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Multi-Mineral Element Profiles in Genuine and "Bathing" Cultured Chinese Mitten Crabs (*Eriocheir sinensis*) in Yangcheng Lake, China

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Abstract: The authentication of high-quality fishery products originating from specific geographical regions is urgently needed worldwide. Chinese mitten crabs (*Eriocheir sinensis*), originating from Yangcheng Lake, are prime counterfeiting targets for the same reasons. Foreign crabs that are cultured briefly in the lake, known as "bathed" crabs, are illegally marketed as natives, negatively impacting the product quality. To establish a method for distinguishing "bathed" and genuine crabs, in this paper we conduct a comparative investigation by an Agilent 7500ce ICP-MS on multi-mineral element profiling of the third pereiopod from genuine and one month deliberately "bathing" cultured crabs. The profiles of 11 elements were significantly different between the genuine and foreign crabs before and after bathing. The discriminant analysis reached 100% accuracy to separate the genuine and "bathed" crabs into different groups. Bathing culture was unable to converge element profiles between the genuine and foreign crabs. The biogeochemical profiles can be effective for distinguishing "bathed" crabs.

Keywords: *Eriocheir sinensis*; "bathing" culture; foreign crab; mineral element; habitat; Yangcheng Lake; biogeochemical profile

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1. Introduction

The Chinese mitten crab or Chinese hairy crab (*Eriocheir sinensis*) is classified under Crustacea, Decapoda, Grapsidae, and *Eriocheir*, and is native to the East Pacific coast near the Korean Peninsula [1]. This crab species has inadvertently invaded large numbers of European and American regions in the past 100 years [2,3]. This species is particularly rich in mineral elements, crude proteins, vitamins [4], and other nutrients, and has extremely high nutritional value. Therefore, it has become a traditional Chinese precious aquatic product and an economically important aquatic animal. In 2017, the total annual production of Chinese mitten crabs reached 750,000 t [5]. These crabs are cultured primarily in the Yellow, Liaohe, and Yangtze River basins, with the latter being the most predominant region for their culture [6]. As the Yangtze River Basin is the main Chinese mitten crab-producing area, Jiangsu Province has an annual production of approximately 350,000 t, constituting ~50% of the country's total output [5].

In 2005, the former General Administration of Quality Supervision, Inspection, and Quarantine recognized the crab harvested from this region as "Yangcheng Lake Hairy Crab", to preserve the quality and protect against counterfeiting technologies. At present, a coding system with labels is used to ensure that the crabs sold commercially originate from the lake. However, in actual practice, crabs are often sold with incomplete label entries or with imitation labels. Among the counterfeit products, "bathed" crabs are the most common. These market-sized adult crabs are housed and cultured in Yangcheng Lake for a very short period (e.g., few days) before being sold as native crabs from the lake. The annual output of Yangcheng Lake crabs is only 2000 t, whereas the sales volume of the Yangcheng Lake-labeled crabs in the market exceeds 800,000 t, similar to the market returns of the Mexican Interdonato lemon and Thai jasmine rice and highlights the degree of counterfeited crabs being sold [7,8].

The composition and content of mineral elements in various habitats are characteristically unique [9,10], and influence agricultural and fishery habitats significantly, creating distinct differences in products from different regions [11–13]. Mineral element tracing is an effective and widely used method to trace the origin of aquatic products [11,12]. The otolith microchemistry of *Clarias batrachus* distinguishes it from the other groups of fishes found in the Ganges River system [14]. The microchemical analysis of otoliths of *Prochilodus lineatus* effectively differentiates between populations from three different producing areas of the La Plata River Basin [15]. Carter et al. combined mineral elements with isotopes and compared Australian prawns with those imported from neighboring countries and obtained a good discrimination rate [16]. Additionally, mineral elements can also distinguish between cultured and wild catfish [17]. Previously, we reported that the patterns of mineral elements in the third pereiopod of *E. sinensis* from different lakes were significantly different and could be used to determine the geographical origins of crabs with high accuracies (90–100%) [18–20].

Although "bathed" crabs are the most common counterfeit way, none of the studies have confirmed the exact differences between them and genuine Yangcheng Lake crabs. Due to the lack of corresponding direct research and data, although consumers know the existence of "bathed" crabs, no identification method currently exists to effectively control their sales. This has resulted in the large brand and economic losses for businesses from the Yangcheng Lake selling genuine crabs. Thus, based on the aforementioned scientific literature, we hypothesized that mineral elements in the "bathed" crabs would retain their original characteristics, even after short-term exposure to Yangcheng Lake water, and would be incomparable to those in crabs native to the lake. The mineral element signatures can effectively distinguish the "bathed" crabs from genuine ones and reinforce the rights and interests of consumers. In this paper, we used a multi-element analysis method to compare the mineral composition of crabs native to Yangcheng Lake with deliberately "bathing" cultured crabs. We exposed the "bathed" crabs to the lake water for a month (that was much longer than several-day period of "bathing" culture for aforementioned "bathed" crabs) to ensure the accuracy of the comparative testing. Our findings provide a theoretical basis for the application of using mineral composition profiles for the identification of "bathed" crabs. Through this study, we have improved the origin traceability model used in the *E. sinensis* crabs and provided a method for the identification of adulteration in other aquatic products.

2. Materials and Methods

2.1. Sample Collection

On 13 September 2018, two groups of *E. sinensis* crabs (foreign crabs) were sampled in the purse seine area of another lake in the Yangtze River system. One group of foreign crab samples (n = 20, "bathed" crabs before bathing culture (BCB)) were transported to the laboratory for mineral element profiling prior to bathing. Another group of the foreign crabs (subjected to "bathing") was transported into a large cage (5 m × 5 m; 31°26.8111′ N, 120°49.4606′ E) and housed in a large-scale purse seines (100 m × 142 m) in Yangcheng Lake where similar sized genuine crab had already reared. On the same day, one Yangcheng Lake group (n = 20, Yangcheng Lake genuine crabs from September (YCS)) of crabs was sampled for a comparative study of basal mineral element composition between the two groups of BCB and YCS prior to the bathing of the foreign crabs. After continuous culture of the aforementioned group of foreign crabs for one month in Yangcheng Lake, the group of one month-"bathed" foreign *E. sinensis* crabs (n = 20, "bathed" crabs after one-month of bathing culture (BCA)) were sampled on 16 October 2018 with the group of genuine crabs

(n = 20, Yangcheng Lake genuine crabs from October (YCO)) from the same purse seines. All crabs sampled in October were brought back to the laboratory for a repeat comparative study on mineral element background levels between the two groups following the foreign crab bathing in Yangcheng Lake. All crabs sampled in the present study were market-sized adult individuals. For each group of samples in the present study, 20 crabs (10 each for female and male crab) of similar specifications (e.g., body weight, carapace length, carapace width, body height, especially in October) were selected (Table 1). Additionally, all genuine Yangcheng Lake crabs used met the requirements of "Yangcheng Lake Originated Chinese Mitten Crabs" as defined in the national standard of GB/T19957-2005 [21].

Table 1. Morphometric information of Chinese mitten crab (*Eriocheir sinensis*) populations (n = 20, mean ± SD).

Geographical Origin	Body Weight (g)	Carapace Length (mm)	Carapace Width (mm)	Body Height (mm)	Water Contents of the Third Pereiopod (%)
BCB	108.1 ± 20.8^{ab}	57.4 ± 2.6^{a}	64.8 ± 3.6^{a}	31.86 ± 1.33^{a}	56.9 ± 5.0^{c}
BCA	111.4 ± 20.1^{ab}	56.4 ± 2.0^{ab}	63.0 ± 3.2^{a}	30.83 ± 1.02^{ab}	60.6 ± 0.9^{ab}
YCS	101.9 ± 13.2^{b}	55.6 ± 3.1^{b}	63.1 ± 3.6^{a}	30.72 ± 1.89^{b}	62.3 ± 3.8^{a}
YCO	116.9 ± 26.1^{a}	57.5 ± 3.3^{a}	64.0 ± 4.0^{a}	31.34 ± 2.03^{ab}	59.3 ± 3.8^{b}

Different superscript letters in each column indicate significant differences ($p < 0.05$); "Bathed" crabs before bathing culture (BCB); "Bathed" crabs after one-month of bathing culture (BCA); Yangcheng Lake genuine crabs from September (YCS); and Yangcheng Lake genuine crabs from October (YCO).

2.2. Sample Pretreatment

According to Luo et al. [18], the entire left third pereiopod [22] (i.e., walking leg, a non-lethal sample material that consists of outer exoskeleton and inner muscle tissues) of each *E. sinensis* specimen was used as the source material for the elemental analyses, employing the approach described in our Chinese Patent (Code: ZL 201010220154.3). The pereiopods were collected non-lethally from the crabs and were stored in polyethylene bags at −20 °C in the laboratory until analysis.

The pereiopod samples were defrosted, washed six times with ultrapure water, and dried for 24 h at 80 °C in an oven until a constant weight was attained. The dried samples were pulverized using a Carnelian mortar and pestle, and then stored under dry conditions prior to analysis.

2.3. Element Analysis

The dry pereiopod samples (0.1 ± 0.005 g) were treated with 5 mL of nitric acid (GR, Merck, Darmstadt, Germany), and each mixture was gently digested using a microwave digestion instrument (ETHOS A T260, Milestone, Milan, Italy). The cooled digests were diluted to 100 mL using ultra-pure water and transferred to acid-washed tubes prior to analysis. An inductively coupled plasma mass spectrometer (Agilent 7500ce ICP-MS, Agilent, Santa Clara, CA, USA) was used to determine the contents of the mineral elements Na, Mg, Al, K, Ca, Fe, Mn, Cu, Zn, Sr, and Ba. The efficiency and reproducibility of the elemental analyses were checked by spike recoveries of the standard solutions; the recoveries ranged from 96–110%. The recovery rates of Na, Mg, K, Ca, Fe, Cu, and Zn were also determined using the standard sample of dogfish liver (DOLT-5 Fish Liver, National Research Council of Canada, Ottawa, Ontario, Canada), which were found to be between 95–113%.

2.4. Statistical Analysis

Prior to the analysis, data were checked for normality distribution and variance homogeneity using SPSS 23.0 (IBM SPSS Statistics Inc., Chicago, IL, USA). The SPSS 23.0 was also used to perform one-way analysis of variance (a one-way ANOVA), least significant difference test, multiple comparison analysis, and linear discriminant analysis (LDA). Pairwise statistical significance was set at $p < 0.05$.

3. Results

3.1. Mineral Elements in the Third Pereiopods

The measurement results of the third pereiopod elements of samples from YCS, YCO, BCB, and BCA groups are shown in Table 2. The mineral content of the Yangcheng Lake crabs followed the order Ca > Mg > Na > K > Fe > Sr > Ba > Zn > Mn > Al > Cu, whereas the Fe content of the samples from BCB was higher (Fe > Mg > Na > K). The Fe content of samples from BCA was significantly lower than that of samples from BCB ($p < 0.05$). The contents of the third pereiopod of BCA samples were Mg > Na > Fe > K, and the Al content was slightly larger than that of Mn. According to the one-way ANOVA, the 11 elements determined in this study were significantly different between the samples from YCS and BCB ($p < 0.05$) groups. Between samples from YCO and BCA, only Na and Mn were not significantly different, whereas all other elements were significantly different ($p < 0.05$). After one-month of bathing, samples from BCA had only four elements (Al, Fe, Cu, and Zn) significantly different from the BCB samples ($p < 0.05$).

Table 2. The content of mineral elements in the third pereiopod of Chinese mitten crabs (*Eriocheir sinensis*). (mean ± SD, mg/kg, dry weight).

Elements	BCB	BCA	YCS	YCO
Na	7430 ± 1968 ^{a}	6906 ± 430 ^{ab}	6685 ± 972 ^{b}	6370 ± 894 ^{b}
Mg	10,583 ± 1056 ^{a}	11,129 ± 1280 ^{a}	6714 ± 1110 ^{c}	7544 ± 927 ^{b}
Al	44.3 ± 12.9 ^{c}	77.9 ± 24.4 ^{a}	58.5 ± 30.0 ^{b}	27.0 ± 7.7 ^{d}
K	4867 ± 390 ^{bc}	5088 ± 352 ^{b}	5733 ± 590 ^{a}	4765 ± 262 ^{c}
Ca	154,273 ± 8353 ^{a}	156,577 ± 8792 ^{a}	132,632 ± 12,574 ^{c}	146,989 ± 7762 ^{b}
Mn	59.5 ± 17.5 ^{b}	68.6 ± 16.3 ^{b}	80.7 ± 25.6 ^{a}	57.5 ± 10.8 ^{b}
Fe	11,924 ± 623 ^{a}	6335 ± 370 ^{b}	3612 ± 350 ^{c}	3453 ± 242 ^{c}
Cu	16.0 ± 5.5 ^{b}	21.4 ± 5.5 ^{a}	22.3 ± 6.2 ^{a}	14.2 ± 7.6 ^{b}
Zn	76.5 ± 5.1 ^{c}	80.7 ± 5.7 ^{b}	86.6 ± 8.6 ^{a}	75.1 ± 4.0 ^{c}
Sr	1116 ± 93 ^{a}	1081 ± 107 ^{a}	868 ± 120 ^{c}	992 ± 62 ^{b}
Ba	478 ± 63 ^{ab}	497 ± 68 ^{a}	404 ± 34 ^{c}	450 ± 48 ^{b}

Different superscript letters in each row indicate significant differences ($p < 0.05$); "Bathed" crabs before bathing culture (BCB); "Bathed" crabs after one-month of bathing culture (BCA); Yangcheng Lake genuine crabs obtained in September (YCS); and Yangcheng Lake genuine crabs obtained in October (YCO).

3.2. Discriminant Analysis

The LDA of samples from YCS, YCO, BCB, and BCA was carried out using all 11 elements. Six elements (i.e., Fe, Mg, Al, Ca, K, and Cu) were selected by stepwise discriminant method in the process of linear discriminant analysis to establish the discriminant function. The discrimination results (Table 3, Figure 1) revealed significant differences between the foreign and genuine crabs before and after bathing in the same month, which was accurately identified using LDA. For "bathed" crabs, the accuracy of discrimination was 100%. In contrast, the changes between samples from YCS and YCO were insignificant. From the scatter diagram, the trend of changes in mineral elements in the third pereiopod of Yangcheng Lake crab plateaued and the discriminant analysis could not accurately distinguish samples from YCS and YCO.

Table 3. Linear discriminant analysis results based on the third pereiopod mineral elements of "bathed" Chinese mitten crabs (*Eriocheir sinensis*).

Geographical Origin	Predicted Group (Original/Cross-Validation)				Discriminant Accuracy (%) (Original/Cross-Validation)
	BCB	BCA	YCS	YCO	
BCB	20/20	0/0	0/0	0/0	100/100
BCA	0/0	20/20	0/0	0/0	100/100
YCS	0/0	0/0	17/16	3/4	85/80
YCO	0/0	0/0	0/1	20/19	100/95

"Bathed" crabs before bathing culture (BCB); "Bathed" crabs after one-month of bathing culture (BCA); Yangcheng Lake genuine crabs obtained in September (YCS); and Yangcheng Lake genuine crabs obtained in October (YCO).

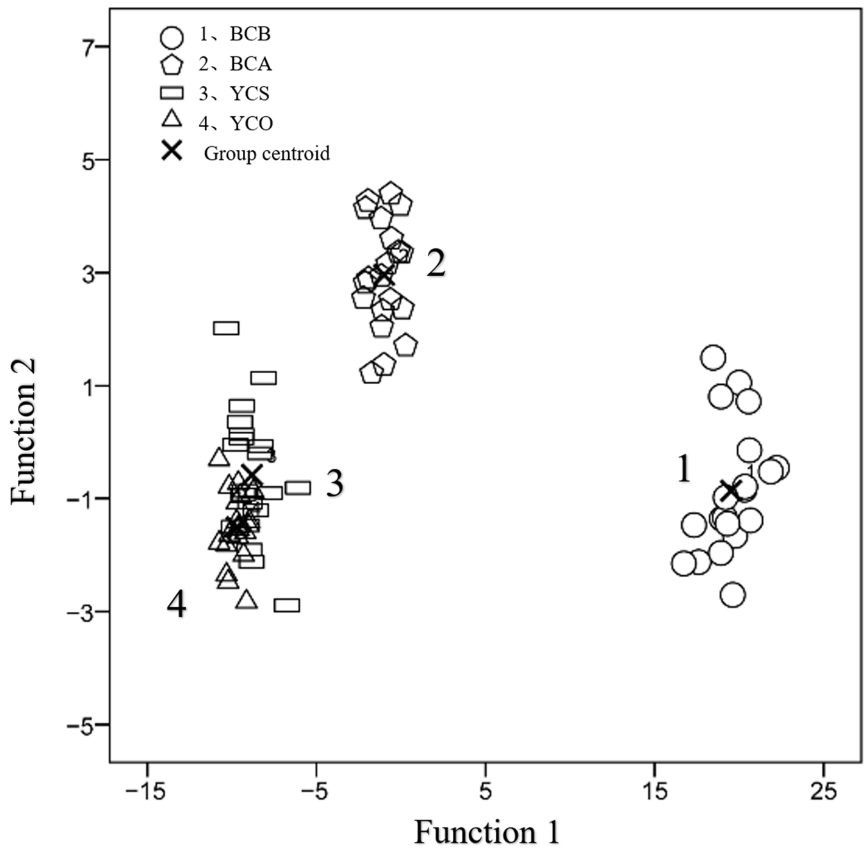

Figure 1. Scatter diagram of linear discriminant analysis based on the third pereiopod mineral elements of Chinese mitten crabs (*Eriocheir sinensis*); "Bathed" crabs before bathing culture (BCB); "Bathed" crabs after one month of bathing culture (BCA); Yangcheng Lake genuine crabs from September (YCS); and Yangcheng Lake genuine crabs from October (YCO).

4. Discussion

Mislabeling and adulteration are widespread in aquatic products worldwide [23–25]. Yangcheng Lake is the most famous aquaculture region of origin for the Chinese mitten crab that is known for its high water quality, shallow water and hard bottom, and abundant water grass. This lake provides ideal habitats for crab growth, giving Yangcheng Lake crab meat a clean and slightly sweet taste. "Yangcheng Lake Hairy Crab" remain the best-selling variety of hairy crabs in China, and are so reputed for the taste that people willingly pay high prices (USD 10–40 per individual vs. USD 1–5 per individual from other regions) for them [26]. Consumption of market-sized Chinese mitten crabs generally starts in September each year, with the peak period falling between October and November. During this time, "bathed" crabs are sold in large amounts. To optimize profits, cheaper crabs from other lakes are used to produce "bathed" crabs. Until September *E. sinensis* crab would reach the terminal molt and reach sexual maturity [27]. All crabs used in this study were adults [28,29], and molt did not influence biogeochemical profiles of the crabs in the present study. In addition, no significant differences were found in morphometrics parameters between BCA and YOC groups (Table 1). Noteworthily, foreign crabs had different mineral compositions from those native to Yangcheng Lake. There were significant differences between the samples from YCS and BCB for the mineral elements measured before bathing. After bathing, samples from BCA and BCB had four significantly different ($p < 0.05$) elements: Al, Fe, Cu, and Zn (Table 2). Luo et al. reported that the element characteristics could be accurately identified for *E. sinensis* crabs from multiple origins [18]. This demonstrates that the mineral elements of crabs from various regions presents with unique characteristics derived from their respective cultured areas. It is possible that the elemental characteristics in the genuine crabs may reflect more than half a year of bio-assimilation and bio-accumulation, as until the start of this study in September, the crab groups of YCS and BCB had already cultured in Yangcheng Lake and in another lake for at least half a year, respectively, from coin-sized juvenile to market-sized adults. Our findings revealed that even a one-month bathing culture was unable to converge the elemental profiles of concentration and composition in foreign crabs to crabs originated in Yangcheng Lake.

Linear discrimination analysis (LDA) is a widely used pattern recognition method for the authentication of fishery and aquaculture products [12]. In this study, "bathed" foreign crabs were accurately identified using LDA with 100% discrimination accuracy (Figure 1). It is noteworthy that the elemental patterns in foreign crabs changed to a large extent in the target tissue of the third pereiopods after a one-month bath period, and it could easily be used for successful differentiation of "bathed" crabs from the Yangcheng Lake originated crabs (e.g., the loss of Fe might be the main driver) (Table 2, Figure 1).

The aforementioned results indicate that the time-dependent changes in the mineral element content of the Yangcheng Lake crabs were stable, whereas those of "bathed" crabs changed substantially due to local adaptation. The patterns of elemental bioavailability in the latter were significantly different from those of the original crabs, i.e., the one-month "bathed" crabs did not completely converge to the element bioavailability characteristics of the genuine crabs. To the best of our knowledge, this is the first study to demonstrate that multi-mineral element profiles can potentially distinguish "bathed" crabs from the genuine ones, originating from Yangcheng Lake. We utilized the LDA multivariate statistical approach to generate these comparative mineral composition profiles. Our study offers a rare and important point of reference for the identification of adulteration in other commercially important aquatic products.

As third pereiopod consists of exoskeleton and muscle at the same time, it is still not known how much of the difference in elemental patterns between genuine and "bathing" cultured crabs can be attributed more to the former hard or the latter soft tissues. Because certain elements are typically incorporated into proteins and accumulated into soft tissues, whereas some elements are especially involved in the substitution with calcium ions in the calcite crystals and accumulated into the hard tissues of invertebrates [30], further studies will be necessary to clarify the contribution degree of each tissue type to the crab authentication according to elemental function roles and metabolism characteristics. Especially, hard tissues may need to be focused on, as trace elements of exoskeleton can effectively be used to discriminate adult crustacean stocks between regions with different water mass characteristics [31]. Moreover, future work will involve more studies of the "bathed" and genuine Yangcheng Lake crabs, focusing on more corresponding theoretical basis and development of even more robust methods for authenticity and traceability testing based on a combination of multi-mineral element profile with other features such as geometric morphometrics, as well as organic nutrient and flavor characteristics.

5. Conclusions

The mineral element profiles of deliberately "bathing" cultured crabs in Yangcheng Lake were investigated. Based on the element analysis of the third pereiopod of the genuine Yangcheng Lake and "bathed" crabs, we accurately distinguished between the two groups with a discriminating rate of 100%. We effectively proved that the one month bathing period was unable to converge the biogeochemical profiles in foreign crabs to those seen in the genuine crabs. These findings suggested that LDA approach might be extremely useful for the classification of "bathed" crabs. The element fingerprint analysis of the crab can be applied to recognize other counterfeited (including "bathed") fishery products.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fishes7010011/s1.

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Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Ethics Committee of Animal Care and Use Committee of the Freshwater Fisheries Research Center at the Chinese Academy of Fishery Sciences. The analysis was carried out following the Guidelines for the Care and Use of Laboratory Animals set by the Animal Care and Use Committee of the Freshwater Fisheries Research Center (2003WXEP61). All operations were carried out with field permit no. 20181AC1128.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available in supplementary material here.

Conflicts of Interest: The authors declare no conflict of interest.

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