Global Metabolomics of Fireflies (Coleoptera: Lampyridae) Explore Metabolic Adaptation to Fresh Water in Insects

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Simple Summary: To date, little is known about the molecular mechanisms of aquatic adaptation in invertebrates, particularly in insects, which are the most abundant and diverse species. How aquatic insects adapt to freshwater environments remains largely unknown. Fireflies have terrestrial and aquatic lineages according to the habits of their larvae, and provides a good opportunity to explore aquatic adaptation of insects. We generated adult and larval metabolomes of two firefly species (aquatic *Aquatica leii* and terrestrial *Lychnuris praetexta*), and then a set of metabolites and their pathways involved in freshwater adaptation of aquatic firefly species were investigated by intraspecific and interspecific metabolomics comparisons, as well as by functional enrichment analysis. These molecules and pathways were primarily involved in oxidative stress/xenobiotics/immunity response, energy metabolism, sense function, and morphological attributes related to the freshwater lifestyle of aquatic *A. leii*. This study suggests that abundance-level changes in metabolites contributed to freshwater adaptation of fireflies, and provides insights into the metabolic mechanisms of aquatic adaptation in insects.

Abstract: Aquatic insects are well-adapted to freshwater environments, but metabolic mechanisms of such adaptations, particularly to primary environmental factors (e.g., hypoxia, water pressure, dark light, and abundant microbes), are poorly known. Most firefly species (Coleoptera: Lampyridae) are terrestrial, but the larvae of a few species are aquatic. We generated 24 global metabolomic profiles of larvae and adults of *Aquatica leii* (freshwater) and *Lychnuris praetexta* (terrestrial) to identify freshwater adaptation-related metabolites (AARMs). We identified 110 differentially abundant metabolites (DAMs) in *A. leii* (adults vs. aquatic larvae) and 183 DAMs in *L. praetexta* (adults vs. terrestrial larvae). Furthermore, 100 DAMs specific to aquatic *A. leii* larvae were screened as AARMs via interspecific comparisons (*A. leii* vs. *L. praetexta*), which were primarily involved in antioxidant activity, immune response, energy production and metabolism, and chitin biosynthesis. They were assigned to six categories/superclasses (e.g., lipids and lipid-like molecules, organic acids and derivatives, and organoheterocyclic compound). Finally, ten metabolic pathways shared between KEGG terms specific to aquatic fireflies and enriched by AARMs were screened as aquatic adaptation-related pathways (AARPs). These AARPs were primarily involved in energy metabolism, xenobiotic biodegradation, protection of oxidative/immune damage, oxidative stress response, and sense function (e.g., glycine, serine and threonine metabolism, drug metabolism-cytochrome P450, and taste transduction), and certain aspects of morphology (e.g., steroid hormone biosynthesis). These results provide evidence suggesting that abundance changes in metabolomes contribute to freshwater adaptation of fireflies. The metabolites identified here may be vital targets for future work to determine the mechanism of freshwater adaptation in insects.

Keywords: aquatic firefly; metabolic profile; fresh water; metabolic adaptation
1. Introduction

 Compared with terrestrial habitats, fresh water possesses a relatively lower content of oxygen (hereafter referred to as hypoxia) and light, water pressure, as well as more abundant pathogenic bacteria communities [1,2]. These factors have greatly affected the evolution of aquatic animals and their ecological strategies. In long-term adaptation, aquatic animals have undergone significant changes in morphology, behavior, and physiology, especially regarding respiratory patterns and swimming style [3,4]. So far, the existing studies on aquatic adaptation of animals have focused on vertebrates, particularly cetaceans (whales, dolphins, and porpoises) [5]; others include loons [6], soft shell turtle (Trionychidae) [7], fish [8,9], and marine invertebrates such as corals and green crabs [10,11]. Overall, these investigations focused on aquatic adaptations of animals regarding behavior, morphology, physiology, and genetic/molecular basis (DNA and RNA); however, the exploration of the metabolic mechanisms behind aquatic adaptation is lacking.

 Despite the accumulation of metabolite information and the wide use of mass-spectrometric techniques in metabolomics, only a few studies have explored the metabolic mechanisms underlying aquatic adaptations of animals. For example, body color is an important ecological trait in aquatic animals, influencing their survival [11,12]. Metabolomics analysis of body color formation in the leopard coral grouper (Plectropomus leopardus) showed greater melanin synthesis activity in black compared to red-colored groups [11]. When tambaqui juveniles (Colossoma macropomum) faced hypoxia, anaerobic metabolism rapidly adjusted glucose and lactate metabolism, providing adequate energy [13]. Overall, little is known about the metabolic mechanisms of aquatic adaptation in insects as the most abundant and diverse animal.

 Aquatic insects (defined by having at least one aquatic life stage) make up 80% of total aquatic animal diversity [1,14]. Most aquatic insects inhabit freshwater environments and suffer from more extreme physiological challenges (e.g., hypoxia, water pressure, and diminished light) than terrestrial insects [1]. Compared to their terrestrial counterparts, aquatic insects present adaptive morphological characteristics, such as a streamlined dorsum, a flattened and smooth body, and a closed tracheal system with air bubbles and gills for oxygen intake [14]. Moreover, the developmental transition from aquatic larvae to terrestrial adults involves several physiological performance-related changes, such as increased flight muscle performance in odonates [15] and sodium chloride (NaCl) transport across the cuticle in Aedes mosquitoes [16]. This implies that metabolic changes are key to freshwater adaptation of aquatic insects, and indicates different metabolic mechanisms between aquatic and terrestrial groups.

 The Lampyridae family (Insecta: Coleoptera: Cantharoidae), commonly known as fireflies, includes at least 2000 recorded species belonging to 100 genera, widely distributed and found in tropical, subtropical, and temperate zones [17,18]. They undergo complete metamorphosis. Their developmental stages include eggs, larvae, pupae, and adults. According to larval habitat preferences, firefly species can be divided into terrestrial (e.g., genera Asymmetricata, Lychnuris and Pteroptyx), aquatic (Luciola and Aquatica), and semi-aquatic lineages (Pygoluciola) [19]. Among them, terrestrial groups contain the vast majority of species (more than 98%). Moreover, compared to terrestrial and semi-aquatic firefly larvae, aquatic species exhibit adaptations to freshwater environments in larval morphology (e.g., branched tracheal gills and both smooth and soft bodies) and behaviour (e.g., swimming) [19,20]. In recent years, comparative transcriptomic analysis of fireflies has been conducted to explore their molecular adaptation to freshwater environments [14]. Genome-scale evolutionary constraints, adaptive signals to the freshwater environment in the sequence and expression levels of candidate genes related to ATP metabolism, immune and hypoxia responses, and insect-specific morphology have all been uncovered in aquatic fireflies. The above uncovered morphological, behavioural, genetic characteristics indicate that aquatic firefly species (Lampyridae) are a suitable model for investigating freshwater adaptation of insects. Currently, despite investigations on adaptive behavior, molecular basis, and morphology of the aquatic firefly have been conducted, metabolic mechanisms
remain largely unknown. Even when extended to other insect groups, few studies have explored insects’ adaptation to water environment at a metabolomics level. Fireflies provide an opportunity to explore the metabolic adaptations of insects to freshwater environments.

Here, we sequenced and intraspecifically and interspecifically compared the adult and larval metabolic profiles of two firefly species (aquatic *Aquatica leii* and terrestrial *Lychnuris praetexta*). Then, metabolites with significant changes in *A. leii* were identified by comparing differentially abundant metabolites (DAMs) between both species. Finally, functional analyses (e.g., category division and metabolic pathway enrichment) were performed on candidate metabolites involved in freshwater adaptations.

### 2. Materials and Methods

#### 2.1. Insect Materials

Larvae (4th–6th instars) and adults of *A. leii* and *L. praetexta* were raised as previously described (Supplementary information in Zhang et al., 2020). *A. leii* freshwater larvae (ALL) and terrestrial adults (ALA) were randomly collected, to a total of 50 4th instar, 50 5th instar, 30 5th instar larvae, and 20 female and 20 male adults. We additionally randomly collected larvae (LPL) and adults (LPA) of *L. praetexta* inhabiting terrestrial environments across the entire life cycle, to a total of 10 4th instar, 6 5th instar, and 5 6th instar larvae, and 20 female and 20 male adults. Each collected sample per developmental stage was put in a 10 mL plastic centrifuge tube and then stored at −80 °C. The sampling was independently repeated six times as six biological replicates. The intestines were removed from all individuals to avoid disturbance of residual foods and gut microbes. A flow diagram of the experiments and data analyses is shown in Figure 1.

#### 2.2. Metabolite Extraction

For metabolite extraction of each replicate, 50 mg of firefly tissue at each developmental stage was mixed with 400 µL of pre-cooled extracting solution (methanol:water = 3:1, V/V) in a 2 mL Eppendorf tube, and then a 6 mm diameter grinding bead was added. After
grinding for 6 min at 50 Hz at 10 °C in a Wonbio-96c high-throughput tissue crusher (Wanbo Biotechnology, Shanghai, China), the samples were extracted at 4 °C at 40 kHz for 30 min using a controlled-temperature ultrasonic cleaning machine (SBL-10TD, Scientz Biotechnology, Ningbo, China). The samples were placed at −20 °C for 10 min for proteins to precipitate. After centrifugation at 30,000 × g at 4 °C for 15 min (refrigerated centrifuge 5430R, Eppendorf, Hamburg, Germany), the supernatant of each developmental stage of 4th–6th instars of A. leii samples was equally mixed as ALL samples and used for further global metabolomics analysis. Adult A. leii, 4th–6th instars of L. praetexta, and adult L. praetexta samples were also similarly processed as ALA, LPL, and LPA samples, respectively. In total, the samples were used to generate 24 metabolic profiles (i.e., ALL_1–6, ALA_1–6, LPL_1–6, and LPA_1–6).

In addition, to avoid systematical and technical errors caused by metabolome sequencing, 5 µL from each collected supernatant sample was mixed into a 2 mL Eppendorf tube as a quality control sample (QC; n = 3). The QC samples were sequenced parallel to the experimental samples, which represented the whole sample set, and were injected at regular intervals to monitor stability in the sequencing process.

2.3. Global Metabolomics Analysis

Global metabolomics analysis was implemented using liquid chromatography with tandem mass spectrometry (LS-MS/MS) as previously described [21], using contained an ultrahigh performance liquid chromatography (UHPLC) system (Vanquish, Thermo Fisher Scientific, Waltham, MA, USA) with a UPLC HSS T3 column (100 mm × 2.1 mm, 1.8 µm) coupled to a Q Exactive HF-X mass spectrometer (Orbitrap MS, Thermo Fisher Scientific). The gradient elution parameters were set as follows: 0–3.5 min: 100% phase A (95% water/5% acetonitrile + 0.1% formic acid), 0.4 mL/min (mobile phase velocity); 3.5–5 min: 75.5% A–24.5% B (5% water/47.5% acetonitrile/47.5% isopropanol + 0.1% formic acid), 0.4 mL/min; 5–5.5 min: 35% A–65% B, 0.4 mL/min; 5.5–7.4 min: 100% B, 0.4 mL/min; 7.4–7.6 min: 100% B, 0.6 mL/min; 7.6–7.8 min: 48.5% A–51.5% B, 0.6 mL/min; 7.8–9 min: 100% A, 0.5 mL/min; 9–10 min: 100% A, 0.4 mL/min. The auto-sampler temperature was set to 4 °C, and the injection volume was 2 µL. The range for m/z detection was 70–1050. Q Exactive HF-X mass spectrometer was used for its ability to acquire MS/MS spectra in information-dependent acquisition (IDA) mode, controlled by the acquisition software (Xcalibur, Thermo Fisher Scientific). In this mode, the acquisition software continuously evaluates the full scan MS spectrum. The electrospray ionization (ESI) source conditions were set as follows: sheath gas flow rate of 50 arb, aux gas flow rate of 13 arb, capillary temperature 325 °C, normalized collision energy 204,060 eV, full MS resolution at 60,000, resolution (MS/MS) at 7500, collision energy at 10/30/60 in normalized collisional energy (NCE) mode, and spray voltage at (+) 3.5 kV or (–) 3.5 kV. Raw data of the 24 metabolic profiles of ALL, ALA, LPL, and LPA (n = 6) were obtained by collecting data in positive and negative ion modes.

2.4. Data Preprocessing

The raw data were converted into the mzXML format by using ProteoWizard, and processed using R package XCMS (version 3.2) for peak detection, extraction, alignment, and integration, as previously described [21,22]. The software generated a data matrix for the raw data, including sample information, retention time (RT), mass-to-charge ratio (m/z) values, and peak intensity. Metabolic features that were detected at least in 70% of samples were retained. After filtering, minimum metabolite values were assessed for samples in which the metabolite levels fell below the lower limit of quantitation. Peak annotation was carried out using Compound Discover v3.0 (Thermo Fisher Scientific) and OSI-SMMS v1.0 (software system for rapid identification and analysis of small molecular compounds in metabolomics; Dalian Chem Data Solution Information Technology, Dalian, China), integrated with the mzcloud database (HighChem LLC, Bratislava, Slovakia). An internal standard was used for data QC (reproducibility). Metabolic features with relative standard
deviation (RSD) of QC > 30% were discarded. Following normalization procedures and imputation, statistical analysis was performed on log-transformed data to identify significant differences in metabolite levels between comparable groups. Subsequently, the acquired MS/MS spectra were matched against metabolome databases including HMDB (Hydrogen Mitigation Design Basis, http://www.hmdb.ca/, accessed on 28 April 2022), KEGG (Kyoto Encyclopedia of Genes and Genomes, http://www.genome.jp/kegg/, accessed on 28 April 2022), and an in-house MS/MS database (Biotree Biotec Co., Ltd. Shanghai, China). The MS/MS spectra match score was calculated using a dot-product algorithm and ranged from 0 to 1. The cutoff for match score was set as 0.4. The data were normalized by the internal standards before statistical analysis. The Pearson correlation coefficient was calculated between each sample pair to evaluate the repeatability of the biological replicates. Principal Component Analysis (PCA) was performed to separate and classify the sample groups based on metabolic profiles using SIMCA-P+ software v16 (Sartorius Stedim Data Analytics AB, Umea, Sweden).

2.5. Identification of Differential Abundant Metabolites (DAMs)

The significance of each DAM between larvae and adults of each firefly species was evaluated using unpaired Wilcoxon tests. The p-values were further corrected by the Benjamini-Hochberg adjustment method (false discovery rate, FDR < 0.05), |log2FC(fold change)| > 1, and VIP (the variable importance of projection) > 2. The metabolites involved in freshwater adaptation of larval A. leii were detected as previously described (Zhang et al., 2020). Briefly, the DAMs of L. praetexta were intersected with that of A. leii, thus excluding non-responding metabolites to fresh water in larval A. leii. Subsequently, DAMs that were only detected in A. leii (A. leii-specific DAMs) were retained as metabolites that presented abundance changes to freshwater environments; these metabolites were identified as aquatic adaptation-related metabolites (AARMs) in A. leii larvae.

2.6. HMDB Classification and KEGG Functional Enrichments

According to the HMDB database annotation information, AARMs were divided and assigned to three levels: superclass, class, and subclass. In addition, the identified differential metabolites were further analyzed to determine the relevant biological pathways using MetaAnaylst 5.0 (http://www.metaboanalyst.ca/, accessed on 3 May 2022). Benjamini-Hochberg adjustment was employed as the FDR correction method. To detect reliable KEGG pathways specifically involved in freshwater adaptation of larval A. leii, two methods (i.e., DAM-KEGG-intersection and AARM-KEGG) were used, as previously described (Zhang et al., 2020). In the DAM-KEGG-intersection method DAMs of L. praetexta and A. leii were subjected to KEGG enrichment analysis, their results were intersected, and the pathways specific to A. leii were obtained. In the AARM-KEGG method, the AARMs above obtained were directly used for KEGG enrichment. The results shared by both methods were considered as the final KEGG metabolic pathways associated with freshwater adaptation of A. leii (namely, aquatic adaptation-related pathways, AARPs).

3. Results
3.1. Overview of Sequencing Data

A total of 26,140 peaks (13,643 and 12,497 peaks in positive (ESI+) and negative (ESI−) ion mode, respectively) were detected by the mass spectrometer. Through further screening, 20,276 peaks (10,433 peaks in ESI+ and 9,843 peaks in ESI−) were obtained. By further screening peaks, a total of 2928 metabolites (2030 metabolites in ESI+ and 938 metabolites in ESI−) were identified. The correlation value was close to 1 in the ESI+ (Figure 2A) and ESI− (Figure 2B), indicating robust sampling and metabolome sequencing. The PCA results showed cluster samples in each group, and a clear separation among five groups (including four experimental and one QC groups), both in the ESI+ (Figure 3A) and ESI− (Figure 3B), suggesting significant differences of metabolite profiles among the five groups.
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in ESI−) were identified. The correlation value was close to 1 in the ESI+ (Figure 2A) and ESI− (Figure 2B), indicating robust sampling and metabolome sequencing. The PCA reconstruction was performed on the constructed dataset in ESI+ and ESI− scan modes for the first two components (including four experimental and one QC groups), both in the ESI+ (Figure 3A) and ESI− (Figure 3B), suggesting significant differences of metabolite profiles among the five groups. The PCA scatter plot scores in ESI+ (Figure 2A) and ESI− (Figure 2B), indicating robust sampling and metabolome sequencing. The PCA reconstruction was performed on the constructed dataset in ESI+ and ESI− scan modes for the first two components (including four experimental and one QC groups), both in the ESI+ (Figure 3A) and ESI− (Figure 3B), suggesting significant differences of metabolite profiles among the five groups. The PCA scatter plot scores in ESI+ (Figure 2A) and ESI− (Figure 2B), indicating robust sampling and metabolome sequencing. The PCA reconstruction was performed on the constructed dataset in ESI+ and ESI− scan modes for the first two components (including four experimental and one QC groups), both in the ESI+ (Figure 3A) and ESI− (Figure 3B), suggesting significant differences of metabolite profiles among the five groups.

3.2. Analysis of Aquatic Adaptation-Related Metabolites (AARM) in A. leii Larvae

In total, 100 AARMs were identified (Figure 4). According to their annotation information and references (Table 1), 17 AARMs, including 15 up- and 2 down-regulated metabolites, were classified into functional categories involving their antioxidant activity. A total of 22 AARMs (17 up- and 5 down-regulated) were identified to be associated with immunity; in particular (some of them cover multiple immune functions), 10 metabolites were involved in anti-inflammatory response (e.g., 12a-hydroxy-3-oxocholadienic acid, 21-deoxycortisol, and 3-formyl-6-hydroxyindole), 10 metabolites involved in anticancer response (e.g., capsianoside H, 5′-deoxy-5-fluorocytidine, and glutamylproline), four metabolites involved in antibacterial response (e.g., agavoside G, annoglabasin F, and 2,3-Dihydroabscisic alcohol), two metabolites involved in antiviral response (1-(3-Furanyl)-6,7-dihydroxy-4,8-dimethyl-1-nonanone and 23-trans-p-Coumaroyloxytormentic acid), two metabolites involved in immune responses (2-hydroxyestrone sulfate and 19-oxoandrost-4-ene-3,17-dione), and two metabolites involved in immunomodulatory effects (Canarigenin 3-[glucosyl-(1->4)-6-deoxy-alloside] and Dynorphin A (6–8)). In addition, 18 AARMs were related to energy metabolism, 15 of which showed up-regulation, while three were down-regulated. Two AARMs were typically related to chitin biosynthesis in response to freshwater environments, including (2E,8Z)-Decadiene-4,6-diyne-1-yl-3-methylbutanoate and 25-Hydroxyxachysterol3. The exact biological function of the 41 remaining AARMs identified in this study was not reported in previous publications.
Table 1. Aquatic adaptation-related metabolites (AARMs) with function information. #: According to references.

| Function | Specific Functions # | Id          | Metabolites | Log2FC (ALL/ALA) | FDR         | References |
|----------|----------------------|-------------|-------------|------------------|-------------|------------|
| Antioxidants |                      |             |             |                  |             |            |
| Anti-Oxidant |                     | metab_23671 | Asparaginyl-Tyrosine | 2.64 | $6.82 \times 10^{-3}$ | [23] |
| Anti-Oxidant |                     | metab_9504  | Cinchonidine   | 1.41 | $7.84 \times 10^{-3}$ | [24] |
| Anti-Oxidant |                     | metab_17388 | Cynaroside A   | 3.66 | $6.82 \times 10^{-3}$ | [25] |
| Anti-Oxidant |                     | metab_18889 | Leu-Asp-Glu-Lys | 1.78 | $6.82 \times 10^{-3}$ | [23] |
| Anti-Oxidant |                     | metab_22119 | Lys-Gln-Asp-Lys | 8.56 | $6.82 \times 10^{-3}$ | [23] |
| Anti-Oxidant |                     | metab_2651  | Lys-Glu-Ser-Leu-Ser | 1.37 | $7.84 \times 10^{-3}$ | [23] |
| Active oxygen regulator |                   | metab_6098  | Pekeberrygenin  | 1.85 | $7.84 \times 10^{-3}$ | [26] |
| Active oxygen regulator |                 | metab_8499  | Priverogenin A | 1.69 | $7.84 \times 10^{-3}$ | [26] |
| Active oxygen regulator |                 | metab_10795 | Tyr-Glu-Asp    | −1.60 | $7.84 \times 10^{-3}$ | [23] |
| Anti-Oxidant |                     | metab_3751  | Tyr-Phe-Glu    | 1.90 | $7.84 \times 10^{-3}$ | [23] |
| Anti-Oxidant |                     | metab_2367  | Tyr-Pro-Trp    | 1.88 | $7.84 \times 10^{-3}$ | [23] |
| Antioxidant |                     | metab_11607 | Val-His-Tyr-Tyr | 3.07 | $7.84 \times 10^{-3}$ | [23] |
| Antioxidant |                     | metab_25229 | Inosinic acid  | 1.23 | $6.82 \times 10^{-3}$ | [27] |
| Antioxidant |                     | metab_13246 | Histidinyl-2,3-Dihydroabscisic acid | 2.02 | $7.84 \times 10^{-3}$ | [23] |
| Antioxidant |                     | metab_9495  | N(6)-(Octanoyl)lysine | 1.85 | $7.84 \times 10^{-3}$ | [23] |
| Antioxidant |                     | metab_18186 | 6-Hydroxysandoricin | 2.57 | $6.82 \times 10^{-3}$ | [23] |
| Antioxidant |                     | metab_3786  | Gamma-Glutamylleucine | 10.80 | $7.84 \times 10^{-3}$ | [28] |

Immunity

| Function | Specific Functions # | Id          | Metabolites | Log2FC (ALL/ALA) | FDR         | References |
|----------|----------------------|-------------|-------------|------------------|-------------|------------|
| Anticancer |                     | metab_10041 | Capsianoside H | 2.83 | $7.84 \times 10^{-3}$ | [29] |
| Antibacterial |                  | metab_9908  | (R)-Roemerine | −2.57 | $7.84 \times 10^{-3}$ | [30] |
| Antiviral |                     | metab_8329  | 1-(3-Furanyl)-6,7-dihydroxy-4,8-dimethyl-1-nonanone | 1.58 | $7.84 \times 10^{-3}$ | [31] |
| Antiinflammatory/Energy consumption |                   | metab_21033 | 12a-Hydroxy-3-oxocholadienic acid | 1.37 | $6.82 \times 10^{-3}$ | [32] |
| Antiinflammatory/Anticancer/Antibacterial |                   | metab_22266 | 2,3-Dihydroabscisic alcohol | 3.54 | $6.82 \times 10^{-3}$ | [33] |
| Antiinflammatory |                 | metab_9179  | 21-Deoxy cortisol | 1.96 | $7.84 \times 10^{-3}$ | [34] |
| Antiinflammatory/Immune responses |                   | metab_23834 | 2-Hydroxyestrone sulfate | −1.13 | $6.82 \times 10^{-3}$ | [35] |
| Antiinflammatory |                   | metab_15702 | 3-Formyl-6-hydroxyindole | −1.36 | $6.82 \times 10^{-3}$ | [36] |
| Antiinflammatory |                   | metab_22693 | 3-Sulfodeoxycholic acid | 1.89 | $6.82 \times 10^{-3}$ | [37] |
| Antiinflammatory |                   | metab_12280 | 5′-Deoxy-5-fluorocytidine | 6.87 | $7.84 \times 10^{-3}$ | [38] |
| Antiinflammatory/Antibacterial/Anti-inflammatory/Anticancer/Antibacterial/Anti-inflammatory |   | metab_5304  | 6-Succinoaminopurine | 1.30 | $6.82 \times 10^{-3}$ | [39] |
| Antiinflammatory |                   | metab_1497  | Agavoside G | 1.45 | $7.84 \times 10^{-3}$ | [40] |
| Anticancer/Antiinflammatory/Anticancer/Antibacterial/Anti-inflammatory |   | metab_5350  | Canarigenin 3-[glucosyl-(1→4)-6-deoxy-alloside] | 1.82 | $7.84 \times 10^{-3}$ | [42] |
| Immunomodulatory/Anti-oxidant |                  | metab_4216  | Dynorphin A (6–8) | 2.40 | $7.84 \times 10^{-3}$ | [43] |
| Immunomodulatory |                  | metab_20976 | Ganoderic acid MF | 1.46 | $6.82 \times 10^{-3}$ | [44] |
| Anticancer/Antimicrobial/anti-inflammatory |       | metab_9442  | Isolindleyin | 1.46 | $7.84 \times 10^{-3}$ | [45] |
| Immune responses |                  | metab_13613 | 19-Oxoadrost-4-ene-3,17-dione | 2.26 | $7.84 \times 10^{-3}$ | [46] |
| Antiviral |                     | metab_16386 | Coumaroyloxytormentic acid | 4.34 | $6.82 \times 10^{-3}$ | [47] |
| Antiinflammatory/Anticancer/Anti-oxidant |                   | metab_11225 | Gamma-Glutamyl-S-methylcysteiny1-beta-alanine | −6.43 | $7.84 \times 10^{-3}$ | [48] |
| Anticancer |                     | metab_5186  | Glutamylproline | 1.06 | $7.84 \times 10^{-3}$ | [49] |
| Antiinflammatory |                 | metab_19040 | Lactosykeramide (d18:1/12:0) | 3.38 | $6.82 \times 10^{-3}$ | [50] |
Table 1. Cont.

| Function                  | Specific Functions # | Id             | Metabolites                                         | Log2FC (ALL/ALA) | FDR            | References |
|---------------------------|----------------------|-----------------|-----------------------------------------------------|------------------|----------------|------------|
| Energy                    | metab_11739          | Pro-Thr-Thr-Phe | 2.11                                               | 7.84 × 10⁻³      | [51]           |            |
| Energy                    | metab_5455           | Pro-Trp-Phe     | 1.37                                               | 7.84 × 10⁻³      | [51]           |            |
| Energy                    | metab_24676          | 6,7-Dimethyl-8-(1-D-ribityl)llumazine             | 2.09                                               | 6.82 × 10⁻³      | [52]           |            |
| Energy                    | metab_1299           | Ala-Leu-Leu     | 2.74                                               | 7.84 × 10⁻³      | [51]           |            |
| Energy                    | metab_5032           | Gly-Leu-Leu     | 1.98                                               | 7.84 × 10⁻³      | [51]           |            |
| Energy                    | metab_2274           | Val-Leu-Val-Phe | 1.31                                               | 7.84 × 10⁻³      | [51]           |            |
| Energy                    | metab_10699          | Ala-Ala-Trp-Ile | 1.92                                               | 7.84 × 10⁻³      | [51]           |            |
| Energy                    | metab_10810          | Biliverdin      | 10−1.8                                             | 7.84 × 10⁻³      | [53]           |            |
| Energy metabolism/Antibacterial/Immune reaction | metab_19325 | Bisnorchoic acid | 3.24                                               | 6.82 × 10⁻³      | [32]           |            |
| ATP enzyme inhibitor      | metab_4095           | Cyclopiazonic acid | −1.51                                              | 7.84 × 10⁻³      | [54]           |            |
| Energy                    | metab_23404          | Gamma-L-Glutamyl-L-pipeolic acid         | 2.61                                               | 6.82 × 10⁻³      | [51]           |            |
| Energy                    | metab_11280          | Gly-Ile-Val     | 2.23                                               | 7.84 × 10⁻³      | [51]           |            |
| Energy                    | metab_10695          | Ile-Ile-Val     | 1.70                                               | 7.84 × 10⁻³      | [51]           |            |
| Energy                    | metab_10648          | Ile-Phe-Phe-Thr | 2.08                                               | 7.84 × 10⁻³      | [51]           |            |
| Energy                    | metab_2709           | Thr-Val-Val     | 1.57                                               | 7.84 × 10⁻³      | [51]           |            |
| Energy                    | metab_9753           | Trp-Phe         | 1.78                                               | 7.84 × 10⁻³      | [51]           |            |
| Energy                    | metab_12349          | Val-Leu-Ser     | 2.05                                               | 7.84 × 10⁻³      | [51]           |            |
| Energy                    | metab_5070           | 8(R)-HETE       | −8.13                                              | 7.84 × 10⁻³      | [55]           |            |
| Morphology                | metab_401            | 25-Hydroxytachysterol3 (2E,8Z)-Decadiene-4,6-diyn-1-yl-3-methylbutanoate | 1.02 | 7.84 × 10⁻³      | [56]           |            |
| Morphology                | metab_9436           | 5229 Inosinic acid | 1.23                                               | 6.82 × 10⁻³      | ×             |            |

Figure 4. Venn diagram of the number of DAMs shared by *A. leii* and *L. praetexta*.

3.3. HMDB Class Analysis of Aquatic Adaptation-Related Metabolites (AARM) in *A. leii* Larvae

In total, 53 AARMs were annotated in the HMDB database (Table S4), and were divided into six superclasses (Figure 5), including lipids and lipid-like molecules, organic acids and derivatives, organoheterocyclic compounds, organic oxygen compounds, benzenoids, and nucleosides, nucleotides, and analogues. At the class level, the lipids and lipid-like molecules included prenol lipids, steroids and steroid derivatives, fatty acyls, sphingolipids, and glycerophospholipids (Figure 5A). A total of 32 AARMs belonged to the lipid and lipid-like molecules superclass; most of them belonged to the prenol lipids class, with 12 up-regulated and 2 down-regulated metabolites. For steroids and steroid derivatives, 10 AARMs (i.e., eight up- and two down-regulated metabolites) were included. All the six AARMs belonging to the fatty acyl class showed up-regulation. The sphingolipids and glycosphingolipids classes identified only one member each, presenting up-regulation abundance in response to fresh water in aquatic *A. leii* larvae. Within the six superclasses, organic acids and derivatives contained the second largest number (12) of AARMs. This superclass included carboxylic acids and derivatives (10 ARMS, from which eight were up- and two were down-regulated), keto acids and derivatives, and peptidomimetics (Figure 5B). The last two categories included only one member each, presenting down-regulation abundance. Regarding the other four superclasses (Figure 5C),
organoheterocyclic compounds included imidazopyrimidines (1 up-regulated AARM), benzazepines (1 up- and 1 down-regulated AARM), indoles and derivs (1 down-regulated AARM), and tetrapyroles and derivatives (1 down-regulated AARM). In organic oxygen compounds superclass, one class—organooxygen compounds—included two up-regulated AARMs. For the benzenoids and the nucleosides, nucleotides, and analogues superclasses, each covered only one class: benzene and substituted derivatives, and purine nucleotides, respectively. All the AARMs included in these two superclasses were up-regulated in aquatic \( A. \) \( leii \) larvae.

![Figure 5](image_url)

**Figure 5.** Heat maps of differentially abundant metabolites between \( A. \) \( leii \) larvae and adults. (A) Metabolic levels of AARM contained in lipids and lipid-like molecules; (B) Metabolic levels of AARM contained in organic acids and derivatives; (C) Metabolic levels of AARM contained in organoheterocyclic compounds, organic oxygen compounds, benzenoids, and nucleosides, nucleotides, and analogues. Each line represents a differentially abundant metabolite, and each row represents a sample. Different colors represent different abundances, with darker colors indicating higher abundance.

### 3.4. Pathway Analysis of Aquatic Adaptation-Related Metabolites (AARM) in \( A. \) \( leii \) Larvae

Through the DAM-KEGG-intersection method described to detect reliable KEGG pathways specifically involved in freshwater adaptation of larval \( A. \) \( leii \), we observed that DAMs (aquatic larvae vs. adults) were significantly enriched to 14 KEGG pathways (Table S5), such as antifolate resistance (map01523), riboflavin metabolism (map00740), and taste transduction (map04742). Meanwhile, DAMs in \( L. \) \( praetexta \) (terrestrial larvae vs. terrestrial adults) were significantly enriched to eight KEGG pathways (Table S6), such as insect hormone biosynthesis (map00981), folate biosynthesis (map00790), and histidine metabolism (map00340). Furthermore, 10 KEGG pathways specific to \( A. \) \( leii \) were identified (Figure 6), such as antifolate resistance (map01523), riboflavin metabolism (map00740), and taste transduction (map04742) (Table 2). In addition, via the AARM-KEGG method we applied, all the above obtained AARMs were enriched to 13 metabolic pathways, such as antifolate resistance (map01523), arachidonic acid metabolism (map00590), and
biosynthesis of cofactors (map01240) (Table S7). Finally, the results of intersection analysis showed that all 10 metabolic pathways obtained by the DAM-KEGG-intersection method were included in the results obtained by the AARM-KEGG method (Figure S1). Both methods obtained 10 consistently enriched pathways, which were identified as target pathways (AARPs) involved in freshwater adaptation of A. leii (Table 2).

![Venn diagram](image)

**Figure 6.** Venn diagram of KEGG terms enriched by DAMs between larvae and adults from A. leii and L. praetexta.

| Id      | Description                              | FDR       |
|---------|------------------------------------------|-----------|
| map01523| Antifolate resistance                     | 1.05 × 10⁻¹|
| map00740| Riboflavin metabolism                    | 1.06 × 10⁻¹|
| map04742| Taste transduction                        | 1.06 × 10⁻¹|
| map00600| Sphingolipid metabolism                  | 1.15 × 10⁻¹|
| map00564| Glycerophospholipid metabolism           | 1.43 × 10⁻¹|
| map00260| Glycine, serine and threonine metabolism | 1.50 × 10⁻¹|
| map00230| Purine metabolism                         | 1.92 × 10⁻¹|
| map00140| Steroid hormone biosynthesis             | 7.86 × 10⁻²|
| map0982 | Drug metabolism-cytochrome P450           | 8.20 × 10⁻²|
| map00780| Biotin metabolism                         | 1.03 × 10⁻¹|

**Table 2.** Aquatic adaptation-related pathways (AARPs) identified in aquatic A. leii.

### 4. Discussion

#### 4.1. Metabolite Function Linked to Freshwater Adaptation

Antioxidants protect animal cells against DNA and cell damage, cytotoxicity, and apoptosis under hypoxia [58] by reversing the increase in reactive oxygen species (ROS) that can occur in circumstances such as water hypoxia that trigger more ROS and toxicity than in terrestrial environments [59]. Overall, in the present study, most antioxidants were found to be up-regulated in aquatic A. leii larva compared to adults, likely a mechanism for ROS elimination resulting from hypoxia. In particular, several oligopeptides (2–10 amino acids) detected in this study possess antioxidant activity [23], such as those generated by hydrolysis of porcine collagen [60] and isolated from skipjack tuna (Katsuwonus pelamis) dark muscle [61]. In addition, pokeberrygenin and priverogenin A as triterpenoids were ROS modulators of cells [26]. Cynaroside A is a flavonoid with proven antioxidant activity, contributing to the adaptation of the Tibetan sheep to high-altitude cold and hypoxia [25]. Treatments with both low and high amounts of inosinic acid eliminated mouse lumenal oxidative stress [27]. The increasing abundance of these four metabolites likely contributed to the elimination of ROS generated by hypoxia under fresh water. Energy metabolism and antioxidation jointly regulated the response of organisms to environmental stress, as previously reported in Strigomonas culicis [62] and in large yellow croaker (Larimichthys crocea) [63]. This indicates that abundance changes of antioxidation-related metabolites accompanied by energy metabolism contributed to scavenging ROS. This is useful for the maintenance of a normal ROS level, assisting in the adaptation of A. leii larvae to hypoxia in fresh water.
In addition, the animal immune response is highly sensitive to abiotic and biotic stresses such as hypoxia, water pressure, and water pollutants/microbes. For example, tissue oxygen deficit caused by the exogenous pollutant fipronil, an important commonly used insecticide, could induce nonspecific immunity in common carp (*Cyprinus carpio*) [64], and the immune efficacy of *Eogammarus posjeticus* significantly changed under various water pressures [65]. When fishes (e.g., *Odontesthes bonariensis*, *Oncorhynchus mykiss*, *Oryzias melastigma*) were exposed to organic pollutants, the metabolism of 6-succinoaminopurine significantly changed to enhance immune defense [39]. Canarigenin 3-[glucosyl-(1->4)-6-deoxy-alloside] belonging to saponin has been found to have anti-inflammatory and antioxidant functions, and to affect cell membrane permeability [42]. In the present study, many AARMs were found to be involved in immune functions, most of which showed up-regulation. Therefore, these immune-related AARMs probably contributed to the adaptation of aquatic firefly to hypoxia, water pressure, and pollutant exposure/microbial invasion in fresh water. Meanwhile, energy metabolism was essential for the antimicrobial response, elimination of inflammation and injured cells, and other biological functions [66]. Previous studies found a balance between energy production and metabolism to be involved in the response of animals to hypoxia and pressure in water [67,68]. In particular, biliverdin is an important metabolite that participates in mitochondrial energy consumption [53], while the oligopeptides Thr-Val-Val and Val-Leu-Ser, and gamma-L-glutamyl-L-pipecolic acid are involved in ATP production [51]. Bisnorcholic acid participates in energy metabolism and antibacterial and immune responses [32]. In the current study, several AARMs were found to be related to energy production and consumption, which might be key for maintaining the dynamic balance of energy in freshwater adaptation of aquatic firefly, as similarly reported from transcriptomics perspectives [14].

Cuticle chitin biosynthesis (including the chitin-containing exoskeleton) contributes to an adaptation to the increasing water pressure and both the size and morphology of the open tracheal system (the chitin-containing lining of the tracheal tube) in insects [14]. In this study, two detected AARMs were related to insect-specific freshwater adaptation morphology. Namely, (2E,8Z)-Decadiene-4,6-diyn-1-yl-3-methylbutanoate and 25-Hydroxytachysterol3 participate in cuticle formation [56,57]. This result suggests that these two AARMs may have contributed to the required adaptation in respiration, movement, and resistance to water pressure of aquatic firefly. In addition, tyrosinase inhibitor (isolindleyin) and tyrosine (6-hydroxysandoricin) impact melanin synthesis [45]. In this study, the increased isolindleyin and decreased 6-hydroxysandoricin impacted melanin synthesis involved in light response, which may be an adaptive characteristic of aquatic firefly to low light under water.

4.2. HMDB Categories Linked to Freshwater Adaptation

More than half of AARMs were assigned to various categories at different levels, according to the HMDB database. Lipids and lipid-like molecules included the largest number of AARMs, and most of them were up-regulated in aquatic *A. leii* larvae compared with adults. Previous studies reported a greater amount of lipids and lipid-like molecules in aquatic than in terrestrial insects [69]. In general, lipids and lipid-like molecules are structural components of the membrane [70], and can affect the morphological structure and composition of cell membranes involved in tolerance against water pressure [71]. Abundance change of lipids and lipid-like metabolites may be related to the adaptation of *A. leii* larvae to water pressure.

Interestingly, steroids and steroid derivatives belonging to lipids and lipid-like molecules have been widely found in aquatic crustaceans, some of which act as hormones used to regulate the molting of arthropods [72]. AARMs involved in steroids and steroid derivatives may have facilitated the formation of a suitable chitin-containing exoskeleton, contributing to an adaptation to water pressure in *A. leii*. Organic acids are intermediate products of metabolism, and play important roles in various cellular biochemical pathways, such as energy metabolism and detoxification [73]. A study reported that the metabolism of organic
acids in *Callosobruchus chinensis* larvae increased when exposed to hypoxia [74]. In addition, several studies proved that carboxylic acids and derivatives, keto acids and derivatives, and peptidomimetics present antibacterial and anti-inflammatory activity [75–77]. Therefore, an altered metabolism of organic acids and derivatives contribute to hypoxia adaptation and immune responses of *A. leii* in fresh water. In addition, nucleosides, nucleotides, and analogues are a diverse group of endogenous metabolites widely present in organisms [78]. DNA can be more easily damaged under water than terrestrial environmental stress (e.g., water pressure and hypoxia) [79]. In the present study, the increasing abundance of AARMs assigned to nucleosides, nucleotides, and analogues likely improved the ability of aquatic firefly to efficiently repair DNA. In addition, a previous study reported an increased abundance of organic oxygen compounds in *Anopheles sinensis* larvae under deltamethrin exposure [80], and organoheterocyclic compounds were described as immunomodulatory metabolites in yogurt [81]. AARMs categorized as organic oxygen and organoheterocyclic compounds in the current study likely contributed to freshwater adaptation of aquatic firefly by adjusting immune-related and toxic-related metabolism.

4.3. Metabolic Pathways Linked to Freshwater Adaptation

Metabolites belonging to the glycine, serine, and threonine metabolism pathway significantly changed in *Callosobruchus chinensis* larvae when exposed to hypoxia [74]. Together with the antioxidant function of this pathway [82], the glycine, serine, and threonine metabolic pathway may have enhanced the antioxidant capacity of aquatic firefly tissues under freshwater hypoxia. The antifolate resistance pathway, one AARP identified in this study, was reported to be significantly changed in high-altitude adaptation of Tibetans, adjusting folate metabolism [83], also contributing to differences in the metabolic rate of folic acid at different oxygen concentrations [84]. Moreover, riboflavin supplementation improved energy metabolism efficiency in mice exposed to acute hypoxia [85]. The antifolate resistance pathway thus endorsed hypoxia adaptation of *A. leii* larvae in freshwater environments by promoting riboflavin-mediated energy metabolism. In addition, purine metabolism is an important pathway in mammalian oxidative stress adaptation [86]. For example, an increased capacity for purine recycling and ATP synthesis from inosine monophosphate was detected in ischemic tissues of bottlenose dolphins (*Tursiops truncatus*) during diving [87]. In the present study, purine metabolism was identified as an AARP, indicating its key role in oxidative stress adaptation of not only mammals but insects, via increased purine utilization and ATP production. Hypoxia induced ROS in male rats with an immune response, while antioxidant biotin (e.g., vitamin E) could block this increase and avoid immune injury [88]. Biotin metabolism, here identified as an AARP, expanded a key function of biotin as protectant of oxidative/immune damage in freshwater adaptation of animals. In addition, drug metabolism-cytochrome P450, identified as an AARP, and a well-documented KEGG term involving xenobiotic biodegradation and metabolism, responded to the benzo(a)pyrene stress in *A. leii* larvae [89], suggesting that detoxification-related pathways probably regulate hypoxia adaptations of aquatic fireflies by balancing oxygen use.

Several studies have shown differences in olfaction triggered by chemosensory-related genes (e.g., ionotropic receptors involved in smell and taste) between terrestrial Lepidoptera and aquatic Trichoptera insects [90]. Indeed, the taste transduction pathway was identified as an AARP in *A. leii* in the present study. This evidence shows the importance of metabolic pathways involved in the olfaction and taste functions for freshwater adaptation of insects. In addition, as a key pathway that impacted the timing of molting and metamorphosis of insects [91], the steroid hormone biosynthesis pathway was found to be involved in freshwater adaptation in *A. leii*. This reflects how the aquatic lifestyle of insects may benefit from adaptive changes in timing of molting and metamorphosis.
5. Conclusions

This study explored the metabolic mechanisms regarding adaptation of insects to freshwater environments using global metabolomic profiling of the Lampyridae family. We documented candidate adaptive changes in the metabolites involved in antioxidant activity, immunity, energy metabolism, and morphological attributes (e.g., exoskeleton and the tracheal system) related to the freshwater lifestyle. These metabolites were assigned to categories linked to responses to freshwater environmental stressors (e.g., hypoxia, water pressure, limited light, and the abundant microbes). Furthermore, we also identified metabolic pathways that likely contribute to firefly freshwater adaptation. The single species used for freshwater and terrestrial fireflies in this study likely limited the applicability of information. Thus, to expand the base of evidence supporting the metabolite candidates obtained here, it is important to sequence metabolic profiling of more firefly species in the future.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/insects13090823/s1. Figure S1: In the Venn diagram, (A) represents the results obtained by DAM-KEGG-intersection method, (B) represents the results obtained by AARM-KEGG method, as described in detail in Section 2.6. Table S1: The list of DEMs between adult and larval A. leii. Table S2: The list of DEMs between adult and larval L. praetexta; Table S3: The list of aquatic A. leii-specific DAMs between larvae and adults (AARMs). These metabolites did not show differential expression in L. praetexta. Table S4: HMDB enriched from A. leii DEMs. Table S5: KEGG pathways enriched from A. leii DEMs. Table S6: KEGG pathways enriched from L. praetexta DEMs. Table S7: KEGG pathways enriched from A. leii specific DEMs.

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References
1. Dijkstra, K.D.B.; Monaghan, M.T.; Pauls, S.U. Freshwater biodiversity and aquatic insect diversification. *Ann. Rev. Entomol.* 2014, 59, 143–163. [CrossRef][PubMed]
2. Ntougias, S.; Polkowski, Z.; Nikolaki, S.; Dionyssopoulou, E.; Stathopoulou, P.; Doudoumis, V.; Ruman, M.; Kozak, K.; Namieśnik, J.; Tsiamis, G. Bacterial community structures in freshwater polar environments of Svalbard. *Microb. Environ.* 2016, 31, 401–409. [CrossRef][PubMed]
3. Mitterboeck, T.F.; Fu, J.; Adamowicz, S.J. Rates and patterns of molecular evolution in freshwater versus terrestrial insects. * Genome* 2016, 59, 968–980. [CrossRef][PubMed]
4. Lushchak, V.I. Environmentally induced oxidative stress in aquatic animals. *Aquat. Toxicol.* 2011, 101, 13–30. [CrossRef]
5. Wang, Z.; Yang, G. A literature review on the molecular mechanism underlying secondary aquatic adaptation of cetaceans. *J. China West Norm. Univ. (Nat. Sci.)* 2016, 37, 25–38.
6. Gayk, Z.G.; Le Duc, D.; Horn, J.; Lindsay, A.R. Genomic insights into natural selection in the common loon (*Gavia immer*): Evidence for aquatic adaptation. *BMC Evol. Biol.* 2018, 18, 64. [CrossRef]
7. Escalona, T.; Weadick, C.J.; Antunes, A. Adaptive patterns of mitogenome evolution are associated with the loss of shell scutes in turtles. *Mol. Biol. Evol.* 2017, 34, 2522–2536. [CrossRef]
8. Jones, F.C.; Grabberr, M.G.; Chan, Y.F.; Russell, P.; Mauceli, E.; Johnson, J.; Swofford, R.; Pirun, M.; Zody, M.C.; White, S.; et al. The genomic basis of adaptive evolution in threespine sticklebacks. *Nature* 2012, 484, 55. [CrossRef]
9. Rastorguev, S.M.; Nedoluzhko, A.V.; Gruzdeva, N.M.; Boulygina, E.S.; Tsygankova, S.V.; Oshchepkov, D.Y.; Mazur, A.M.; Prokhorochuk, E.B.; Skryabin, K.G. Gene expression in the three-spined stickleback (Gasterosteus aculeatus) of marine and freshwater ecotypes. *Acta Nat.* 2018, 10, 66–74. [CrossRef]

10. Wang, H.; Tang, L.; Wei, H.; Lu, J.; Mu, C.; Wang, C. Transcriptomic analysis of adaptive mechanisms in response to sudden salinity drop in the mud crab, *Scylla paramamosain*. *BMC Genom.* 2018, 19, 421. [CrossRef]

11. Zhu, X.; Hao, R.; Tian, C.; Zhang, J.; Zhu, C.; Li, G. Integrative transcriptomics and metabolomics analysis of body color formation in the leopard coral grouper (*Plectropomus leopardus*). *Front. Mar. Sci.* 2021, 8, 726102. [CrossRef]

12. Brassch, I.; Brunet, F.; Volff, J.-N.; Schartl, M. Pigmentation pathway evolution after whole-genome duplication in fish. *Genome Biol. Evol.* 2009, 1, 479–493. [CrossRef] [PubMed]

13. do Carmo Neves, L.; Favero, G.C.; Beier, S.L.; Ferreira, N.S.; Palheta, G.D.A.; de Melo, N.F.A.C.; Luz, R.K. Physiological and metabolic responses in juvenile *Colossoma macropomum* exposed to hypoxia. *Fish Physiol. Biochem.* 2020, 46, 2157–2167. [CrossRef] [PubMed]

14. Zhang, Q.L.; Li, H.W.; Dong, Z.X.; Yang, X.J.; Lin, L.B.; Chen, J.Y.; Yuan, M.L. Comparative transcriptomic analysis of fireflies (Coleoptera: Lampyridae) to explore the molecular adaptations to fresh water. *Mol. Ecol.* 2020, 29, 2676–2691. [CrossRef] [PubMed]

15. Bybee, S.; Cordoba-Aguilar, A.; Duryea, M.C.; Futahashi, R.; Hansson, B.; Lorenzo-Carballa, M.O.; Schilder, R.; Stoks, R.; Suvorov, A.; Svennson, E.I.; et al. Odonata (dragonflies and damselflies) as a bridge between ecology and evolutionary genomics. *Front. Zool.* 2016, 13, 46. [CrossRef] [PubMed]

16. Hine, R.M.; Rouhier, M.F.; Park, S.T.; Qi, Z.; Piermarini, P.M.; Beyenbach, K.W. The excretion of NaCl and KCl loads in mosquitoes. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 2014, 307, R837–R849. [CrossRef]

17. Fu, X.; Li, J.; Tian, Y.; Quan, W.; Zhang, S.; Liu, Q.; Liang, F.; Zhu, X.; Zhang, L.; Wang, D. Long-read sequence assembly of the firefly *Pyrocoelia pectoralis* genome. *GigaScience* 2017, 6, gxi112. [CrossRef]

18. Lewis, S.; Cratsley, C. Flash signal evolution, mate choice, and predation in fireflies. *Ann. Rev. Entomol.* 2008, 53, 293–321. [CrossRef]

19. Fu, X.; Ballantyne, L.; Lambkin, C. The external larval morphology of aquatic and terrestrial Luciliinae flies (Coleoptera: Lampyridae). *Zootaxa* 2012, 3405, 1–34. [CrossRef]

20. Fu, X.; Wang, Y.; Lei, C. Adaptive external morphology and swimming behavior in the aquatic firefly, *Luciola substriata*. *Entomol. Knowl.* 2005, 42, 419–423.

21. Jin, H.; Ma, H.; Gan, N.; Wang, H.; Li, Y.; Wang, L.; Song, L. Non-targeted metabolomic profiling of filamentous cyanobacteria *Aphanizomenon flos-aquae* exposed to a concentrated culture filtrate of *Microcystis aeruginosa*. *Harmful Algae* 2022, 111, 102170. [CrossRef] [PubMed]

22. Ni, Y.; Zhang, X.; Zhang, H.; Xu, T.; Zhu, L.; Storey, K.B.; Chen, Q. Metabolic responses of plasma to extreme environments in overwintering Tibetan frogs *Nanorana parkeri*: A metabolome integrated analysis. *Front. Zool.* 2021, 18, 41. [CrossRef] [PubMed]

23. Xu, N.; Chen, G.; Liu, H. Antioxidative categorization of twenty amino acids based on experimental evaluation. *Molecules* 2017, 22, 2066. [CrossRef] [PubMed]

24. Noriega, P.; Solà, M.; Barukcic, A.; García, K.; Osorio, E. Cosmetic antioxidant potential of extracts from species of the *Cinchona* pubescens (Vahl). *Int. J. Phyt. Nat. Ingred.* 2015, 2015, 2, 14. [CrossRef]

25. Liu, X.; Sha, Y.; Lv, W.; Cao, G.; Guo, X.; Pu, X.; Wang, J.; Li, S.; Hu, J.; Luo, Y. Multi-omics reveals that the rumen transcriptome, microbiome, and its metabolome co-regulate cold season adaptability of tibetan sheep. *Front. Microbiol.* 2022, 13, 859601. [CrossRef]

26. Ling, T.; Boyd, L.; Rivas, F. Triterpenoids as reactive oxygen species modulators of cell fate. *Chem. Res. Toxicol.* 2022, 35, 569–584. [CrossRef]

27. Wada, A.; Higashiyama, M.; Kurihara, C.; Ito, S.; Tanemoto, R.; Mizoguchi, A.; Nishii, S.; Inaba, K.; Sugihara, N.; Hanawa, Y.; et al. Protective effect of luminal uric acid against indomethacin-induced enteropathy: Role of antioxidant effect and gut microbiota. *Dig. Dis. Sci.* 2022, 67, 121–133. [CrossRef]

28. Metstruy, S.J.; Karhunen, V.; Edwards, M.H.; menni, C.; Geisendorfer, T.; Huber, A.; Reichel, C.; Dennison, E.M.; Cooper, C.; Spector, T.; et al. Metabolomic signatures of low birthweight: Pathways to insulin resistance and oxidative stress. *PLoS ONE* 2018, 13, e0194316. [CrossRef]

29. Chilczuk, B.; Marciniak, B.; Stochmal, A.; Pecio, L.; Kontek, R.; Jackowska, I.; Materska, M. Anticancer potential and capsinoides identification in lipophilic fraction of sweet pepper (*Capsicum annuum* L.). *Molecules* 2020, 25, 3097. [CrossRef]

30. Agnihotri, V.K.; ElSohly, H.N.; Khan, S.I.; Jacob, M.R.; Joshi, V.C.; Smillie, T.; Khan, I.A.; Walker, L.A. Constituents of *Nelumbo nucifera* leaves and their antimarial and antifungal activity. *Phytomed. Lett.* 2008, 1, 89–93. [CrossRef]

31. Hilmarsson, H.; Kristmundsdottir, T.; Thormar, H. Virucidal activities of medium- and long-chain fatty alcohols, fatty acids and monoglycerides against herpes simplex virus types 1 and 2: Comparison at different pH levels. *APMIS* 2005, 113, 58–65. [CrossRef] [PubMed]

32. de Aguiar Vallim, T.Q.; Tarling, E.J.; Edwards, P.A. Pleiotropic roles of bile acids in metabolism. *Cell Met.* 2013, 17, 657–669. [CrossRef] [PubMed]

33. Chadwick, M.; Trewn, H.; Gawthorp, F.; Wagstaff, C. Sesquiterpenoids lactones: Benefits to plants and people. *Int. J. Mol. Sci.* 2013, 14, 12780–12805. [CrossRef] [PubMed]
34. Li, R.; Guo, L.X.; Li, Y.; Chang, W.Q.; Liu, J.Q.; Liu, L.F.; Xin, G.Z. Dose-response characteristics of Clematis triterpenoid saponins and clematichines in rat model of rheumatoid arthritis by liquid chromatography/mass spectrometry-based serum and urine metabolomics. *J. Pharm. Biom. Anal.* 2017, 136, 81–91. [CrossRef]

35. Cutolo, M.; Sulli, A.; Straub, R.H. Estrogen metabolism and autoimmunity. *Autoimmun. Rev.* 2012, 11, A460–A464. [CrossRef]

36. Yu, D.; Du, J.; Pu, X.; Zheng, L.; Chen, S.; Wang, N.; Li, J.; Chen, S.; Fan, S.; Shen, B. The gut microbiome and metabolites are altered and interrelated in patients with rheumatoid arthritis. *Front. Cell. Infect. Microbiol.* 2021, 11, 763507. [CrossRef]

37. Feng, P.; Li, Q.; Liu, L.; Wang, S.; Wu, Z.; Tao, Y.; Huang, P.; Wang, P. Crotetin prolongs recovery period of DSS-induced colitis via altering intestinal microbiome and increasing intestinal permeability. *Int. J. Mol. Sci.* 2022, 23, 3832. [CrossRef]

38. Vainchtein, L.D.; Rosing, H.; Schellens, J.H.; Beijnen, J.H. A new, validated HPLC-MS/MS method for the simultaneous determination of the anti-cancer agent capetitabine and its metabolites: 5′-deoxy-5-fluorocytidine, 5′-deoxy-5-fluorouridine, 5-fluorouracil and 5-fluorodihydrouracil, in human plasma. *Biom. Chromatogr.* 2010, 24, 374–386. [CrossRef]

39. Alvarez-Munoz, D.; Farré, M. A snapshot of biomarkers of exposure for environmental monitoring. In *Environmental Metabolomics: Applications in Field and Laboratory Studies to Understand from Exposure to Metabolome*; Elsevier: Amsterdam, The Netherlands, 2020; pp. 311–338.

40. Xiao, G.; Shao, X.; Zhu, D.; Yu, B. Chemical synthesis of marine saponins. *Nat. Prod. Rep.* 2019, 36, 769–787. [CrossRef]

41. Wang, L.; Li, D.; Wang, C.; Zhang, Y.; Xu, J. Recent progress in the development of natural ent-kaurane diterpenoids with anti-tumor activity. *Mini-Rev. Med. Chem.* 2011, 11, 910–919. [CrossRef]

42. Rao, A.V.; Gurfinkel, D.M. The bioactivity of saponins: Triterpenoid and steroidal glycosides. *Mini-Rev. Med. Chem.* 2014, 14, 1138–1155. [CrossRef] [PubMed]

43. Pomorska, D.K.; Gach, K.; Janecka, A. Immunomodulatory effects of endogenous and synthetic peptides activating opioid receptors. *Mini-Rev. Med. Chem.* 2011, 11, 349–355. [CrossRef] [PubMed]

44. Liu, R.M.; Zhong, J.J. Ganoderic acid Mf and S induce mitochondria mediated apoptosis in human cervical carcinoma HeLa cells. *Phytomedicine* 2011, 18, 349–355. [CrossRef] [PubMed]

45. Lee, J.Y.; Kim, J.; Nam, Y.J.; Kim, H.J.; No, K.T. Isolindleyin exerts anti-melanogenic effects in human epidermal melanocytes via direct binding to tyrosinase. *Biochem. Biophys. Res. Commun.* 2021, 534, 802–807. [CrossRef] [PubMed]

46. Xi, J.; Ding, D.; Zhu, H.; Wang, R.; Su, F.; Wu, W.; Xiao, Z.; Liang, X.; Zhao, Q.; Hong, Z.; et al. Disturbed microbial ecology in Alzheimer’s disease: Evidence from the gut microbiota and fecal metabolome. *BMC Microbiol.* 2021, 21, 226. [CrossRef]

47. Vardhan, S.; Sahoo, S.K. Exploring the therapeutic nature of limonoids and triterpenoids against SARS-CoV-2 by targeting nsp13, nsp14, and nsp15 through molecular docking and dynamics simulations. *J. Tradit. Complementary Med.* 2022, 12, 44–54. [CrossRef]

48. Lu, Y.; Wang, J.; Soladoye, O.P.; Aluko, R.E.; Fu, Y.; Zhang, Y. Preparation, receptors, bioactivity and bioavailability of γ-glutamyl peptides: A comprehensive review. *Trends Food Sci. Technol.* 2021, 113, 301–314. [CrossRef]

49. Silveira-Dorta, G.; Martin, V.S.; Padron, J.M. Synthesis and antiproliferative activity of glutamic acid-based dipeptides. *Amino Acids* 2015, 47, 1527–1532. [CrossRef]

50. Yu, W.; Ying, J.; Wang, X.; Liu, X.; Zhao, T.; Yoon, S.; Zheng, Q.; Fang, Y.; Yang, D.; Hua, F. The involvement of lactosylceramide in drastic changes in oxygen availability. *Comp. Biochem. Physiol. Part A Mol. Integr. Physiol.* 2013, 165, 384–404. [CrossRef]

51. Seidler, N.W.; Jona, I.; Vegh, M.; Martonosi, A. Cyclopiazonic acid is a specific inhibitor of the Ca2+-ATPase of sarcoplasmic reticulum. *J. Biol. Chem.* 1989, 264, 17816–17823. [CrossRef]

52. Xue, H.; Huo, Y.; Hu, Y.; Zhang, J.; Deng, C.; Zhang, J.; Wang, X. The role of ALOX15B in heat stress-induced apoptosis of porcine sertoli cells. *J. Tradit. Complementary Med.* 2021, 1148–1155. [CrossRef] [PubMed]

53. Ogasawara, Y.; Dairi, T. Biosynthesis of oligopeptides using ATP-grasp enzymes. *Chemistry* 2017, 680–693. [CrossRef]

54. Chen, T.C.; Persons, K.S.; Lu, Z.; Mathieu, J.S.; Holick, M.F. An evaluation of the biologic activity and vitamin D receptor binding affinity of the photoisomers of vitamin D3 and previtamin D3. *J. Nutr. Biochem.* 2000, 11, 638–645. [CrossRef] [PubMed]

55. Shum, M.; Shintre, C.A.; Althoff, T.; Gutierrez, V.; Segawa, M.; Saxberg, A.D.; Martinez, M.; Adamson, R.; Young, M.R.; Faust, B.; et al. ABCB10 exports mitochondrial biliverdin, driving metabolic maladaptation in obesity. *Sci. Transl. Med.* 2021, 13, eabd1869. [CrossRef] [PubMed]

56. Seidler, N.W.; Jona, I.; Vegh, M.; Martonosi, A. Cyclopiazonic acid is a specific inhibitor of the Ca2+-ATPase of sarcoplasmic reticulum. *J. Biol. Chem.* 1989, 264, 17816–17823. [CrossRef]

57. Xue, H.; Huo, Y.; Hu, Y.; Zhang, J.; Deng, C.; Zhang, J.; Wang, X. The role of ALOX15B in heat stress-induced apoptosis of porcine sertoli cells. *J. Tradit. Complementary Med.* 2021, 1148–1155. [CrossRef] [PubMed]

58. Chen, T.C.; Persons, K.S.; Lu, Z.; Mathieu, J.S.; Holick, M.F. An evaluation of the biologic activity and vitamin D receptor binding affinity of the photoisomers of vitamin D3 and previtamin D3. *J. Nutr. Biochem.* 2000, 11, 638–645. [CrossRef] [PubMed]

59. Rizzo, W.B. Fatty aldehyde and fatty alcohol metabolism: Review and importance for epidermal structure and function. *J. Pharm. Biom. Anal.* 2017, 136, 81–91. [CrossRef]

60. Ao, J.; Li, B. Amino acid composition and antioxidant activities of hydrolysates and peptide fractions from porcine collagen. *Food Sci. Technol. Int.* 2012, 18, 425–434. [CrossRef]
61. Chi, C.F.; Hu, F.Y.; Wang, B.; Li, Z.R.; Luo, H.Y. Influence of amino acid compositions and peptide profiles on antioxidant capacities of two protein hydrolysates from Skipjack Tuna (Katsuwonus pelamis) dark muscle. Mar. Drugs 2015, 13, 2580–2601. [CrossRef]

62. Bombaca, A.C.S.; Brunoro, G.V.F.; Dias-Lopes, G.; Ennes-Vidal, V.; Carvalho, P.C.; Perales, J.; d’Avila-Levy, C.M.; Valente, R.H.; Menina-Barreto, R.F.S. Glycolytic profile shift and antioxidant triggering in symbiont-free and H2O2-resistant Strigomonas culicis. Free Rad. Biol. Med. 2020, 146, 392–401. [CrossRef]

63. Zeng, L.; Ai, C.X.; Zheng, J.L.; Zhang, J.S.; Li, W.C. Cu pre-exposure alters antioxidant defense and energy metabolism in large yellow croaker Larimichthys crocea in response to severe hypoxia. Sci. Total Environ. 2019, 687, 702–711. [CrossRef][PubMed]

64. Chen, J.; Liu, N.; Li, B.; Zhang, H.; Zhao, Y.; Cao, X. The effects of fipronil exposure on oxidative stress, non-specific immunity, autophagy, and apoptosis in the common carp. Environ. Sci. Pollut. Res. 2021, 28, 27799–27810. [CrossRef][PubMed]

65. Chen, J.; Liu, H.; Cai, S.; Zhang, H. Comparative transcriptome analysis of Eucantherina posseticus at different hydrostatic pressure and temperature exposures. Sci. Rep. 2019, 9, 3456. [CrossRef][PubMed]

66. Bird, L. Getting enough energy for immunity. Nat. Rev. Immunol. 2019, 19, 269. [CrossRef]

67. Cho, H.Y.; Loretti, E.; Shih, M.C.; Perata, P. Energy and sugar signaling during hypoxia. New Phytol. 2021, 229, 57–63. [CrossRef]

68. Yancey, P.H. Cellular responses in marine animals to hydrostatic pressure. [CrossRef]

69. Zhao, M.; Wang, C.Y.; Sun, L.; He, Z.; Yang, P.L.; Liao, H.J.; Feng, Y. Edible aquatic insects: Diversities, nutrition, and safety. Foods 2021, 10, 3033. [CrossRef]

70. Harayama, T.; Riezman, H. Understanding the diversity of membrane lipid composition. Nat. Rev. Mol. Cell Biol. 2018, 19, 281–296. [CrossRef]

71. Chengappa, P.; Sao, K.; Jones, T.M.; Petrie, R.J. Intracellular pressure: A driver of cell morphology and movement. Int. Rev. Cell. Mol. Biol. 2018, 337, 185–211.

72. Moussian, B. Recent advances in understanding mechanisms of insect cuticle differentiation. Insect Biochem. Mol. Biol. 2010, 40, 363–375. [CrossRef][PubMed]

73. Sauer, S.W.; Okun, J.G.; Hoffmann, G.F.; Koelker, S.; Morath, M.A. Impact of short- and medium-chain organic acids, acylcarnitines, and acyl-CoAs on mitochondrial energy metabolism. Biochim. Biophys. Acta 2008, 1777, 1276–1282. [CrossRef][PubMed]

74. Cui, S.; Wang, L.; Qiu, J.; Liu, Z.; Geng, X. Comparative metabolomics analysis of Callosobruchus chinensis larvae under hypoxia, hypoxia/hypercapnia and normoxia. PLoS Negl. Trop. Dis. 2017, 11, 1267–1276. [CrossRef][PubMed]

75. Vazquez, J.A.; Duran, A.; Rodriguez-Amado, I.; Prieto, M.A.; Rial, D.; Murado, M.A. Evaluation of toxic effects of several carboxylic acids on bacterial growth by toxicodynamic modelling. Microb. Cell Factories 2011, 10, 100. [CrossRef]

76. Martinez, O.F.; Duque, H.M.; Franco, O.L. Peptidomimetics as potential anti-virulence drugs against resistant bacterial pathogens. Front. Microbiol. 2022, 13, 831037. [CrossRef]

77. Spada, V.; Ferranti, P.; Chianese, L.; Salimei, E.; Addeo, F.; Picariello, G. Antibacterial potential of donkey’s milk disclosed by untargeted proteomics. J. Prot. 2021, 231, 104007. [CrossRef]

78. Jordheim, L.P.; Durantel, D.; Zoulim, F.; Dumontet, C. Advances in the development of nucleoside and nucleotide analogues for cancer and viral diseases. Nat. Rev. Mol. Cell Biol. 2021, 229, 42–521. [CrossRef]

79. Olcina, M.; Lecane, P.S.; Hammond, E.M. Targeting hypoxic cells through the DNA damage response. Clin. Cancer Res. 2010, 16, 5624–5629. [CrossRef]

80. Li, Y.S.; Tang, J.X.; Li, J.L.; Liang, C.; Zhang, M.H.; Wu, J.Y.; Wang, G.X.; Zhu, G.D.; Cao, J. Study on emergency metabolic changes and acyl-CoAs on mitochondrial energy metabolism. Insect Biochem. Mol. Biol. 2018, 100, 101. [CrossRef]

81. Yang, S.; Yan, D.; Zou, Y.; Mu, D.; Li, X.; Shi, H.; Luo, X.; Yang, M.; Yue, X.; Wu, R.; et al. Fermentation temperature affects yogurt quality: A metabolomics study. Food Biosci. 2021, 42, 101104. [CrossRef]

82. Wu, X.; Liu, C.; Yang, S.; Shen, N.; Wang, Y.; Zhu, Y.; Guo, Z.; Yang, S.Y.; Xing, D.; Li, H.; et al. Glycine-serine-threonine metabolic axis delays intervertebral disc degeneration through antioxidant effects: An imaging and metabonomics study. Oxidative Med. Cell. Longev. 2021, 2021, 5579736. [CrossRef][PubMed]

83. Yang, J.; Jin, Z.B.; Chen, J.; Huang, X.F.; Li, X.M.; Liang, Y.B.; Mao, J.Y.; Chen, X.; Zheng, Z.; Bakshi, A.; et al. Genetic signatures of high-altitude adaptation in Tibetans. Proc. Natl. Acad. Sci. USA 2017, 114, 4189–4194. [CrossRef][PubMed]

84. Delchier, N.; Ringling, C.; Cuvelier, M.E.; Courtois, F.; Rychlik, M.; Renard, C.M. Thermal degradation of folates under varying oxygen conditions. Food Chem. 2014, 165, 85–91. [CrossRef][PubMed]

85. Wang, P.; Fan, F.; Li, X.; Sun, X.; Ma, L.; Wu, J.; Shen, C.; Zhu, H.; Dong, Z.; Wang, C.; et al. Riboflavin attenuates myocardial injury via LSD1-mediated crosstalk between phospholipid metabolism and histone methylation in mice with experimental myocardial infarction. J. Mol. Cell. Cardiol. 2018, 115, 115–129. [CrossRef]

86. Tian, R.; Yang, C.; Chai, S.M.; Guo, H.; Seim, I.; Yang, G. Evolutionary impacts of purine metabolism genes on mammalian oxidative stress adaptation. Zool. Res. 2022, 43, 241–254. [CrossRef]

87. Del Castillo Velasco-Martinez, I.; Hernandez-Camacho, C.J.; Mendez-Rodriguez, L.C.; Zenteno-Savín, T. Purine metabolism in response to hypoxic conditions associated with breath-hold diving and exercise in erythrocytes and plasma from bottlenose dolphins (Tursiops truncatus). Comp. Biochem. Physiol. Part A Mol. Integr. Physiol. 2016, 191, 196–201. [CrossRef]

88. Goswami, A.R.; Ghosh, T. Vitamin E reduces hypoxia-induced immune responses in male rats. High Altitude Med. Biol. 2019, 20, 12–21. [CrossRef]
89. Zhang, Q.L.; Guo, J.; Deng, X.Y.; Wang, F.; Chen, J.Y.; Lin, L.B. Comparative transcriptomic analysis provides insights into the response to the benzo(a)pyrene stress in aquatic firefly (Luciola leii). *Sci. Total Environ.* 2019, 661, 226–234. [CrossRef]

90. Yin, N.N.; Nuo, S.M.; Xiao, H.Y.; Zhao, Y.J.; Zhu, J.Y.; Liu, N.Y. The ionotropic receptor gene family in Lepidoptera and Trichoptera: Annotation, evolutionary and functional perspectives. *Genomics* 2021, 113, 601–612. [CrossRef]

91. Niwa, Y.S.; Niwa, R. Transcriptional regulation of insect steroid hormone biosynthesis and its role in controlling timing of molting and metamorphosis. *Dev. Growth Differ.* 2016, 58, 94–105. [CrossRef]