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Divergent responses in the gut microbiome and liver metabolome to ammonia stress in three freshwater turtles

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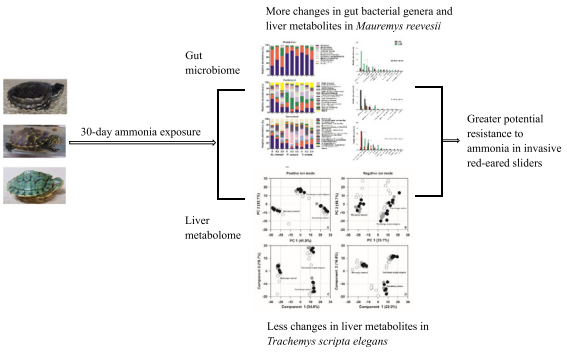
HIGHLIGHTS

- Ammonia exposure altered gut microbial composition and liver metabolite of turtles.
- Microbial and metabolic responses to ammonia stress differed among species.
- More changed bacterial genera and metabolites were found in native species.
- Less changed metabolites reflected a higher ammonia resistance for invasive turtle.

GRAPHICAL ABSTRACT



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ABSTRACT

Ammonia is a common pollutant in aquaculture system, and toxic to all aquatic animals. However, different aquatic animals exhibit diverse physiological responses to high-level ammonia exposure, potentially indicating their divergent resistance to ammonia stress. In this study, juveniles of three freshwater turtles (*Mauremys reevesii*, *Pseudemys nelsoni* and *Trachemys scripta elegans*) were exposed to different concentrations of ammonia (0, 0.3 and 3.0 mg/L) for 30 days, and their swimming, growth performance, gut microbiota, and hepatic metabolites were measured to evaluate the interspecific difference in physiological responses to ammonia stress. Despite no differences in swimming ability, growth rate, and gut microbial diversity, observable changes in microbial community composition and hepatic metabolite profiles were shown in ammonia-exposed turtles. A relatively higher abundance of potentially pathogenic bacteria was found in *M. reevesii* than in the other two species. Moreover, microbial compositions and metabolic responses differed significantly among the three species. *M. reevesii* was, out of the three tested species, the one in which exposure to ammonia had the greatest effect on changes in bacterial genera and hepatic metabolites. Conversely, only a few metabolites were significantly changed in *T. scripta elegans*. Integrating these findings, we speculated that native *M. reevesii* should be more vulnerable to ammonia stress compared to the invasive turtle species. Our results plausibly reflected divergent potential resistance to ammonia among these turtles, in view of differential physiological responses to ammonia exposure at environmentally relevant concentrations.

1. Introduction

Despite naturally occurring in various environments, ammonia nitrogen (ammonia-N) contents in natural waterbodies are generally maintained at relatively low levels (e.g., normally <1.0 mg/L in surface water of major rivers in China, Ma et al., 2020; Zhang et al., 2022). In aquaculture systems, ammonia-N is a common environmental pollutant, that is mainly produced from animal excrement, residual bait, etc. (Ip et al., 2001; Gao et al., 2017). Its content is easy to be excessive in those traditional closed intensive aquaculture ponds. For example, the ammonia-N content in shrimp pond water can reach 6.5 mg/L after several weeks of intensive culture (Chen and Lin,

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1992). The unionized (NH_3) and ionized (NH_4^+) species are the two forms of ammonia-N existing in the water. Of the two, the former easily permeates cell membranes and is largely responsible for ammonia toxicity (Ip et al., 2001; Mooney et al., 2019). It has been well documented that excess ammonia exposure reduces the survival rate and inhibits the growth and feeding performances in some shrimp and fish species (Barimo and Walsh, 2005; Paust et al., 2011; Cui et al., 2017; Vaage and Myrick, 2021). Exposure to concentrations of >20 mg/L of ammonia reduces the immunity of aquatic animals, and makes them more vulnerable to pathogens (Qi et al., 2017; Lv et al., 2021). Even at a low concentration (e.g., 1.0 mg/L, Kim et al., 2015), ammonia exposure may perturb multiple physiological processes (such as inducing antioxidant response and cell apoptosis, altering the expression of immune- and antioxidant-related genes, etc.) in various aquatic organisms (Cui et al., 2017; Gao et al., 2017; Qi et al., 2017; Egnew et al., 2019; Duan et al., 2021; Huang et al., 2021; Ren et al., 2022). Additionally, increasing studies demonstrate that ammonia exposure can alter the composition and function of gut microbiota in crustaceans, fish and turtles, and thus influence their health status (Qi et al., 2017; Ding et al., 2021; Duan et al., 2021; Khan et al., 2021; S.-D. Wang et al., 2021; Yang et al., 2021).

Waterborne ammonia is believed to be toxic to all aquatic animals. However, its toxicity may vary among different species or even within a given species under different environmental conditions (Chen and Lin, 1992; Ip et al., 2001; Kim et al., 2015; Souza-Bastos et al., 2017; Mooney et al., 2019). In other words, the resistance to ammonia stress differs among species or populations (Ip et al., 2001; Mooney et al., 2019; Chen et al., 2022). Aquatic animals may adopt diverse physiological responses to enhance their resistance ability against environmental stress (Ip et al., 2001; Zhang et al., 2015; Peng et al., 2017; Xiao et al., 2020; Zhang et al., 2020). For example, the white-leg shrimp, *Litopenaeus vannamei*, would accelerate the conversion of ammonia and glutamate into glutamine by the action of glutamine synthetase to alleviate ammonia stress (Qiu et al., 2018); and some freshwater turtles (e.g., the Chinese soft-shelled turtle, *Pelodiscus sinensis*) would stimulate active antioxidant responses under ammonia stress, thereby reducing ammonia-induced oxidative damage (Chen et al., 2022). Comparison of various physiological responses to environmental stresses is crucial to understanding the mechanism of differential stress-resistant ability in different species. However, there are only few studies addressing divergent ammonia resistances among species (but see Ruyet et al., 1995; Chen et al., 2022).

With the development of turtle aquaculture, some alien species [such as the red-eared slider turtle *Trachemys scripta elegans* (first introduced in the 1980s), and Florida red-bellied turtle *Pseudemys nelsoni* (in the 1990s)] were introduced into China. *T. scripta elegans* and *P. nelsoni* are native to North American habitats that are similar to those occupied by some Chinese native turtles (such as the three-keeled pond turtle *Mauremys reevesii*). As we know, *T. scripta elegans* has become a successful invasive species in China, as well as other countries in Asia, Africa, and Europe, and poses a serious threat to local native turtles (Lowe et al., 2004). *P. nelsoni* has been cultured in China for more than two decades. However, it has not been documented to establish wild populations, or spread into natural environments. Currently, it is not listed as an invasive species in China, despite being an allochthonous species (Xu et al., 2012). It is generally believed that an alien species becomes invasive depending on whether it has a competitive superiority compared to native species occupying the same ecological niche (Callaway and Ridenour, 2004). Although it has been described that *T. scripta elegans* has a greater resistance to thermal stress than native turtles (Geng et al., 2018), studies focusing on the competitive superiority of invasive species in terms of physiological resistance to various environmental stresses are still scarce. In this study, juveniles of three freshwater turtle species (*M. reevesii*, *P. nelsoni* and *T. scripta elegans*) were empirically exposed to environmentally relevant concentrations of ammonia, and evaluated for the interspecific differences in physiological responses to ammonia stress by measuring the changes in the functional performance, gut microbiota, and liver metabolomic profiles. Specifically, we expected that the sensitivity of physiological responses to ammonia stress would be lower in *T. scripta elegans* than in the other two turtles, and such difference would be reflected by the divergent microbiome and metabolome responses to ammonia exposure among turtle species, and partially indicated the more or less invasive potential of alien turtle species.

2. Material and methods

2.1. Experimental animals

A total of 75 fertilized eggs of three turtle species (25 eggs in each species) were purchased from a private farm in Huzhou city, Zhejiang province, and transported to our laboratory at Hangzhou Normal University in June 2021. Turtle eggs were placed in 25 × 20 × 10 cm^3 plastic containers that filled with moist vermiculite, and then incubated at 28 °C in a temperature incubator (Binder KB240, Binder GmbH, Tuttlingen, Germany). The containers were weighed twice a week, and distilled water was added to the vermiculite to maintain a relatively constant substrate water potential. Newly hatched turtles were reared individually in a glass jar until the yolk sac was absorbed, and then weighed. All glass jars were placed in an artificial climate chamber at a temperature of 24 °C and light: dark photoperiod of 12:12 h (switched on at 6:00, off at 18:00). The general care of hatchlings was described previously by Lu et al. (2021).

2.2. Experimental treatment

Approximately three months after hatching, 15 juvenile turtles were randomly selected from each species, and equally allocated to different aquaria (one single turtle in each aquarium) each containing either control (CTRL), 0.3, or 3.0 mg/L ammonia (5 individuals in each treatment). The concentration of 3.0 mg/L ammonia selected in this study is the maximum dose of the primary standard of the requirement for water discharge from freshwater aquaculture pond (SC/T 9101-2007) and represents an environmentally realistic level in some polluted waterbodies (up to 3.4 mg/L, Ma et al., 2020), while the concentration of 0.3 mg/L represents 1/10 of the above-mentioned level and is close to the values reported in some Chinese rivers (0.22 mg/L, Zhang et al., 2022). The water quality parameters in the experiment were as follows: temperature, 23.95 ± 0.02 °C; pH: 7.02 ± 0.02; electrical conductivity, 114.07 ± 8.63 us/cm; dissolved oxygen, 6.87 ± 0.05 mg/L. Each aquarium contained an equal amount of dechlorinated tap water. After being prepared for experimental treatments with ammonium chloride (NH_4Cl), the ammonia level in each aquarium was measured and adjusted by adding an NH_4Cl stock solution (3.0 g/L, 11.457 g of NH_4Cl was dissolved in 1 L distilled water). During the exposure experiment, turtles were fed daily in the morning, and all uneaten food pellets were removed after approximately two hours. The water for turtle husbandry was renewed every other day. Water samples were collected from each aquarium directly and at 24 h after treatment renewals, and analyzed for ammonia concentrations and other quality parameters using a portable ammonia-N analyzer (Qingdao Juchuang Environmental Protection Group Co., Ltd) and REX DZB-712 multi-parameter water quality analyzer (Shanghai INESA Scientific Instrument Co., Ltd), respectively. The mean values ± standard errors (SE) of detected ammonia concentrations were presented in Supplementary Table S1. The actual ammonia content in exposure waters appeared to increase somewhat on the following day (probably due to the excretion of turtles), and water quality parameters also changed accordingly (Table S1).

2.3. Swimming performance measurement

On the day before ending the exposure, the swimming performance of each turtle was evaluated inside a swimming racetrack (120 × 10 × 20 cm^3) containing 10 cm depth water. The racetrack was placed in a climate-controlled room set at 24 °C, and the temperature of the water was maintained at the corresponding level. After being acclimated for approximately 1 h, the turtle was individually introduced to one end of the racetrack, and then the mid-body was gently tapped with a paintbrush

(always by the second author) to encourage it to swim continuously. The swimming performance of each turtle was recorded using a Panasonic HDC-SD900GK digital video camera, and the videoclips were examined later for the average speed over 50 cm.

2.4. Gut microbiota diversity

After 30-day of exposure, turtles were weighed, and then euthanized on ice and dissected. The liver and gut of each turtle was carefully removed under sterile conditions, rapidly frozen in liquid nitrogen and then stored at −80 °C. The specific growth rate of each individual was calculated using the formula: specific growth rate = $(\ln W_t - \ln W_0) / T \times 100$ %, where W_0 = initial body mass and W_t = final body mass (Kang et al., 2022).

DNA of gut microbiota was extracted using the Qiagen TM QIAamp DNA Stool Mini Kit (Qiagen GmbH). The V3–V4 variable region of the 16 S rRNA gene was amplified using the primers, B341F (5′- CCTACGGG NGGCWGCAG -3′) and B785R (5′- GACTACHVGGGTATCTAATCC -3′). The detailed procedures of PCR amplification and product purification were performed as previously described (Kang et al., 2022). Purified amplicons were sequenced on the Illumina NovaSeq PE250 platform (Illumina Inc., San Diego, USA).

2.5. Hepatic metabolomic profiling

The frozen liver tissue was thawed, and approximately 100 mg from each sample was homogenized at low temperatures. Homogenates were vortexed and centrifuged at 4 °C for 10 min, then supernatants (800 μL from each sample) were transferred into clean centrifuge tubes and dried. Dried residues were dissolved with 200 μL of 50 % acetonitrile solution prepared with 2-amino-3-(2-chloro-phenyl)-propionic acid as an internal standard. Meanwhile, the quality control sample was prepared by mixing an equal volume of each extracted sample. All samples were filtered through a 0.22 μm filtering membrane before liquid chromatography-mass spectrometry (LC-MS) analysis.

LC-MS analysis was performed on a Vanquish UHPLC System (Thermo Fisher Scientific, San Jose, CA, USA) with an ACQUITY UPLC® HSS T3 column (150 × 2.1 mm, 1.8 μm) (Waters, Milford, MA, USA) coupled to a Q Exactive Orbitrap mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA). The column temperature was maintained at 40 °C, the flow rate was set at 250 μL/min, and 2 μL of each sample was added into the separation column. The mobile phase comprised acetonitrile (A) and 5 mM ammonium formate (B) in the negative ion mode, 0.1 % formic acid in acetonitrile (C) and 0.1 % formic acid in water (D) in the positive ion mode. The elution program was: 0–1 min 2 % A, 1–9 min increased to 50 % A, 9–12 min increased to 98 % A, 12–13.5 min maintained at 98 % A, 13.5–14 min decreased to 2 % A, and 14–17 min maintained at 2 % A (negative ion mode); 0–1 min 2 % C, 1–9 min increased to 50 % C, 9–12 min increased to 98 % C, 12–13.5 min maintained at 98 % C, 13.5–14 min decreased to 2 % C, and 14–20 min maintained at 2 % C (positive ion mode). The parameters of mass spectrometry were as follows: sheath gas pressure, 30 arbitrary units; auxiliary gas flow, 10 arbitrary units; spray voltage, −2.50 kV and 3.50 kV for the negative and positive modes respectively; capillary temperature, 325 °C; MS1 scan range, *m/z* 100–1, 000; MS1 resolving power, 60,000 FWHM; data dependent scan number per cycle, 4; MS/MS resolving power, 15,000 FWHM, normalized collision energy, 30 %. Unnecessary information was removed using dynamic exclusion. LC-MS analysis of the hepatic metabolome was completed at Suzhou PANOMIX Biomedical Tech Co., Ltd. (Suzhou, China).

2.6. Data analyses

Data were tested for normality (Kolmogorov–Smirnov test) and homogeneity of variances (Levene's test) before parametric analyses. Two-way analysis of variance (ANOVA) was performed to test the differences in initial body mass, swimming speed, and specific growth rate among turtle species and treatment groups.

For gut microbiota profiling, raw paired-end reads were analyzed, quality-filtered, and processed. Effective sequences were clustered into operational taxonomic units (OTUs) at the 97 % similarity level. Representative sequences were annotated by searching the RDP and SILVA databases and compiled into each taxonomic level, and the α diversity (Shannon-Wiener) index was calculated for each sample. Calculated indices were visualized and subsequent statistical analyses were performed in the R software environment. Non-parametric Kruskal-Wallis tests were performed to test among-group differences in the Shannon-Wiener index and gut microbial composition at each taxonomic level, and principal coordinates analysis (PcoA) was performed to test among-group difference in the relative abundance of OTUs for the β diversity analysis.

The raw data of LC-MS metabolomic profiling were converted into mzXML format and processed using ProteoWizard (v3.0.8789) and XCMS in R, and then analyzed on the MetaboAnalyst web-based platform (Pang et al., 2021). Multivariate unsupervised principal component analysis (PCA) and supervised partial least squares discriminant analysis (PLS-DA) were performed to test among-group differences in hepatic metabolites. Metabolites were identified by searching the mass spectra against available databases [i.e., Human Metabolome Database (HMDB), Metabolite Link (Metlin), MassBank Database]. One-way ANOVAs were performed to test among-group differences in key identified metabolites.

3. Results

3.1. Juvenile swimming speed and growth rate

The mean initial body mass of *M. reevesii* was higher than those of the other two turtles (*M. reevesii*, 12.13 ± 0.48 g; *P. nelsoni*, 7.83 ± 0.21 g; *T. scripta elegans*, 8.29 ± 0.22 g, $F_{2, 36} = 47.46$, $P < 0.001$), but it did not differ among treatment groups in each species (CTRL, 9.65 ± 0.56 g; 0.3 mg/L-exposed, 9.55 ± 0.63 g; 3.0 mg/L-exposed, 9.06 ± 0.63, $F_{2, 36} = 0.84$, $P = 0.440$). No turtles died during the experiment. No significant differences in swimming speed ($F_{2, 36} = 1.45$, $P = 0.248$) and growth rate ($F_{2, 36} = 0.83$, $P = 0.444$) were found among different treatment groups (Fig. 1). *P. nelsoni* juveniles appeared to gain mass faster than the two other turtle species ($F_{2, 36} = 9.10$, $P < 0.001$), and *T. scripta elegans* juveniles had a relatively higher swimming speed ($F_{2, 36} = 4.30$, $P = 0.021$, Fig. 1).

3.2. Gut microbiota composition

A total of 45 gut microbial samples from 3 turtle species were used for the bacterial composition analysis. Each sample generated at least 130,000 effective sequences, and most of the OTUs could be annotated at the family or genus level in each sample. No significant differences in the α diversity of gut microbiota could be found among groups in each species (e.g., Kruskal-Wallis test on the Shannon-Wiener index, all $P > 0.340$, Fig. 2), and no significant separations of bacterial communities among groups were identified by the PcoA of β diversity (Fig. 2).

Further taxonomic analysis showed that Firmicutes (58.2 ± 4.2 %), Bacteroidetes (29.6 ± 3.4 %) and Proteobacteria (8.5 ± 2.3 %) were the most predominant phyla in all samples. Native *M. reevesii* had a higher relative abundance of Bacteroidetes, but lower relative abundance of Firmicutes than alien *P. nelsoni* and *T. scripta elegans*; similarly, obvious among-species differences in the relative abundance could also be observed in some bacterial families (e.g., more Bacteroidaceae, less Peptostreptococcaceae in *M. reevesii*) or genera (e.g., more *Bacteroides*, less *Romboutsia*, *Clostridium_sensu_stricto*, and *Terrisporobacter* in *M. reevesi*; more *Turicibacter* in *P. nelsoni*; more *Anaerosporobacter*, less *Akkermansia* in *T. scripta elegans*) (Fig. 3). However, no significant differences in the relative abundance of predominant bacterial phyla among different treatment groups were observed in each species (Kruskal-Wallis test, all $P > 0.145$, Fig. 3). Among-group differences in the relative abundance were found in only one or a few bacterial families in the three turtles. For example, despite accounting for a very low proportion, the family Pseudomonadaceae was mainly or only found in 3.0 mg/L-exposed turtles (0.09 ± 0.07 % for *M. reevesii*; 0.10 ± 0.10 % for *P. nelsoni*; 0.03 ±

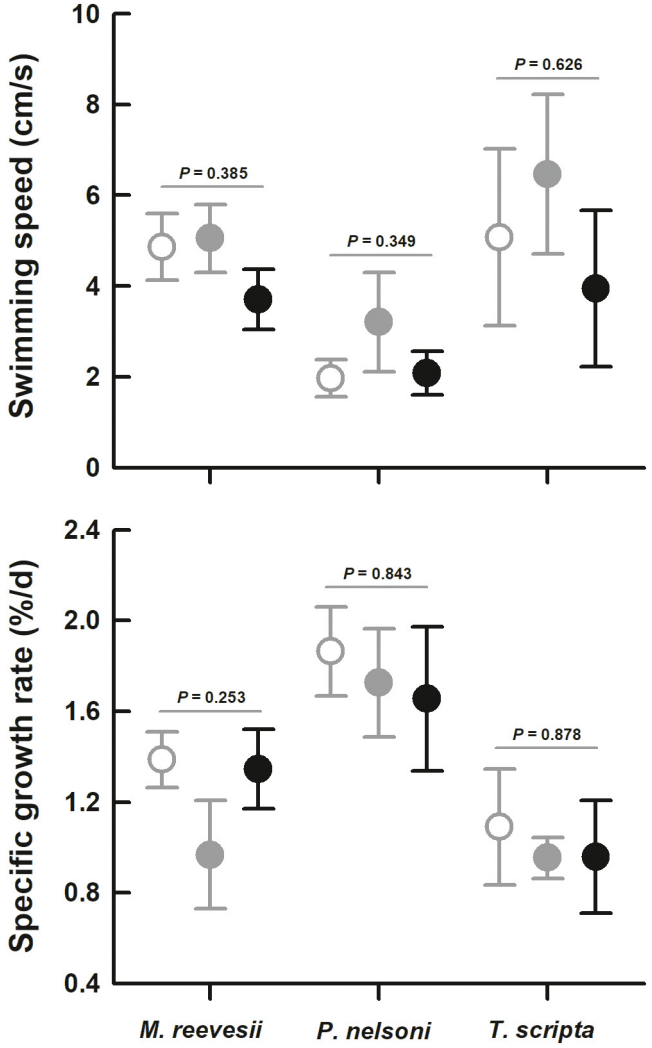

Fig. 1. Swimming speed and specific growth rate of three freshwater turtles (*Mauremys reevesii*, *Pseudemys nelsoni*, and *Trachemys scripta elegans*) exposed to control (grey open circles), 0.3 (grey solid circles), 3.0 mg/L (black solid circles) ammonia.

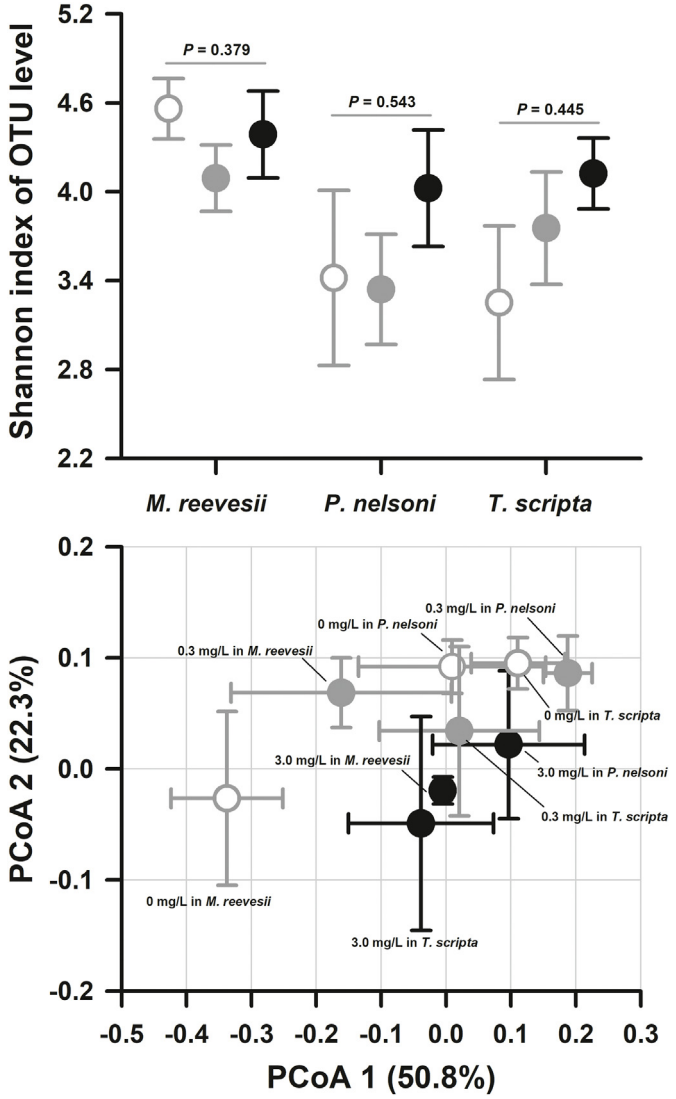

Fig. 2. The Shannon-Wiener index and score plots for principal coordinates analysis (PCoA) for gut microbiota of three freshwater turtles exposed to control (grey open circles), 0.3 (grey solid circles), 3.0 mg/L (black solid circles) ammonia.

0.02 % for *T. scripta elegans*) (Fig. 3). At the genus level, *Bacteroides* (18.3 ± 3.9 %), *Romboutsia* (12.9 ± 3.9 %), *Turicibacter* (3.6 ± 1.0 %), *Parabacteroides* (1.8 ± 0.3 %), and some unclassified genera belonging to Lachnospiraceae (10.9 ± 1.7 %), Bacteroidales (7.1 ± 1.4 %) and Clostridiales (3.9 ± 0.9 %) were the most predominant bacterial genera. Despite only marginally significant differences, the relative abundances of some bacterial genera (such as *Bacteroides*, *Monoglobus* and unclassified genera belonging to Lachnospiraceae or Ruminococcaceae in *M. reevesii*, *Romboutsia* in *P. nelsoni* and *T. scripta elegans*) appeared to decrease with increasing ammonia concentration, whereas those of others (such as *Clostridium_sensu_stricto* and unclassified genera belonging to Bacteroidales in *M. reevesii* and *P. nelsoni*, unclassified genera belonging to Clostridiales in *P. nelsoni*) increased (Fig. 3). The relative abundance of bacterial genera accounting for small proportions were also affected by ammonia exposure in the three turtle species (Fig. 4). Specifically, some genera, such as *Deinococcus*, *Raoultella*, *Anaerorhabdus*, *Myroides* (in *M. reevesii*), *Clostridium_XlVa*, *Lawsonella* (in *P. nelsoni*), *Ottowia*, *Clostridioides* and *Aeromonas* (in *T. scripta elegans*) were found to increase in ammonia-exposed individuals or only in the 3.0 mg/L-exposed group, while a few genera, such as *Amedibacillus*, *Bacilliculturomica* (in *M. reevesii*), *Clostridium_XlVb*, *Blautia* (in *P. nelsoni*), and *Bariatricus* (in *T. scripta elegans*), decreased (Fig. 4). Overall, the significantly-changed bacterial genera differed among the three turtle species; more significantly-changed genera were observed in *M. reevesii* and less in *P. nelsoni* (Fig. 4).

3.3. Hepatic metabolite profile

PCA analysis of hepatic metabolite profiles revealed a distinct separation among different species, but a modest separation among groups in each species, with 54.6 % and 42.4 % of cumulative variance contributions from the first two components in the positive and negative ion modes, respectively (Fig. 5A, B). PLS-DA analysis revealed a more obvious separation among different groups, with 54.5 % and 38.8 % of cumulative variance contributions from the first two latent components, respectively (Fig. 5C, D).

More significantly-changed metabolites due to ammonia exposure were found in *P. nelsoni* and *M. reevesii* than in *T. scripta elegans* (Fig. 6). By searching information in available databases, some metabolites were identified and showed significant differences among species and treatment groups. Some metabolites decreased (e.g., glutamate in the three species, glutamine in *P. nelsoni* and *T. scripta elegans*, proline in *M. reevesii* and *P. nelsoni*); or increased with increasing ammonia concentration (e.g., glutathione in *P. nelsoni*) (Table 1). The changing extent of identified metabolites in response to ammonia exposure differed among species. For example, metabolites related to energy metabolism (e.g., nicotinamide adenine dinucleotide-NAD, reduced nicotinamide adenine dinucleotide-NADH) changed significantly in *P. nelsoni*, but not in *M. reevesii* and *T. scripta elegans*; some amino acids (e.g., isoleucine, proline, valine, citrulline) and other metabolites

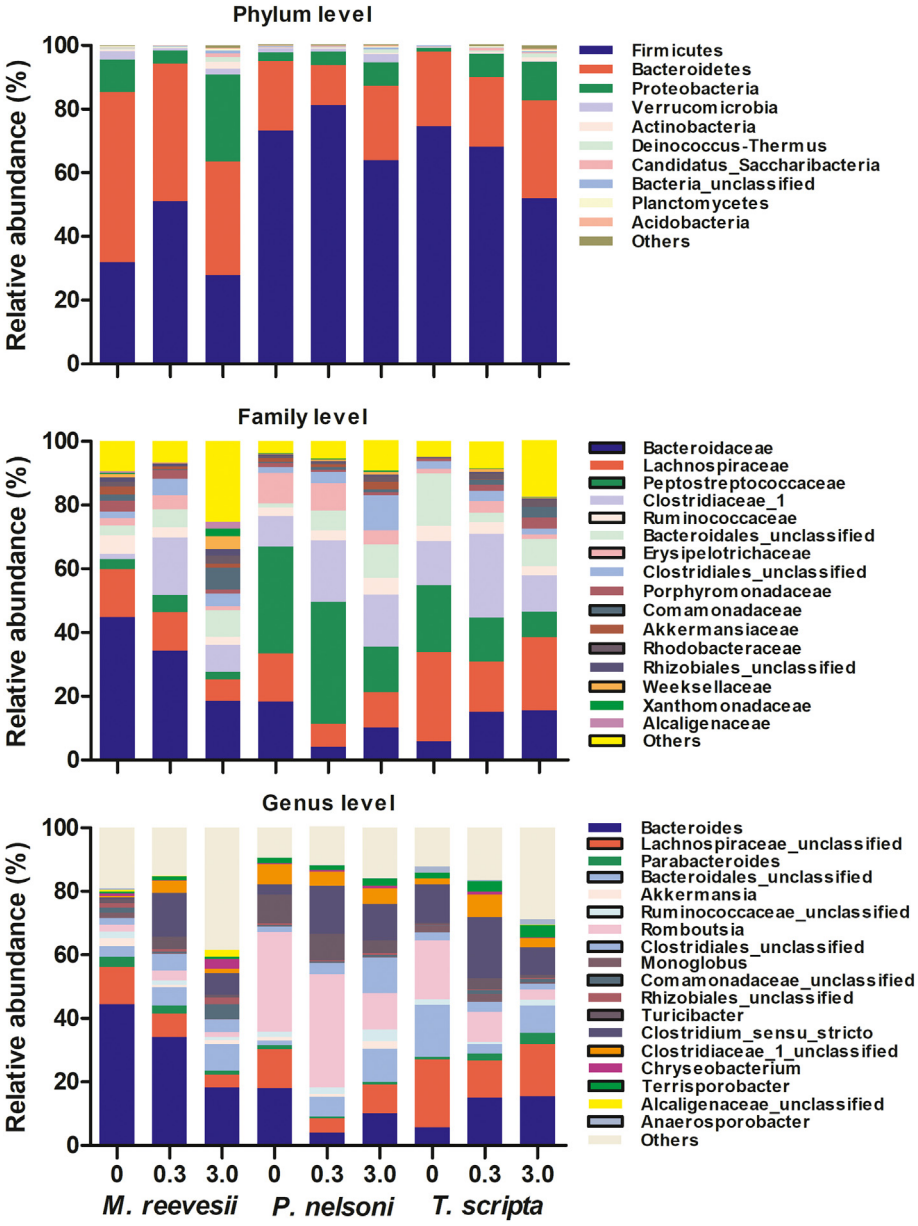

Fig. 3. Relative abundances of the gut microbiota at the phylum, family, and genus levels in different groups of three freshwater turtles.

(e.g., creatine, choline, niacinamide, adenosine monophosphate-AMP, uracil, cytidine) changed significantly in *M. reevesii* and *P. nelsoni*, but not in *T. scripta elegans* (Table 1).

4. Discussion

In this study, we determined the functional, metabolic, and gut microbial responses of three freshwater turtle species exposed to environmentally relevant concentrations of ammonia. Our results would be compared with those in other ammonia-exposed aquatic organisms to explore differential toxic effects of ammonia among different species, and those obtained from studies conducted on turtles to reveal divergent physiological responses to ammonia stress among turtle species. Although behavioral and growth inhibition due to high levels of ammonia exposure has been documented in planarians, crustaceans, fish, etc. (Remen et al., 2008; Miranda-Filho et al., 2009; Paust et al., 2011; Alonso and Camargo, 2015; Vaage and Myrick, 2021), no obvious changes in locomotor and growth performances were observed in the three turtle species, partially because only lower ammonia concentrations were used here. However, we did observe metabolic (or other physiological) changes caused by ammonia exposure in this study, which is similar to findings in other studies (e.g., in crustaceans, Ren et al., 2015; T.-Y. Wang et al., 2021; molluscs, Peng et al., 2017; fish, Qi et al., 2017; Gao et al., 2021; reptiles, Huang et al., 2021). As expected, these responses to ammonia exposure differed significantly among turtle species, possibly reflecting discrepant potential resistance to ammonia stress among these three species.

Similar microbial diversity among turtle species possibly indicated a limited impact of exposure to low levels of ammonia on the intestinal bacterial community in these species. This was not consistent with previously described gut microbiome changes induced by exposure to higher levels of ammonia in crustaceans (20 mg/L total ammonia-N in *L. vannamei*, Lv et al., 2021) and fish (50 mg/L total ammonia-N in *Carassius auratus* and 146 mg/L in *Pelteobagrus fulvidraco*; Qi et al., 2017; S.-D. Wang et al., 2021), as well as in turtles (1.418 mg/L unionized ammonia in *T. scripta elegans*, Ding et al., 2021; 200 mg/L total ammonia-N in *M. sinensis*, Khan et al., 2021). Two of the most common bacterial phyla, Firmicutes and Bacteroidetes in the gut play important roles in providing nutrition for the host (Ding et al., 2021; Duan et al., 2021). However, the discordant

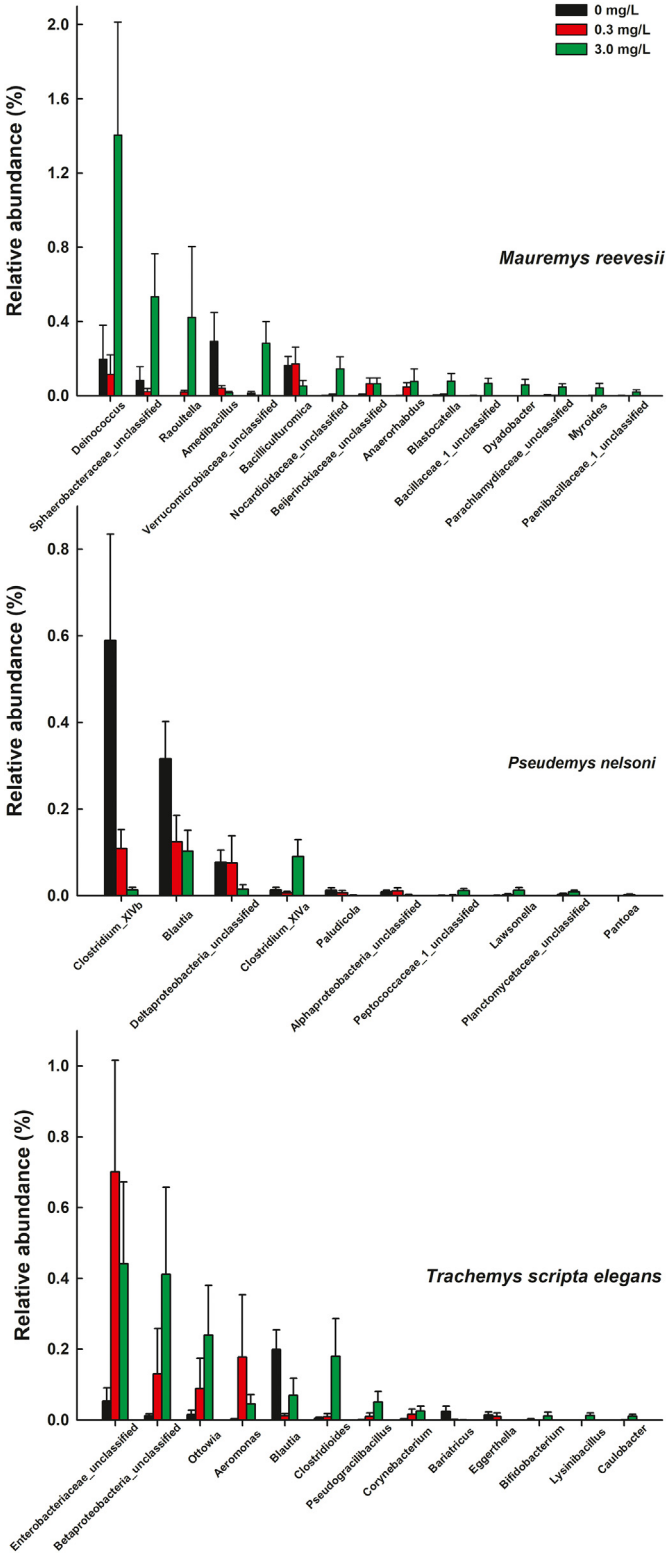

Fig. 4. Relative abundances of significantly-changed bacterial genera in three freshwater turtles.

ratio between them would influence the growth or health of animals (Khan et al., 2021; S.-D. Wang et al., 2021). Compared to the two alien species, relatively more Bacteroidetes or lower Firmicutes/Bacteroidetes ratio in *M. reevesii* might be potentially linked to its growth performance, although it was not observed in this study. Similarly, the potentially pathogenic bacterial family Bacteroidaceae was relatively more abundant, while the beneficial bacterial genera *Romboutsia* and *Clostridium_sensu_stricto* were relatively less abundant in *M. reevesii*, which probably influenced its growth and health (Ding et al., 2021). *Bacteroides* can play an essential role in improving food digestion and pathogen resistance for the host (Wexler, 2007; Duan et al., 2021). Despite no statistical significance, the relative abundance of *Bacteroides* in the 3.0 mg/L ammonia-exposed group was reduced by 59 % relative to the CTRL group, probably indicating a great changeability to ammonia exposure. A significantly reduced abundance of *Bacteroides* has been shown in ammonia-exposed crustacean, fish, and turtle species (Qi et al., 2017; Duan et al., 2021; Khan et al., 2021). Moreover, a lower relative abundance of beneficial bacterial genus *Akkermansia* that related to intestinal barrier function was also shown in *T. scripta elegans* than in the other two turtle species. However, this genus accounted for only a small proportion (<1.0 %). Some representatives of *Turicibacter* have been correlated with host inflammation and are considered pathogenic (Khan et al., 2021). A higher abundance of *Turicibacter* observed in *P. nelsoni* might cause a potential risk of increased intestinal inflammation.

Generally, ammonia exposure increases the susceptibility of crustaceans, fish, and other aquatic animals to pathogens, resulting in an increased risk of opportunistic bacterial infection (Qi et al., 2017; Xue et al., 2020; Khan et al., 2021; Lv et al., 2021). Despite no obvious symptoms of infection and death in ammonia-exposed turtles, some changed bacterial genera, especially increased pathogenic or opportunistic pathogenic bacterial genera, in turtle guts might partly support this view. The responses of specific intestinal bacteria to ammonia exposure varied across different turtle species. For example, a common aquatic pathogenic bacterial genus *Aeromonas* was increased in ammonia-exposed *T. scripta elegans*, but not in *M. reevesii* and *P. nelsoni*, while some other pathogenic bacteria (e.g., *Myroides*, *Clostridium_XlVa*) were increased in ammonia-exposed *M. reevesii* or *P. nelsoni*. Considering the potential relation to host physiology, divergent changes in specific gut bacteria might imply differential response strategies to ammonia stress for the three turtle species. Overall, a higher abundance of pathogenic bacteria and lower abundance of beneficial bacteria observed in *M. reevesii*, together with more significantly-changed bacterial genera in ammonia-exposed individuals might reflect a relatively higher vulnerability of gut microbiota to ammonia exposure in this native species.

The tissue metabolome should be a more sensitive measure than general physiological endpoints (González-Ruiz et al., 2019; Liu et al., 2022). Our hepatic metabolomic profiling showed that a number of amino acids and several metabolites related to energy metabolism were reduced after exposure to low levels of ammonia, indicating ammonia-induced perturbations in amino acid and energy metabolism. Similar metabolic perturbations have been observed in ammonia-exposed crustaceans (*L. vannamei*, Xiao et al., 2019; *Portunus trituberculatus*, Meng et al., 2021; *Eriocheir sinensis*, T.-Y. Wang et al., 2021) and fish (*Oryzias melastigma*, Zhu et al., 2018; *Oreochromis niloticus*, Zhu et al., 2019). Hepatic glutamine levels were decreased in 3.0 mg/L-exposed individuals of *P. nelsoni* and *T. scripta elegans* (Table 1), indicating that the synthesis of glutamine was depressed. However, as a primary mechanism for ammonia assimilation, an increase in glutamine synthesis (including an increase in the activity of glutamine synthetase) in animal tissues can be expected and has been documented in fish and aquatic turtles exposed to high environmental ammonia (Ip et al., 2004, 2008; Sanderson et al., 2010; Sinha et al., 2013; Li et al., 2016; Zhu et al., 2018). On the other side, chronic exposure to low levels of ammonia might conversely lead to a decrease in the activities of enzymes related to nitrogen metabolism, including glutamate dehydrogenase and glutamine synthetase, thus altering protein dynamics and growth in fish species (additional 70 μmol/L total ammonia-N in *Oncorhynchus mykiss*, Linton et al., 1998). Whether the activities of relevant enzymes would change after chronic exposure to low-concentration ammonia in our three turtle species should be confirmed in future studies.

Our results also showed that changed hepatic metabolites differed among the three turtle species, indicating divergent physiological responses to ammonia exposure in these turtles. More amino acids were significantly reduced in ammonia-exposed *M. reevesii* and *P. nelsoni* than in *T. scripta elegans*, suggesting a higher degree of ammonia-induced perturbation of

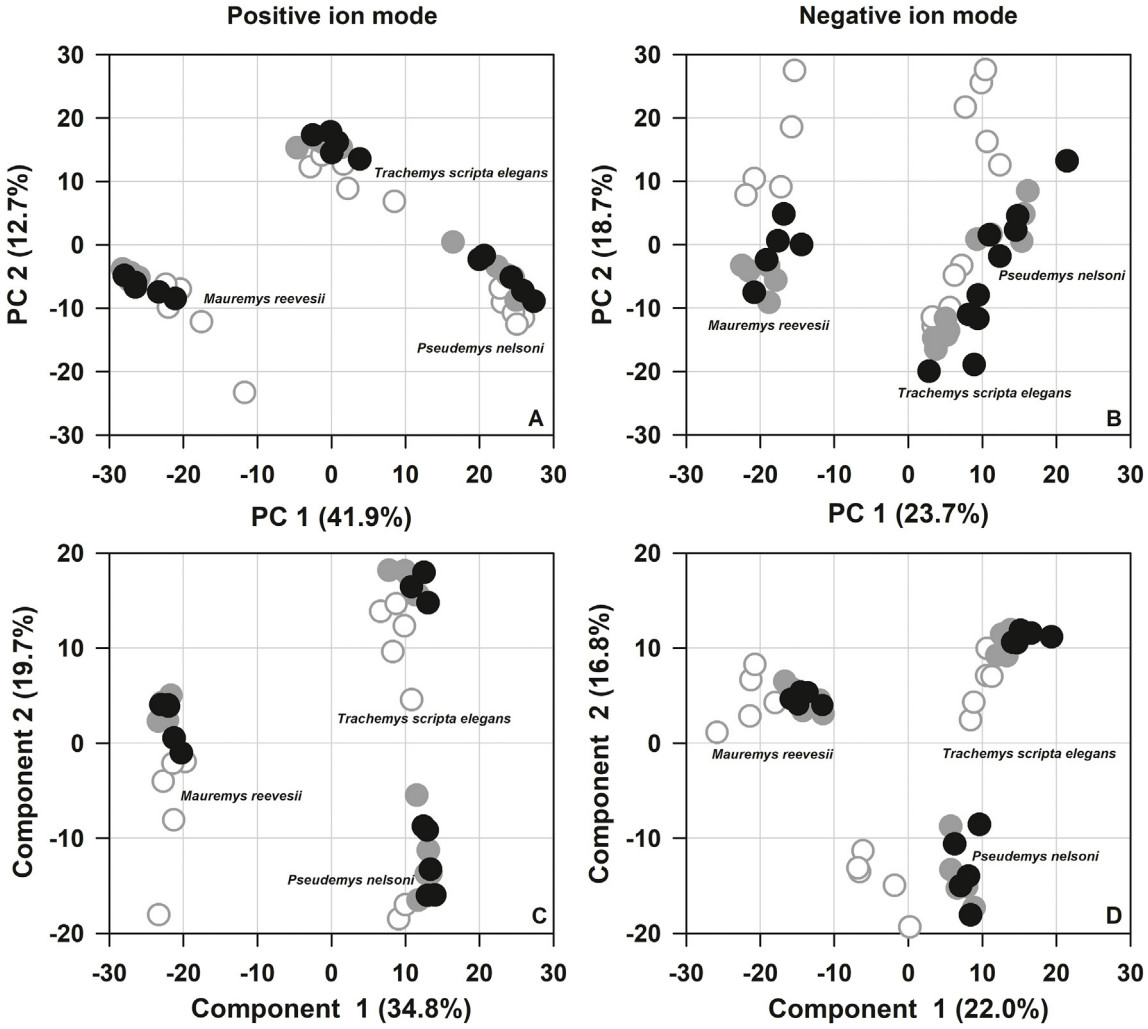

Fig. 5. Score plots for principal component analysis [PCA, in the positive (A) and negative (B) ion mode] and partial least squares discriminant analysis [PLS-DA, in the positive (C) and negative (D) ion mode] analysis of hepatic metabolite profiles showing separation among different groups of three freshwater turtles. Grey open circles: control; grey solid circles: 0.3 mg/L; black solid circles: 3.0 mg/L.

amino acid metabolism in the former two turtle species. Branched-chain amino acids (BCAAs; valine, leucine, and isoleucine) play crucial roles in the regulation of protein synthesis and other nutrient metabolisms (such as glucose) in the liver, and the disorder of BCAAs should be potentially linked to liver injury (Khanna and Gopalan, 2010; Wang and Guo, 2013). Additionally, some metabolites related to energy metabolism (such as creatine, AMP, niacinamide, NAD) were significantly reduced in *M. reevesii* and/or *P. nelsoni*, but not in *T. scripta elegans*. For example, creatine is the main component of energy metabolism, and could promote the circulation of ATP (Ostojic and Forbes, 2022). Its deficiency may lead to low energy output for the body, which might have occurred in *P. nelsoni* and *M. reevesii*. Generally, these results of metabolite changes indicated that the impact of ammonia exposure would be less severe in *T. scripta elegans* than in the other two turtle species.

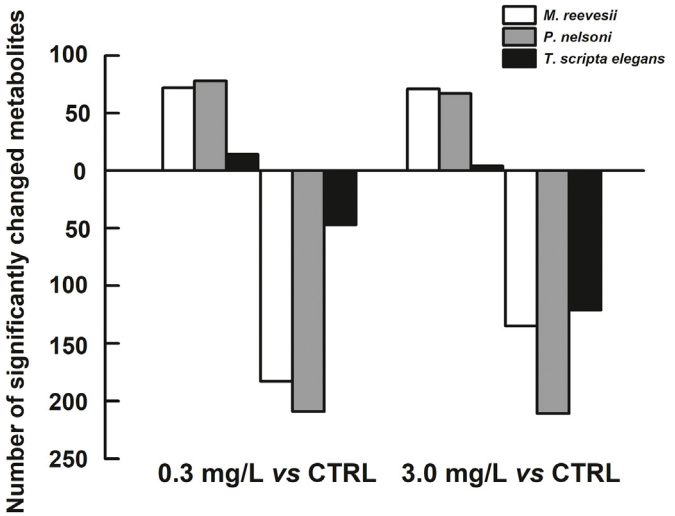

Fig. 6. Number of significantly increased or decreased hepatic metabolites in ammonia-exposed (0.3 and 3.0 mg/L) turtles relative to the control ones.

From another perspective, divergent metabolomic profiles might reflect different potential resistance to ammonia exposure in the three turtle species. As expected, less significantly-changed hepatic metabolites in ammonia-exposed *T. scripta elegans* than in the other two turtle species indicated its lower sensitivity, i.e., greater resistance, to ammonia exposure. It is not surprising that aquatic animals have varying degrees of ammonia resistance, and may adopt various strategies to alleviate ammonia stress (Qiu et al., 2018; Chen et al., 2022). For example, the soft-shelled turtles, *P. sinensis*, would reduce the production of endogenous ammonia and rapidly regulate the activities of antioxidant enzymes to alleviate ammonia toxicity; while three-keeled pond turtles, *M. reevesii*, appeared to have a basic level of antioxidant defense under ammonia exposure (Ip et al., 2008; Chen et al., 2022). As a species having successfully invaded across the world, greater potential resistance to ammonia stress for *T. scripta*

Table 1
Several identified hepatic metabolites, and their fold changes (FC) and associated *P*-values (* $P < 0.05$, ** $P < 0.01$) in three freshwater turtles exposed to control (CTRL), 0.3, 3.0 mg/L ammonia.

Metabolites	*Mauremys reevesii*				*Pseudemys nelsoni*				*Trachemys scripta elegans*			
	0.3 mg/L vs CTRL		3.0 mg/L vs CTRL		0.3 mg/L vs CTRL		3.0 mg/L vs CTRL		0.3 mg/L vs CTRL		3.0 mg/L vs CTRL	
	Log_2(FC)	*P*	Log_2(FC)	*P*	Log_2(FC)	*P*	Log_2(FC)	*P*	Log_2(FC)	*P*	Log_2(FC)	*P*
Amino acid metabolism												
Isoleucine	−1.33	**	−0.84	*	−1.12	**	−1.15	**	−0.38		−0.70	
Leucine	1.28		0.13		0.54		−0.21		0.77		−1.51	*
Proline	−0.46		−2.14	*	−2.72	*	−8.76	**	0.14		3.24	
Valine	−0.90	*	−0.85	*	−1.22	**	−1.32	**	−0.12		−0.61	
Tryptophan	−1.23		−0.56		−1.17	**	−1.18	**	−0.63		−0.80	
Citrulline	−1.89	**	−1.21	*	−2.10	**	−1.84	**	−0.87		−0.97	
Glutamine	−0.21		−0.19		−0.53		−1.09	*	−0.88		−1.90	**
Glutamate	−1.28	*	−2.38	**	−0.79		−1.58	*	−0.86		−2.91	**
Antioxidant system												
Glutathione	0.94		0.80		4.28	**	5.48	**	0.48		0.20	
Energy metabolism												
Creatine	−1.49	**	−1.40	**	−1.27	**	−1.58	**	−0.04		−0.65	
Niacinamide	−0.12		−1.43	*	0.30		−1.38	*	0.20		−0.15	
NAD	−0.92		−0.82		−1.02	*	−1.07	*	−0.09		−0.49	
NADH	0.01		−0.28		1.52	*	1.96	**	0.41		0.37	
Nucleotide metabolism												
AMP	−2.31	**	−1.50	**	−1.27	*	−1.14	*	0.21		−0.88	
Uracil	−0.58		−1.40	*	0		−1.18		−1.06		−0.94	
Uridine	−1.33	*	−1.21	*	−1.27	**	−1.56	**	−0.77		−1.17	*
Cytidine	−6.41	*	−3.79		−2.4	**	−2.13	**	−0.12		0.74	
Related to lipid metabolism and neurotransmission												
Choline	−0.77	*	−0.67	*	−0.26		−0.50	*	−0.27		−0.65	

elegans can be expected. Such a feature would make *T. scripta elegans* more adaptable to novel environments, and confer its competitive superiority over native species. Another alien species, *P. nelsoni*, did not seem to be so highly invasive. This situation might be associated with its higher sensitivity to ammonia stress. As an alternative strategy, *P. nelsoni* may adopt different responses of antioxidant defense against ammonia stress. Glutathione plays an important role in biological detoxification processes, and primarily contributes to removing excess reactive oxygen species (ROS) in the liver (Falfushynska et al., 2018; Lu et al., 2022). When exposed to external toxins, tissue glutathione levels and the activities of relevant enzymes (e.g., glutathione-S transferase and glutathione reductase) may change accordingly (Li and Qi, 2019). In this study, hepatic glutathione was increased significantly in ammonia-exposed *P. nelsoni*, but not in *M. reevesii* and *T. scripta elegans*. An increased glutathione level might be due to the activation of the glutathione-related detoxification system. It was observed only in *P. nelsoni*, possibly suggesting that the glutathione-related detoxification system might be its primary antioxidant defense strategy against environmental stresses.

5. Conclusion

In this study, gut microbiome and liver metabolome profiles were determined to evaluate the potential effects of chronic exposure to environmentally relevant concentrations of ammonia in three freshwater turtle species. Despite no significant difference in gut microbial diversity, microbial community composition and hepatic metabolite profiles exhibited some observable changes in ammonia-exposed individuals, reflecting potential toxic effects of ammonia (even at lower concentrations) in these turtle species. Interestingly, the changes in gut microbial composition and hepatic metabolites differed among the three species. Considering the relatively higher abundance of potentially pathogenic bacteria, more bacterial genera and hepatic metabolites being changed significantly in native *M. reevesii* might reflect its more vulnerability under ammonia stress. Contrarily, only a very small number of metabolites (in spite of some pathogenic bacteria in the gut being increased) were changed in *T. scripta elegans*, indicating its greater potential resistance to ammonia stress. Greater potential resistance to stresses may allow *T. scripta elegans* to adapt to novel environments quickly and to exhibit greater invasive potential when released out of its natural regions. Certainly, due to the limitation of a small sample size (only three turtle species used in this study), it was still difficult to draw a general conclusion about the probable link between the physiological sensitivity to ammonia stress and invasive potential of alien species based on current comparison data. Turtle species may adopt different defense strategies to cope with ammonia stress. Significantly elevated hepatic glutathione levels only occurred in *P. nelsoni*, indicating that the glutathione-related detoxification system was primarily adopted by this species. Unexpectedly, *T. scripta elegans* individuals did not exhibit a significantly higher growth rate than native *M. reevesii*. We only conducted the exposure experiments over one month. Long-term consequences of ammonia-induced pathogenic prevalence and metabolic disorder on the individual growth of these turtles should still be investigated in future studies. Overall, our results might reveal divergent physiological response patterns and reflect differential potential resistances to ammonia stress in three freshwater turtles.

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CRediT authorship contribution statement

Qin-Yuan Meng: Conceptualization, Writing-Original draft preparation. **Dong-Mei Mo**: Investigation, Software. **Han Li**: Methodology, Investigation, Data curation. **Wan-Ling Wang**: Data curation, Validation. **Hong-Liang Lu**: Supervision, Writing-Reviewing and Editing.

Data availability

Data will be made available on request.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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