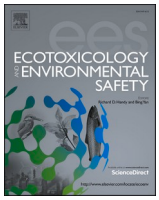
Ecotoxicology and Environmental Safety 260 (2023) 115095

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

journal homepage: www.elsevier.com/locate/ecoenv



Embryonic development, hatchling performance and metabolic profile after egg exposure to environmentally relevant levels of chlorpyrifos in an aquatic turtle

Jia-Meng Yang, Hong-Liang Lu *, Jia-Hui Liu, Xin-Ru Qian, Guang-Li Fu, Jian-Fang Gao *

Herpetological Research Center, School of Life and Environmental Sciences, Hangzhou Normal University, Hangzhou 311121, Zhejiang, China

ARTICLE INFO

Edited by Dr Yong Liang

Keywords:
Pelodiscus sinensis
Organophosphorus insecticide
Embryonic exposure
Physiological performance
Antioxidant response
Hepatic metabolite

ABSTRACT

The extensive use of organophosphorus insecticides poses a threat to the survival of non-target organisms. Ecotoxicological outcomes of embryonic exposure to insecticides are rarely evaluated in various oviparous species. In this study, soft-shelled turtle (*Pelodiscus sinensis*) eggs were incubated in moist substrate containing different levels (0, 2, 20 and 200 μg/kg) of chlorpyrifos to investigate its toxic effects on embryonic development and survival, and hatchling physiological performance. Chlorpyrifos exposure had no significant impacts on embryonic development rate and egg survival in *P. sinensis*. Similarly, embryonic chlorpyrifos exposure neither obviously affected the size and locomotor performance of hatchlings, nor changed the activities of superoxide dismutase and catalase, and content of malondialdehyde in their erythrocytes. Based on liquid chromatography-mass spectrometry analysis, minor metabolic perturbations related to amino acid, lipid and energy metabolism in hatchlings after embryonic chlorpyrifos exposure were revealed by hepatic metabolite profiling. Overall, our results suggested that embryonic exposure to environmentally relevant levels of chlorpyrifos had only a limited impact on physiological performances of hatchlings, although it would result in a potential risk of hepatotoxicity in *P. sinensis*.

1. Introduction

As a common organophosphorus insecticide, chlorpyrifos (CPF) is widely applied in agriculture for pest control worldwide (Eaton et al., 2008; John and Shaike, 2015). CPF that applied to farmland can easily infiltrate into the soil with the water flow, combine with the soil particles and have a long half-life (Ubaid ur Rahman et al., 2021). Consequently, a large amount of CPF residues could be detected in various terrestrial environments (Zhang et al., 2012; Dar et al., 2019). For example, the residual CPF amount in the soils of paddy and vegetable fields can achieve a high level in China and some other parts of Asia (Zhang et al., 2012; Fu et al., 2015; Bhandari et al., 2021). CPF has been shown to be moderately to highly toxic to wildlife and other non-target organisms (Huang et al., 2020; Ubaid ur Rahman et al., 2021). CPF exposure would cause behavioral disorder, inhibit growth, reduce reproductive performance and survival probability in various organisms from aquatic insects to mammals (insect, Callaghan et al., 2001; reptile, Amaral et al., 2012; fish, Qiu et al., 2017; crustacean, Taylor et al., 2019; amphibian, Costa et al., 2021; mammal, Sun et al., 2023).

The embryonic development stage is a highly vulnerable period for oviparous species. Various environmental factors, including pollutants, may influence the development and survival of embryos, as well as the phenotype and fitness of hatchlings (Deeming, 2004). CPF exposure has been documented to slow the developmental rate and increase the mortality of embryos, reduce the body size and induce morphological abnormality of hatchlings in aquatic organisms, including crustaceans, fish and amphibians (Jin et al., 2015; Qiu et al., 2017; Kharkongor et al., 2018). Additionally, CPF exposure is shown to disrupt the embryonic neural development, and inhibit the angiogenesis in bird species (Slotkin et al., 2008; Crump et al., 2021; Desforges et al., 2021). Reptilian embryos in eggs that deposited within underground nests may suffer adverse impacts of soil insecticide residuals, including CPF. However, studies considering such impacts are quite limited in oviparous reptiles so far (but see de Solla et al., 2014; Odetti et al., 2020).

The Chinese soft-shelled turtle, *Pelodiscus sinensis*, is the most commonly cultured freshwater turtle species in China, and reared near agricultural paddy fields, where the soil can be readily contaminated with various agricultural chemicals, including organophosphorus

* Corresponding authors.
E-mail addresses: honglianglu@hznu.edu.cn (H.-L. Lu), gaojf308@hznu.edu.cn (J.-F. Gao).

https://doi.org/10.1016/j.ecoenv.2023.115095
Received 25 February 2023; Received in revised form 11 May 2023; Accepted 29 May 2023
Available online 31 May 2023

insecticides. For example, residual CPF in paddy soils in southeast China can reach up to 0.16 mg/kg (Fu et al., 2015), and it may be absorbed by turtle eggs (de Solla and Martin, 2011). Previous studies have shown that embryonic CPF exposure does not necessarily have a significant influence on egg survival, hatchling abnormality and size, but may markedly disturb biochemical and physiological processes in oviparous reptiles (de Solla et al., 2014; Odetti et al., 2020). Over recent years, the application of various (including biochemical, proteomic, transcriptomic, metabonomic) analytical techniques provides convenience for a comprehensive evaluation of physiotoxicological effects of environmental pollutant exposure on target and non-target organisms (Fang and Gonzalez, 2014; Basova et al., 2022; Peycheva et al., 2022). In this study, we investigated the alterations in hatchling functional performances and hepatic metabolites following exposure to environmentally relevant levels of CPF during the embryonic period in *P. sinensis*. Considering these findings from metabolomic profiling of CPF-exposed non-target animals, including fish, birds and mammals (Zhao et al., 2016; Wu et al., 2019; Perez-Fernandez et al., 2020; Desforges et al., 2021; Legrand et al., 2021), diverse physiological and metabolic perturbations in *P. sinensis* hatchlings would be expected after embryonic CPF exposure.

2. Material and methods

2.1. Chemical reagents and assay kits

Technical grade chlorpyrifos (CPF, CAS no. 2921–88–2) was purchased from Beijing Huawei Ruike Chemical Co., LTD Ltd (Beijing, China), and was dissolved in methanol and diluted with distilled water to create concentrated stock solutions of 0.02, 0.2, and 2.0 mg/mL for the exposure experiment. Stock solutions were made weekly and stored in a dark place in a refrigerator at 4 °C. Assay kits for detecting the hemoglobin content, erythrocyte malondialdehyde (MDA) content, superoxide dismutase (SOD) and catalase (CAT) activities were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

2.2. Turtle eggs exposure

In June 2021, a total of 120 new-laid *P. sinensis* eggs were collected from a private farm, and then transferred to the lab and weighted. Turtle eggs were randomly allocated and incubated in 12 containers (10 eggs in each container) filled with moist vermiculite (2 g water/1 g vermiculite, Lu et al., 2022). Before eggs were moved into containers, stock solutions of CPF were diluted with distilled water, sprayed into incubation substrate, and mixed well with substrate to get initial levels of 2, 20, or 200 μg (3 containers in each treatment group) of CPF per kg of moist vermiculite, respectively. The concentration of 200 μg/kg CPF that selected here is close to the value reported for contaminated paddy soils (0.16 mg/kg, Fu et al., 2015). The control and treatment groups received an equal volume of methanol. All containers were placed in a climate-controlled room that set at 28 °C. The incubation substrate was changed weekly, and diluted CPF solutions (or methanol solution) were sprayed in to achieve the corresponding levels.

2.3. Locomotor ability test

A total of 67 healthy hatchling turtles were collected approximately 6 weeks later (Table 1). Hatchlings were weighed upon emergence. Five turtles without visible lesion were randomly selected from each group, and euthanized on ice and sacrificed to collect blood and liver samples for subsequent measurements of erythrocyte antioxidant responses and hepatic metabolites. Another 10 individuals without visible lesion from each group, whose yolk sacs have been absorbed, were used to measure the locomotor performance of hatchlings. Locomotor performance was evaluated inside a glass-made racetrack (120 × 10 × 20 cm^3), that was placed in a climate-controlled room that set at 28 °C. The racetrack was filled with 10 cm depth of water that maintained at 28 °C. After being acclimated for approximately 1 h, turtles were individually introduced into one end of a racetrack, and encouraged to swim continuously. Their swimming performances were filmed by a laterally-placed Panasonic HDC-SD900GK digital video camera, and video clips were later examined for the maximal speed over 25 cm. All animal procedures were conducted in accordance with current Chinese laws on animal welfare and research, and approved by the Animal Care and Ethics Committee of Hangzhou Normal University.

Table 1
Egg survival and incubation length (mean value ± standard error) under embryonic exposure to environmentally relevant levels of chlorpyrifos in *Pelodiscus sinensis*.

Chlorpyrifos treatment (μg/kg)	Number of incubated eggs	Egg survival (%)	Incubation length (d)
0	30	53.3 (16/30)	41.2 ± 0.4
2	30	56.7 (17/30)	41.4 ± 0.3
20	30	60.0 (18/30)	41.3 ± 0.2
200	30	53.3 (16/30)	41.3 ± 0.1

2.4. Blood sample collection

For those sacrificed turtles, blood samples (approximately 0.2 mL from each individual) were gathered immediately from the neck-chest section, and livers were extracted, rapidly frozen in liquid nitrogen and stored at − 80 °C before measuring hepatic metabolites. Blood samples were transferred into heparin sodium anticoagulation tubes, and centrifuged at 2000 rpm for 5 min at 4 °C. Erythrocytes were separated, then washed with saline solution and precipitated by centrifugation two times. Finally, erythrocytes were haemolysed by adding 9 volumes of ice-cold distilled water. Before measuring antioxidant responses, erythrocyte lysis solution would be diluted.

2.5. Erythrocyte antioxidant analysis

Following the manufacturer's protocols of assay kits, hemoglobin content was determined at 540 nm using a hemoglobin assay kit C021–1–1 based on the hemiglobincyanide method, SOD activity was determined at 450 nm using a SOD assay kit A001–3–1 based on the water-soluble tetrazolium-1 method, CAT activity was determined at 405 nm using a CAT assay kit A007–1–1 based on the ammonium molybdate colorimetric method, and MDA content was determined at 532 nm using a MDA assay kit A003–1–2 based on the thibabituric acid method. All assays were executed using a BioTek ELX 800 microplate spectrophotometer (BioTek Instrument Inc., USA).

2.6. Hepatic metabolomic analysis

Liver tissue was weighed from each sample (approximately 100 mg), homogenized, vortexed and centrifuged at low temperatures. The supernatant after centrifugation was transferred into a clean centrifuge tube, and dried using an Eppendorf Concentrator Plus (Eppendorf AG, Germany). The dried residue was stored at − 80 °C, finally dissolved with pre-chilled 2-chlorobenzalanine/50% acetonitrile solution and filtrated through 0.22 μm filtering membrane before liquid chromatography-mass spectrometry (LC-MS) analysis. Detailed procedures for LC-MS and data processing were performed as previously described (Lu et al., 2022). Briefly, LC analysis was performed on a Vanquish UHPLC System (Thermo Fisher Scientific Inc., USA), which was equipped with an Acquity UPLC® HSS T3 column (Waters Acquity, USA). After chromatographic separation, mass spectrometric analysis was performed on the Q Exactive (Thermo Fisher Scientific Inc., USA) with ESI ion source. Generated raw LC-MS data were converted into mzXML files using ProteoWizard, processed with the XCMS software in

R, and then analyzed on the MetaboAnalyst web-based platform. Hepatic metabolites were identified by searching the mass spectra against available databases [i.e., Human Metabolome Database (HMDB), Metabolite Link (Metlin), MassBank Database].

2.7. Data analysis

Preliminary analyses showed that there were no significant container effects on egg-hatching success [Cochran-Mantel-Haenszel chi-squared (χ^2) test with CPF treatment as the stratification factor], and hatchling size, swimming speed and other examined parameters [mixed model analysis of variance (ANOVA) with incubation container identity as the random factor] (all $P > 0.05$), so this factor was excluded in subsequent analyses. One-way ANOVA or analysis of covariance (ANCOVA) was performed to determine the differences in the initial egg mass, hatchling body mass, swimming speed, and erythrocyte antioxidant parameters among different treatment groups. Pearson χ^2 test was performed to determine the difference in egg-hatching success among groups. For hepatic metabolomic data, principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA) were performed to compare the metabolic variations among groups after being normalized.

3. Results

3.1. Egg survival, hatchling size and locomotor performance

There was no significant among-group difference in initial egg mass ($F_{3, 116} = 1.00$, $P = 0.397$). The egg-hatching success ($\chi^2 = 0.37$, $df = 3$, $P = 0.946$) and incubation length ($F_{3, 63} = 0.07$, $P = 0.977$) did not differ among different treatment groups (Table 1). CPF exposure during the embryonic period was shown to have no significant effects on hatchling body mass and swimming speed (Fig. 1).

3.2. Erythrocyte antioxidant activity of hatchlings

No significant impacts of embryonic CPF exposure on erythrocyte antioxidant enzymatic activities and MDA content could be observed in this study (Fig. 2).

3.3. Hepatic metabolomic profile of hatchlings

PCA analysis of hepatic metabolites revealed a modest separation among groups, with 46.4% and 39.2% of cumulative variance contributions from the first two components in the positive and negative ion modes, respectively (Fig. 3). PLS-DA analysis was used to further characterize these differences, and revealed a more obvious separation among groups, with 37.0% and 32.0% of cumulative variance contributions from the first two latent components (Fig. 3).

Some differentially-expressed hepatic metabolites due to embryonic CPF exposure were identified by searching the available databases. Some amino acids (such as leucine, histidine, aspartic acid, etc.) increased with increasing CPF exposure concentration (Fig. 4). Similarly, hepatic choline, uridine and myo-inositol also increased, but adenosine diphosphate (ADP), malate, and γ-aminobutyric acid levels decreased with increasing CPF exposure concentration (Fig. 4).

4. Discussion

In this study, we determined egg survival, hatchling functional performance and hepatic metabolomic profiling following embryonic exposure to environmentally relevant levels of CPF in a freshwater turtle species, *P. sinensis*. Slowed embryonic development (or longer incubation length for eggs) and reduced egg survival following embryonic CPF exposure has been observed in some oviparous species, including crustaceans, fish, amphibians and birds (Jin et al., 2015; Kharkongor et al., 2018). However, no evident changes in egg survival and incubation

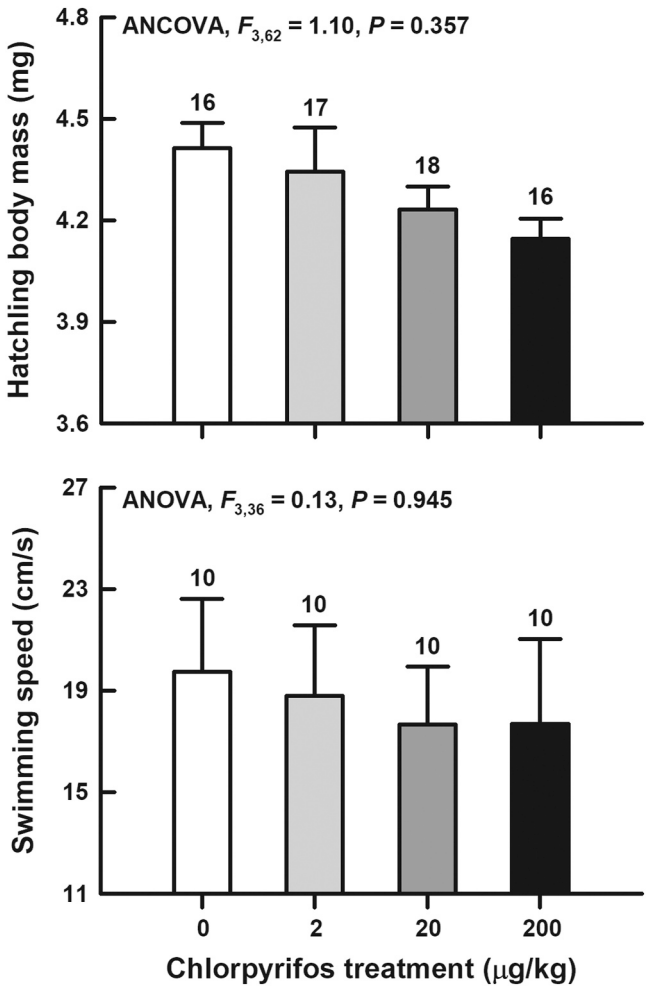


Fig. 1. Mean values (+ standard errors) for body mass and swimming speed of Chinese soft-shelled turtle (*Pelodiscus sinensis*) hatchlings from eggs exposed to 0, 2, 20 and 200 μg/kg chlorpyrifos. Numbers above the error bars were sample sizes.

length after embryonic CPF exposure were found in *P. sinensis* here. Discrepant outcomes from different studies might be due to the difference in used exposure level and treatment method, and difference in mechanical permeability of egg membrane and/or shell between different species. Exposure to environmentally relevant levels of CPF might significantly affect embryonic development and survival in aquatic invertebrate, fish and amphibian species with a highly permeable egg membrane (Jin et al., 2015; Kharkongor et al., 2018), but it fails to cause observable impacts in those species that produced shelled eggs, such as reptiles and birds. Those studies conducted on birds showing discernible effects on embryonic development and survival actually applied the approach of injecting high doses of CPF into the air cell of eggs (Crump et al., 2021; Desforges et al., 2021). Eggshells function as a natural barrier, and might prevent a substantial quantity of soil (or other substrates) insecticide residue from entering eggs, thereby retarding their toxic effects on embryos. Unfortunately, in this study, we did not measure the CPF content in incubating embryos, yolk sacs or hatchlings, and consequently were unable to evaluate the actual amount of CPF that entered eggs, which was expected to be relatively low. Certainly, the CPF levels within eggs (and embryos) would be necessary to be confirmed in future studies in order to further accurately evaluate dose toxic effects of embryonic exposure. Similarly, reduced body size and weakened locomotor ability occurred in fish or amphibian hatchlings after embryonic CPF exposure (Jin et al., 2015; Kharkongor et al., 2018), but such an effect was also not observed in *P. sinensis*. Therefore, the

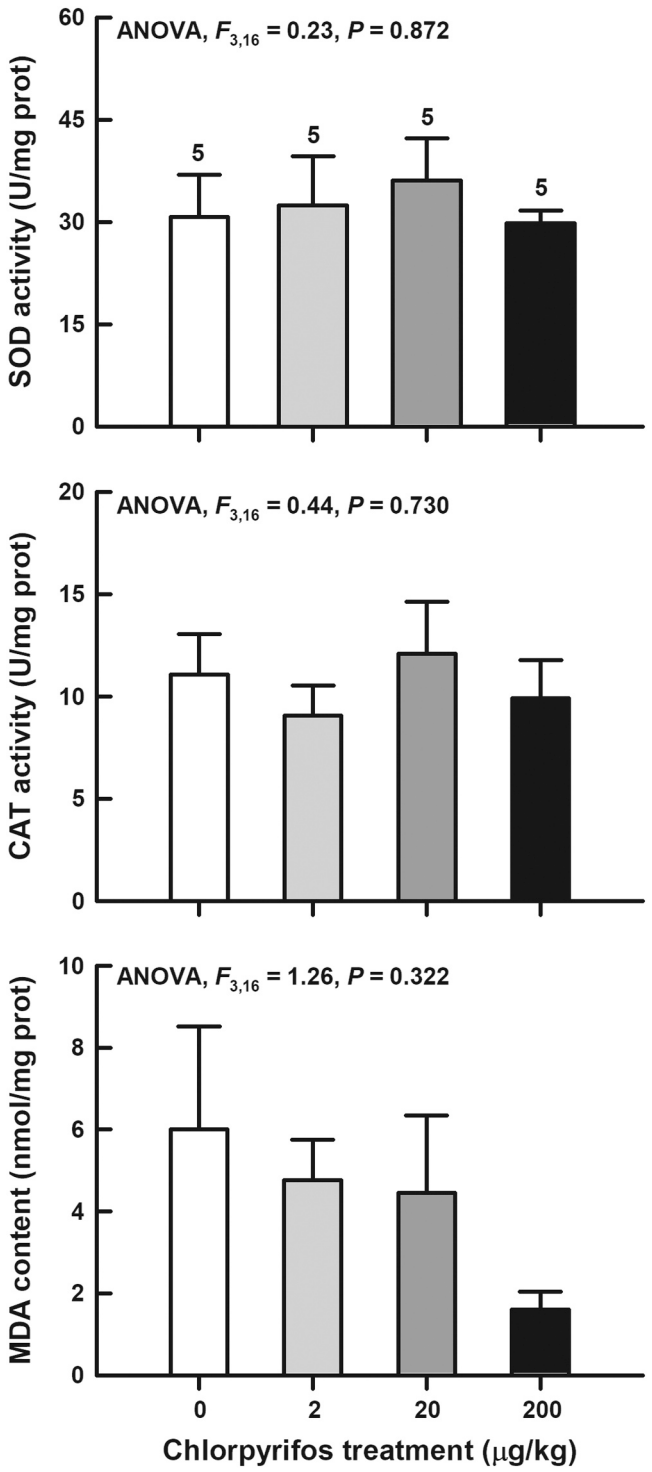


Fig. 2. Mean values (+ standard errors) for erythrocyte SOD and CAT activities, and MDA content in Chinese soft-shelled turtle (*Pelodiscus sinensis*) hatchlings from eggs exposed to 0, 2, 20 and 200 μg/kg chlorpyrifos. Numbers above the error bars were sample sizes.

impact of embryonic exposure to CPF at environmentally relevant levels in incubation substrate on embryonic development, hatchling phenotype and functional performance would be quite limited in those species produced shelled eggs.

Tissue MDA content, SOD and CAT activities are frequently used to indicate oxidative damage or antioxidant responses induced by environmental pollutant exposure in various organisms (Kopjar et al., 2018; Negro et al., 2019; Wang et al., 2019; Alipanah et al., 2022). Significant changes in these parameters have been observed in most previous studies on CPF exposure in different species and in different tissues (Jin et al., 2015; Negro et al., 2019; Alipanah et al., 2022). Contrarily, unremarkable changes were shown in our *P. sinensis* hatchlings from CPF-exposed eggs, probably due to only a limited amount of CPF actually affected on developing embryos, despite not being measured directly here. Certainly, differential antioxidant potential among species might also contribute to diverse antioxidant responses to pollutant exposure.

Metabolic disorders caused by CPF exposure have been observed in various tissues of fish (Gómez-Canela et al., 2017; Wang et al., 2019) and mammal species (Elsharkawy et al., 2013; Zhao et al., 2016; Perez-Fernandez et al., 2020). Functioning as the metabolic center, the liver plays important roles in the metabolism of various compounds, including carbohydrates, amino acids, lipids, etc (Ritter et al., 2020). To explore hepatic metabolic responses to pollutant exposure has been increasingly applied in toxicological tests (Buratti et al., 2011; Wang et al., 2019; Desforges et al., 2021; Lu et al., 2022). However, only two studies to date reported the changes in hepatic metabolites following embryonic CPF exposure in oviparous species (birds, Desforges et al., 2021; Legrand et al., 2021), and both of them measured the metabolomic profiles of embryonic livers. Our study might be the first attempt to empirically investigate metabolic changes in livers of *P. sinensis* hatchlings following embryonic exposure, and contribute to better understanding its long-term impacts at post-hatching periods. Here, multiple metabolic perturbations related to amino acid, lipid, energy metabolism, etc., were shown in *P. sinensis* hatchlings after embryonic CPF exposure. Despite a lack of observable morphological and functional effects, our result confirmed that metabolic perturbations could occur even at environmentally relevant levels. Differently, low-to-no metabolic responses were observed in embryonic livers of CPF-exposed bird embryos in previous studies (Desforges et al., 2021; Legrand et al., 2021).

As one of the intermediates in the tricarboxylic acid (TCA) cycle, hepatic malate level was reduced in livers of *P. sinensis* hatchlings after embryonic CPF exposure, probably reflecting CPF-induced perturbation in the energy metabolism and its potential persistence over a certain period of time (Wang et al., 2019). Additionally, reduced hepatic ADP level might further suggest a potential depletion of the energy-carrying molecule-adenosine triphosphate (ATP) due to embryonic CPF exposure, while increased uridine level might also be linked to the ATP depletion (Zhang et al., 2020). Several amino acids (e.g., leucine, histidine, aspartic acid) were noticed to unexpectedly increase, which was inconsistent with the findings from insecticide-exposed animals (Zhao et al., 2016; Wang et al., 2019). Only considering embryonic CPF exposure in oviparous species, no observable changes in amino acids were found in embryonic livers of birds (Desforges et al., 2021; Legrand et al., 2021). Increased amino acid levels following CPF exposure should be interpreted with caution. However, it would be necessary to carry out further experiments that relate various physiological parameters in order to evaluate the efficacy of above-mentioned situation of some amino acids. Our result also showed an increase in hepatic choline level in hatchlings from CPF-exposed eggs, which might be due to the enhanced synthesis and reduced metabolism of this nutrient. As well-known, CPF inhibits the activity of acetylcholinesterase (AChE), that converts acetylcholine into choline and acetate in nerve tissue of animals (Huang et al., 2020; Silva, 2020). Therefore, a downward trend in choline level should be expected after CPF exposure. Whether there was a compensatory upregulation in choline level in hatchlings after embryonic exposure to low actual levels of CPF or in different tissues should be confirmed in future studies. Additionally, changed hepatic choline level also potentially indicated lipid metabolism disorder induced by CPF exposure, although choline deficiency was commonly associated with lipid accumulation (da Silva et al., 2015). Similarly, CPF-induced neurological disorder could be indicated by reduced

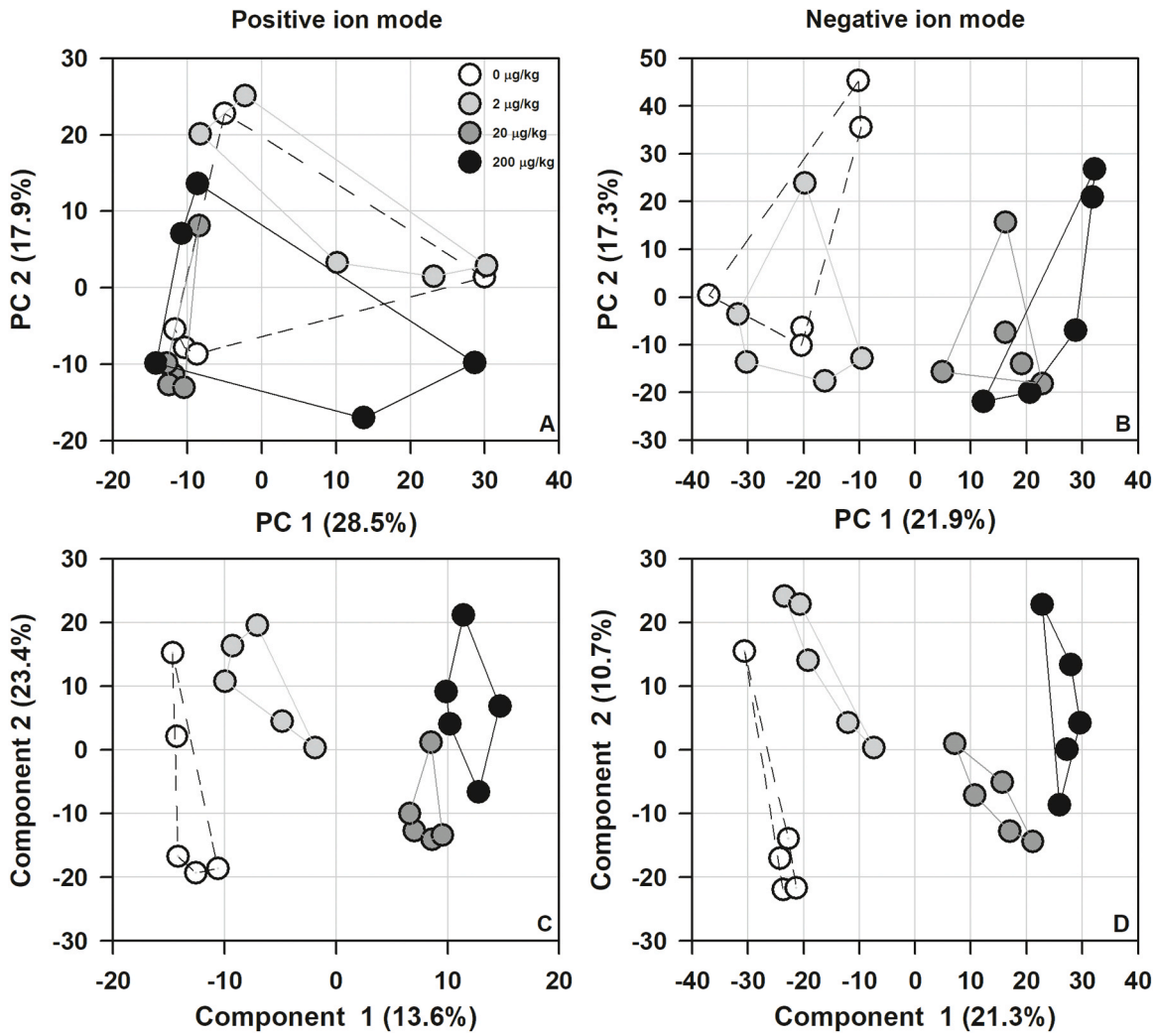


Fig. 3. Score plots for principal component analysis [PCA, in the positive (A) and negative (B) ion mode] and partial least squares discriminant analysis [PLS-DA, in the positive (C) and negative (D) ion mode] showing separation of treatment groups, respectively.

γ-aminobutyric acid (GABA, an inhibitory neurotransmitter) level, which was consistent with the findings in CPF-exposed fish and rats (Wang et al., 2019; Wu et al., 2019). GABA can play a protective role in liver injury. It could be speculated that reduced hepatic GABA level after CPF exposure resulted from the excessive consumption due to exerting its protective effect in injured livers. Moreover, as an organic osmolyte, increased myo-inositol level might be related to a change in intracellular osmotic pressure induced by insecticide exposure, although it should be confirmed in future studies.

5. Conclusion

Potential toxic effects caused by embryonic CPF exposure were evaluated preliminarily via experimental investigation of embryonic survival, hatchling size and physiological performances after egg exposure under environmentally relevant CPF levels. However, due to a lack of investigation of the amount of CPF that entered eggs, actual levels of CPF (and its metabolites) that directly exerted toxicity on developing embryos could not be evaluated currently. The absorption of insecticides by turtle eggs from substrate would increase with increasing exposure levels (de Solla and Martin, 2011). Here, we assumed that the actual amount of CPF that entered eggs would be limited under the protection of rigid eggshells. Consequently, no significant effects on embryonic development and survival, hatchling size, swimming speed and erythrocyte antioxidant ability were observed. Hepatic metabolite profiling revealed multiple, but minor, metabolic perturbations in the livers of hatchlings following embryonic exposure to environmentally relevant levels of CPF in *P. sinensis*. Taken together, only slight toxic effects of embryonic CPF exposure on developing embryos and hatchlings were shown in this study, probably reflecting a certain degree of tolerance to insecticide exposure due to the barrier function of eggshell. Certainly, metabolic perturbations in turtle hatchlings caused by embryonic CPF exposure could be observed, but their potential long-term consequence was currently unable to be predicted and required further investigation.

CRediT authorship contribution statement

Jia-Meng Yang: Investigation, Software, Writing – original draft. **Hong-Liang Lu**: Writing – review & editing. **Jia-Hui Liu**: Methodology, Investigation. **Xin-Ru Qian**: Data curation, Validation. **Guang-Li Fu**: Data curation. **Jian-Fang Gao**: Conceptualization, Supervision.

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.

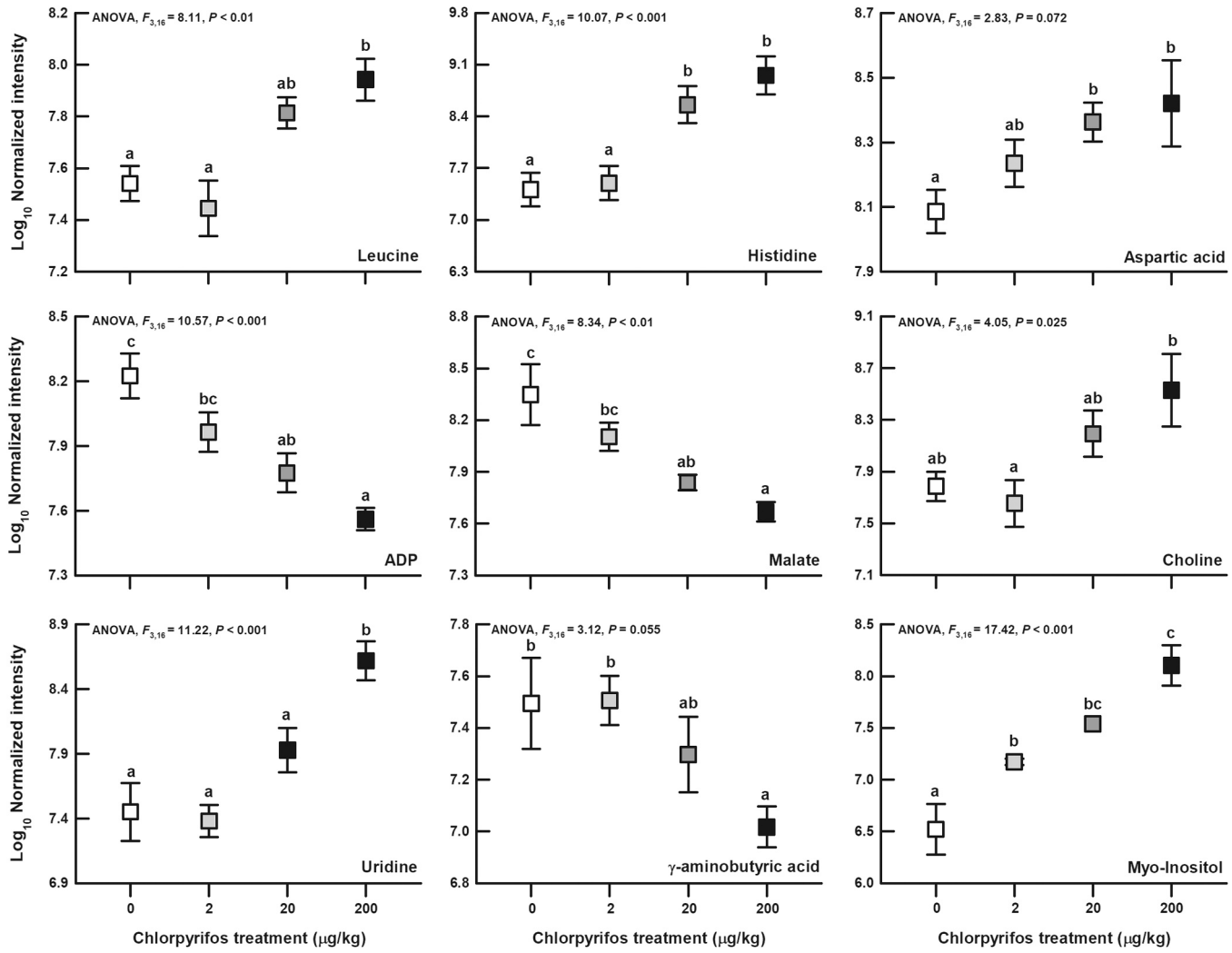


Fig. 4. Mean values (± standard errors) for some identified metabolites in the livers of Chinese soft-shelled turtle (*Pelodiscus sinensis*) hatchlings from eggs exposed to 0, 2, 20 and 200 μg/kg chlorpyrifos. Different letters indicate groups that differ significantly with $\alpha = 0.05$ (a < b < c).

Data Availability

Data will be made available on request.

Acknowledgments

This study was supported by grants from the Natural Science Foundation of Zhejiang Province (LY21C030007).

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