | 2.70E−03    | 1.90E−02    |
| TCA cycle                    | Citric acid                                 |                             | ↓          | 2.0         | 6.20E−04    | 7.10E−03    |
| Biosynthesis of ansamycins   | AminoDHQ                                    |                             | ↓          | 2.1         | 3.03E−03    | 2.00E−02    |
| Amino acid metabolism        | 2-Aminoadipic acid                          |                             | ↑          | 2.1         | 2.10E−03    | 1.70E−02    |
| Phenylalanine metabolism     | 2-Oxo-4-pentenoic acid                      |                             | ↓          | 2.9         | 2.30E−03    | 4.30E−02    |
| POF vs OF                    | Lysine biosynthesis                         | N-acetyl-LL-2,6-diaminopimelic acid | ↑ | 12.2 | 7.22E−07 | 9.39E−06 |
| −                            | N,N-dimethylarginine                        |                             | ↓          | 6.4         | 7.83E−07    | 1.01E−05    |
| −                            | 7a,12a-Dihydroxy-3-oxo-4-cholenolic acid    |                             | ↓          | 11.0        | 4.13E−05    | 1.64E−04    |
| Cysteine and methionine      | Methionine                                  |                             | ↑          | 4.0         | 1.26E−03    | 2.34E−03    |
| metabolism                   | Tryptophan                                  |                             | ↑          | 3.0         | 2.21E−03    | 3.76E−03    |
| −                            | D-Xylulosonic acid                          |                             | ↑          | 6.6         | 3.22E−04    | 6.17E−03    |
| −                            | N-(3-Carboxypropyl)-γ-glutamine             |                             | ↓          | 19.5        | 1.96E−03    | 1.46E−02    |
| −                            | Prostaglandin C1                            |                             | ↓          | 2.8         | 2.60E−03    | 1.79E−02    |
| Arachidonic acid metabolism  | 11-Dehydrothromboxane B2                    |                             | ↓          | 4.4         | 5.21E−03    | 2.06E−02    |
| Valine, leucine, and         | Acetyl lactic acid                          |                             | ↑          | 4.3         | 5.40E−03    | 2.10E−02    |
| isoleucine biosynthesis      |                                             |                             |            |             |             |             |
| Amino acid metabolism        | Creatine                                    |                             | ↓          | 4.9         | 2.60E−02    | 1.2E−01     |
| Arginine and proline         | Creatinine                                  |                             | ↓          | 15.2        | 2.20E−02    | 1.18E−02    |
| metabolism                   | Aspartic acid                               |                             | ↓          | 9.5         | 1.40E−02    | 8.20E−02    |
| −                            | Glutamyl-glutamic acid                      |                             | ↓          | 2.2         | 7.90E−02    | 2.20E−01    |
| Pyrimidine metabolism        | 3-Ureidopropionic acid                      |                             | ↓          | 2.2         | 1.10E−01    | 2.70E−01    |
| Phenylalanine, tyrosine, and  | Pretyrosine                                 |                             | ↓          | 13.0        | 1.40E−03    | 2.50E−02    |
| tryptophan biosynthesis      |                                             |                             |            |             |             |             |
| Fatty acid metabolism        | Palmitic acid                               |                             | ↓          | 1.7         | 3.60E−02    | 1.90E−01    |
One striking difference was found in the comparisons of sea lamprey plasma metabolomes between sexes and maturation states, i.e., males contain hundreds-folds more bile acids than females, and SM contains the highest amounts of bile acids in all groups. Untargeted and targeted analyses showed some variation in fold change for the same bile acid which could be a result of different analytical approaches, including the use of a more sensitive MRM based method and the use of ion pairing agent, TEA, for targeted analysis.

Bile acids/salts are the major end metabolites of cholesterol and are important in lipid, protein, and sugar metabolisms (Hagey et al., 2010). The details of bile acid biosynthetic pathways are only known in mammals such as humans and rodents and have not been resolved in other species, including sea lamprey (Hagey et al., 2010). Based on our metabolomics results and what was available in the literature (Chiang, 2004; Chung-Davidson et al., 2021; Heubi et al., 2007; Pandak & Kakiyama, 2019; Setchell et al., 1988), we posited a putative sea lamprey bile acid synthetic pathway. It appears that SM has evolved a strategy that downregulates the biosynthesis of other lipid species (i.e., fatty acids and leukotrienes) and upregulates cholesterol-derived bile acid biosynthesis. At the same time, SM lowers carnitine that transports long-chain activated fatty acids into the mitochondrial matrix for β-oxidation (Stryer, 1995). Interestingly, citrate carries acetyl groups from mitochondria to the cytosol for fatty acid biosynthesis (Stryer, 1995). SM has higher bile acid levels compared to both PSM and OF, but lower citrate and 12-epi leukotriene B4 compared to PSM, and lower isocitrate compared to OF. Taken together, SM seems to lower fatty acid β-oxidation, limit other lipid biosynthesis, but maximize pheromonal bile acid biosynthesis. On the other hand, PSM swims long distance during migration and searching for suitable spawning ground, which demands ATP consumptions. There is an apparent trade-off between pheromone synthesis and other metabolic pathways, and consistent with many results in life history and chemical ecology of sea lamprey that only SM release sex pheromones that attract OF (Brant et al., 2013; Chung-Davidson et al., 2021; Li et al., 2002).

We found that OF had lower fatty acid metabolism and lower prostaglandin C1 and 11-dehydro thromboxane B2.
compared to POF. Since eicosanoid hormones such as leukotrienes, prostaglandins, and thromboxanes are derived from polyunsaturated fatty acids, it seems that OF downregulated the pathways involved in biosynthesis of the precursors (fatty acids) and the end products (prostaglandins and thromboxanes). Interestingly, arachidonic acid, a precursor for eicosanoid production that modulate steroid synthesis and spermatiation during sexual maturation (Norambuena et al., 2013; Wade et al., 1994), is also critical to producing vitellogenin, a high-density lipoprotein which is synthesized in the liver and transported to the eggs (Norambuena et al., 2013; Tocher, 2003; Wade et al., 1994). The high investment of resources for reproduction is to mobilize lipid reserves from liver and muscles to gonads during sexual maturation and spawning migration (Singh et al., 2012; Sutharshiny & Sivashanthini, 2011; Zaboukas et al., 2006). In the sea lamprey, gonad development occurs when they stop feeding and initiate the atrophy of the digestive tract (Beamish, 1979). Consequently, gonadal growth and the energy expenditure for migration depend on mobilization of lipid and protein reserves accumulated during the parasitic phase (Hardisty, 2006) and are not restored during the process of reproductive migration after feeding has ceased (Martins et al., 2019). A drawback of this study is that we only measured more polar molecules due to the extraction and optimized analytical methods. Further lipidomics analyses will provide more information in lipid metabolisms during sexual maturation in sea lamprey.

Pheromone communication consists of two components: (1) the biosynthesis, storage, and release of the pheromones; (2) the reception and translation of the chemical message into a behavioral pattern (Kittredge & Takahashi, 1972). In a sexual selection context for sea lamprey, the sender is SM, broadcasting information about aspects of its quality towards rival SM and/or OF. Apparently, SM evolved metabolic strategies to produce and release massive amounts of bile acid pheromones to signal spawning. Intersexual selection through female choice is generally regarded the most important driver of signal evolution (Andersson & Iwasa, 1996; Ord et al., 2001). Indeed, OF prefer higher pheromone levels on spawning grounds (Fissette et al., 2020; Johnson et al., 2009). In addition, SM increases 3kPZS release virtually instantaneously upon detection of 3kPZS, indicating presence of competitor males (Fissette et al., 2020). Male signals are typically costly to produce, and thus display frequency may provide a measure of male attractiveness (Davie et al., 2010). The sex- and maturation-dependent metabolomic profiles of sea lamprey support these notions.

High inter-male variation in 3kPZS release and female preference for pheromone plumes with higher 3kPZS concentrations indicate 3kPZS likely guides female mate choice (Buchinger et al., 2017; Fissette et al., 2020; Johnson et al., 2009). Indeed, biosynthesis of 3kPZS and possible precursors appears to be under sexual selection (Buchinger et al., 2019). Interestingly, 3kPZS signaling may also mediate interactions among males, as intrasexual competition increases male 3kPZS release which may have consequences on female mate choice (Fissette et al., 2020). Therefore, changes in metabolomic profiles and chemical signaling strategies resulting from male competition may influence mate choice (Fissette et al., 2020). Our metabolomics results indicate that SM maximizes pheromonal bile acid production, likely benefits reproduction and increase fitness.

In conclusion, adult sea lamprey drastically alter their plasma metabolomic profile and energy expenditure to support migration and reproduction. Spermiating male sea lamprey upregulate bile acid biosynthesis, producing a pheromone known to increase mate attraction and reproductive success.

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**Fig. 2** Putative bile acid synthetic pathway map comparing plasma metabolites in mature male and female sea lamprey. The metabolites in bold letters represent the bile acids quantified by targeted analysis in sea lamprey plasma. The number below each box denotes the fold change for the respective metabolite and the red color indicates upregulation (SM > OF). **OF** ovulatory females, **SM** spermiating males.
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Data availability  The metabolomics and metadata reported in this paper are available via GNPS at https://doi.org/10.25345/CSV29C under the MSV000088417.

Declarations

Conflict of interest  All authors declare that they have no conflict of interest.

Ethical approval  All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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