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BIOLOGICAL SCIENCES

Correlation profile of the heavy metal distribution in the *Pontastacus leptodactylus* tissues

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Abstract: Arsenic (As), copper (Cu), nickel (Ni), zinc (Zn), manganese (Mn), lead (Pb), iron (Fe), aluminum (Al), cadmium (Cd) and chromium (Cr) accumulations were evaluated in the gills, hepatopancreas, exoskeleton, and muscles of *Pontastacus leptodactylus* (crayfish) (Eschscholtz, 1823). The highest metal accumulation was observed in the gills and hepatopancreas. It was detected a negative correlation between Cr-Pb, As-Cr in the muscle tissue. All other metals were displayed positive correlations with each other in the gills, hepatopancreas, and exoskeleton. Strong positive correlations were determined between Ni-Cd and As-Pb in the gills, Ni-Cd, As-Pb and Al-Zn in the hepatopancreas, Zn-Mn and Fe-Cu in the exoskeleton, Ni-Cd, As-Pb, Zn-Mn, Al-Mn, Fe-Cu and Al-Zn in the muscle (r= 1.000). PCA and cluster analysis generally were supported the correlations observed. The correlations between divalent metals may be expressed by the role of divalent metal transporter 1 (DMT1) in the gathering of these metals.

Key words: *Pontastacus leptodactylus*, heavy metals, metal-metal(loid) interactions, correlation.

INTRODUCTION

Heavy metal pollution in the aquatic environment is a major problem. Metals are resistant and have toxic effects on living organisms when they are above their concentration limits. With digestive system, by inhalation, or skin contact, heavy metals may penetrate crustaceans' tissues. Penetration and accumulation of heavy metals in the tissues may cause some debilitating chronic discomfort (Tunca et al. 2013a).

Aquatic organisms can accumulate metals from surrounding water, sediment and food sources into their bodies (Terra et al. 2008). Many indicator species are used to understand environmental quality in the aquatic ecosystems (Alcorlo et al. 2006). Crayfishes are potential indicators for metal accumulation, because of their benthic and solitary life forms, omnivorous nutrition style, long life cycle, slow movement, etc. Besides, they are useful due to large enough to be easily sampled from different body tissues and the ability of accumulating heavy metals.

In various researches, crayfish have been used as bioindicators of metal pollution (Varol & Sünbül 2018, Císar et al. 2018). Many heavy metals cause toxic effects, even in very low amounts, therefore it poses serious threats to human and environmental health (Suarez-Serrano et al. 2010). For this reason, understanding the accumulation of metals in the tissues of crayfish is a very important issue. Previous studies showed that crayfish accumulate significant concentrations of metals

in their tissues and they were found that the amount of metal accumulation is related with metal concentrations in the environment (Guner 2010, Kouba et al. 2010).

Metals accumulating and dispersing in crayfish tissue, both can be affected by metabolic pathways and environmental conditions. In addition, the metabolic effect caused by the presence of a metal can significantly alter the accumulation pattern for reasons such as synergism or antagonism effect. Therefore, this study was conducted to investigate the accumulation profiles in the Yeniçağa Lake *P. leptodactylus* (syn. *Astacus leptodactylus*) to reveal the relation between metal accumulations. The aims of this study were: 1) to study metal contents (As, Cu, Ni, Zn, Mn, Pb, Fe, Al, Cd and Cr) in the crayfish tissues and 2) to understand relationship of the heavy metals in the tissues.

MATERIALS AND METHODS

Study area and sample collection

Ten male crayfish, with total body lengths around 20-25 cm, were caught in February (2018) at Deliler location, Yeniçağa Lake, Bolu (40°046'53.93"N, 32°01'46.51"E) with the help of fishermen with various network pores and special crayfish baskets (Figure 1). The samples were taken from the Deliler location because of the high concentrations of heavy metals in a previous study (Saygı & Yiğit 2012). The samples were brought to the laboratory with plastic freezer and kept in plastic containers at -18°C until dissection.

Sample preparation and analysis

Approximately 1 g of exoskeleton, gills, hepatopancreas and muscle samples were taken from each specimen. Tissue samples were digested with suprapure nitric acid (HNO_3) at 100°C for 60 min and metal concentrations were diluted with deionized water and measurement was done by using ICP-MS (Agilent 7500) (Núñez-Nogueira et al. 2013).

Standard calibration solutions were arranged in 0.1% HNO_3 beginning from 1000 ppm for the metals. Laboratory equipments were cleaned with 1% HNO_3 and rinsed out deionized water. An acid mix solution from each digestion batch was also added to understand the background of pollution. The samples of the blank and control were analysed before and after every 10 samples as well as after the last sample. LUTS-1 (non-defatted lobster

Figure 1. The map showing the sampling locations. (a) Turkey, (b) Yeniçağa Lake, (c) Deliler Location.

hepatopancreas reference material) was used for quality control.

Six standard solutions were prepared for the calibration curves, and the R^2 >0.99 condition was achieved. All readings were repeated three times. A multi-element calibration solution (Bernd Kraft, Germany) was utilized for ICP-MS calibrations, detection limits were as follows; As, 0.04± 0.01 µg/L; Cu, 0.08 ± 0.04 µg/L; Ni, 0.02±0.003 µg/L; Zn, 0.35±0.03 µg/L; Mn, 0.41 ±0.07 µg/L; Pb, 0.46±0.003 µg/L; Fe, 0.82±0.06 µg/L; Al, 0.07 ±0.03 µg/L; Cd, 0.32 ± 0.002 µg/L; and Cr, 0.76± 0.05 µg/L.

Statistical analysis

The Mann Whitney U-test was performed to detect significant accumulation differences between tissues. Spearman Correlation analysis was used to understand the correlations between the heavy metal accumulation in the tissues. The extraction method for Principle Component Analysis was used according to Varimax, with Kaiser Normalization. Cluster Analysis was applied using the Ward's method, on Euclidean distance intervals and Z-score correction (SPSS 20v.).

RESULTS

Heavy metal accumulations were investigated in the tissues of *Pontastacus leptodactylus* from Yeniçağa Lake and concentrations in the tissues are shown in Table I. The correlation of metal accumulations in the tissues in given in Table II.

Principal Component Analysis (PCA) using Varimax normalized rotation and Cluster analysis were calculated for each tissue. PCA indicates that three components explain about 87.468% for the exoskeleton, 74.713% for the gills, 84.565% for the hepatopancreas and 88.055% for the muscle, total variance of the for each tissue in the data set. Zn-Mn-Al show their strongest positive correlational relationship in the first component (PC1) of the exoskeleton and the PC1 explains 43.429% of the total variance with eigenvalue of 4.343. The first component with 31.538% of total variance with an eigenvalue of 3.154 comprises As and Pb with high loadings in the gills. PC1 explains 38.266% of the total variance with eigenvalue of 3.827 comprises As-Pb-Mn with the strongest loadings in the hepatopancreas. Zn-Mn-Al show their highest positive correlational relationship in the PC1 of the muscle and the PC1 explains 41.633% of the total variance with eigenvalue of 4.163 (Table III).

According to Cluster Analysis, results shown in the exoskeleton tissue, unclose three major clusters: (1) Cu-Fe, (2) As-Pb and (3) Zn-Al; in the gills reveal four major clusters: (1) As-Pb, (2) Ni-Cd, (3) Cu-Fe, (4) Zn-Mn, in the hepatopancreas, unclose four major clusters; (1) Ni-Cd, (2) Cu-Fe, (3) Zn-Al, (4) As-Pb and in the muscle, unclose four major clusters; (1) Ni-Cd, (2) Cu-Fe, (3) As-Pb (4) Zn-Mn-Al. These metals are the closest to each other on account of concentration (Figure 2).

Table I. Metal concentrations in crayfish tissues (µg/g dry weight). Data are given as Mean±SE.

	As	Cu	Ni	Zn	Mn	Pb	Fe	Al	Cd	Cr
Exo	8.8±0.7	18.6±3.1	2.7±0.8	67.7±14.4	54.9±12.8	2.9±0.4	43.6±3.1	188.4±14.8	1.4±0.7	7.6±1.7
Gills	15.1±3.2	31.0±5.1	5.1±1.2	113.6±15.9	66.5±9.8	6.1±1.6	64.1±10.3	2334.6±600.2	3.1±1.2	15.6±3.2
Hepato	12.8±3.2	39.1±10.3	5.7±1.4	78.5±9.8	101.6±15.9	4.9±1.6	56.0±5.1	199.5±9.8	2.8±0.7	20.8±2.8
Muscle	6.6±0.9	12.0±4.2	1.0±0.4	35.2±5.6	23.2±5.6	1.8±0.5	37.0±4.2	156.2±5.6	0.7±0.4	4.6±1.5

Table II. Correlations between metal concentrations in the tissues of *P. leptodactylus.*

	Cd	Pb	Mn	Cr	As	Cu	Ni	Zn	Fe	Al
Exoskeleton										
Cd	1									
Pb	0.337	1								
Mn	0.207	0.615	1							
Cr	-0.498	0.000	0.128	1						
As	0.485	0.876**	0.431	-0.148	1					
Cu	0.576	0.227	-0.255	0.140	0.276	1				
Ni	0.018	0.759*	0.572	-0.107	0.765**	-0.299	1			
Zn	0.207	0.615	1.000**	0.128	0.431	-0.255	0.572	1		
Fe	0.576	0.227	-0.255	0.140	0.276	1.000**	-0.299	-0.255	1	
Al	0.212	0.632*	0.997**	0.109	0.448	-0.261	0.585	0.997**	-0.261	1
Gills										
Cd	1									
Pb	0.127	1								
Mn	-0.309	-0.139	1							
Cr	-0.382	-0.176	0.430	1						
As	0.127	1.000**	-0.139	-0.176	1					
Cu	-0.297	0.297	0.406	0.479	0.297	1				
Ni	1.000**	0.127	-0.309	-0.382	0.127	-0.297	1			
Zn	-0.236	-0.285	0.430	-0.212	-0.285	-0.224	-0.236	1		
Fe	-0.321	0.467	0.345	0.285	0.467	0.879**	-0.321	-0.297	1	
Al	-0.418	0.079	-0.055	-0.212	0.079	0.042	-0.418	0.297	0.297	1
Hepatopancreas										
Cd	1									
Pb	-0.104	1								
Mn	0.018	0.569	1							
Cr	-0.321	0.483	0.236	1						
As	-0.104	1.000**	0.569	0.483	1					
Cu	-0.115	0.043	-0.297	0.285	0.043	1				
Ni	1.000**	-0.104	0.018	-0.321	-0.104	-0.115	1			
Zn	0.345	0.440	0.430	0.212	0.440	0.345	0.345	1		
Fe	-0.212	00.000	-0.224	0.042	0.000	0.879**	-0.212	0.406	1	
Al	0.345	0.440	0.430	0.212	0.440	0.345	0.345	1.000**	0.406	1
Muscle										
Cd	1									
Pb	-0.034	1								
Mn	0.140	0.372	1							
Cr	0.266	-0.661*	0.000	1						
As	-0.034	1.000**	0.372	-0.661*	1					
Cu	-0.524	-0.012	-0.442	-0.036	-0.012	1				
Ni	1.000**	-0.034	0.140	0.266	-0.034	-0.524	1			
Zn	0.140	0.372	1.000**	0.000	0.372	-0.442	0.140	1		
Fe	-0.524	-0.012	-0.442	-0.036	-0.012	1.000**	-0.524	-0.442	1	
Al	0.140	0.372	1.000**	0.000	0.372	-0.442	0.140	1.000**	-0.442	1

***. Correlation is significant at the 0.05 level (2-tailed).**
****. Correlation is significant at the 0.01 level (2-tailed).**

Table III. Results from Principal Component Analysis (PCA) for selected metal(loid)s accumulation in the tissues with Cumulative percent variation and eigenvalues

	Exoskeleton			Gills			Hepatopancreas			Muscle		
	PC1	PC2	PC3	PC1	PC2	PC3	PC1	PC2	PC3	PC1	PC2	PC3
As	0.413	0.472	**0.727**	**0.916**	0.037	-0.071	**0.938**	-0.14	-0.052	0.313	**0.812**	-0.405
Cu	-0.114	**0.983**	0.003	0.383	-0.196	**0.788**	0.003	-0.032	**0.959**	-0.744	0.43	0.253
Ni	0.476	-0.041	**0.754**	0.093	**0.875**	-0.283	-0.123	**0.957**	-0.12	0.57	-0.648	-0.298
Zn	**0.975**	-0.08	0.077	-0.62	-0.367	-0.166	0.613	0.707	0.225	**0.855**	0.376	0.348
Mn	**0.97**	-0.071	0.076	-0.511	-0.076	0.581	0.746	0.174	-0.329	**0.855**	0.376	0.348
Pb	0.646	0.327	0.599	**0.916**	0.037	-0.071	**0.938**	-0.14	-0.052	0.313	**0.812**	-0.405
Fe	-0.114	**0.983**	0.003	0.601	-0.275	0.591	-0.047	-0.035	**0.908**	-0.744	0.43	0.253
Al	**0.971**	-0.072	0.103	-0.007	-0.775	-0.082	0.613	0.707	0.225	**0.855**	0.376	0.348
Cd	0.059	**0.815**	0.161	0.093	**0.875**	-0.283	-0.123	**0.957**	-0.12	0.57	-0.648	-0.298
Cr	0.394	0.043	-0.681	-0.176	-0.066	**0.826**	0.595	0.124	0.309	0.147	-0.639	0.53
Cumulative % variation	43.429	74.219	87.468	31.538	60.304	74.713	38.266	63.825	84.565	41.633	75.267	88.055
Eigenvalues	4.343	3.079	1.325	3.154	2.877	1.441	3.827	2.556	2.074	4.163	3.363	1.279

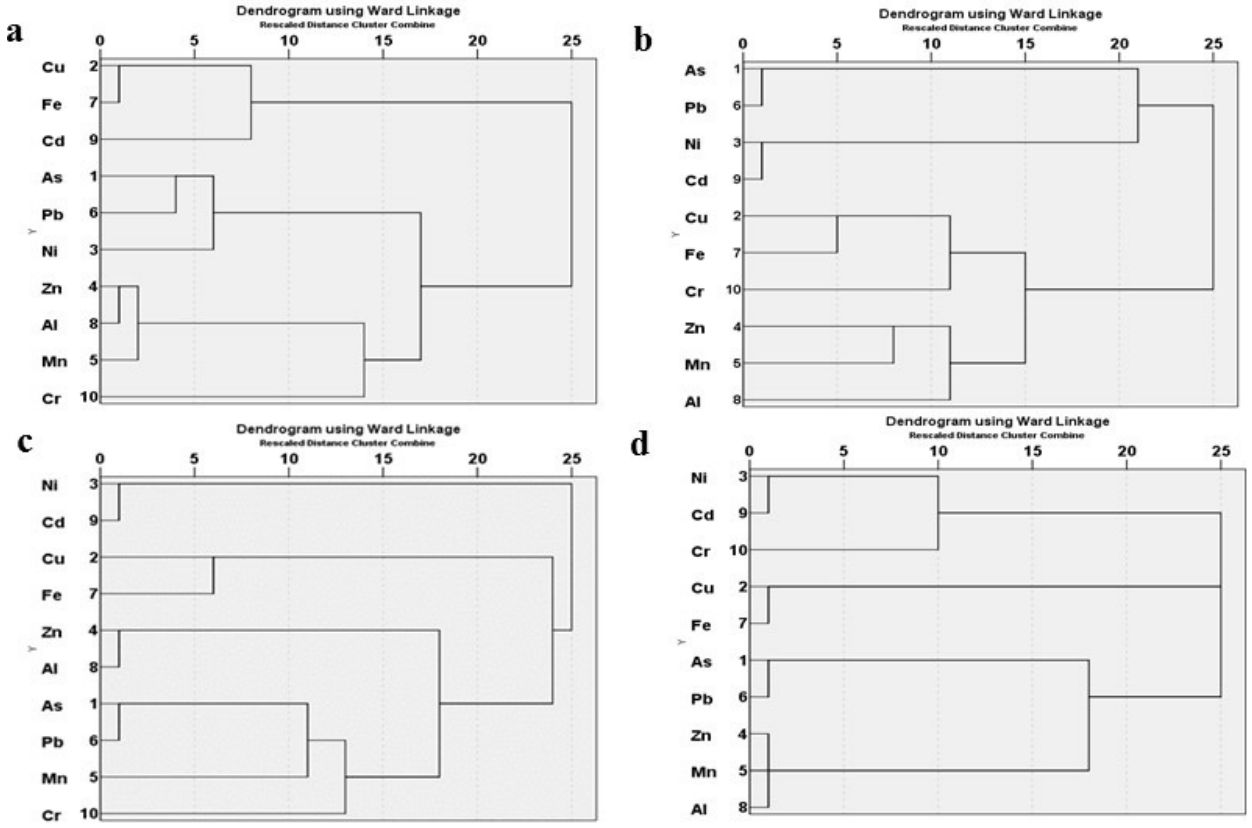

Figure 2. Cluster analysis of (a) exoskeleton, (b) gills, (c) hepatopancreas and (d) muscle tissues.

DISCUSSION

The highest metal(loid) correlations were found in the exoskeleton and the muscle tissues. The most significant correlations between metal(loid)s were observed in muscle tissue. The strong positive correlations were observed between Zn-Mn and Fe-Cu in the exoskeleton, Ni-Cd and As-Pb in the gills, Ni-Cd, As-Pb and Al-Zn in the hepatopancreas, Ni-Cd, As-Pb, Zn-Mn, Al-Mn, Fe-Cu and Al-Zn in the muscle (r= 1.000).

Metal accumulations were detected in all investigated tissues. The highest statistical differences were observed between muscle and gills for all the metal(loid)s (p<0.01). This might be related to the fact that muscle is an internal tissue, while gills are constantly in contact with the external environment. The highest metal(loid) concentrations were found in the hepatopancreas and gills tissues (gills> hepatopancreas> exoskeleton> muscle). Since the gills are constantly in contact with the outside environment (Kurun et al. 2010), it is one of the tissues which displays the highest metal accumulation. The other reason may be that gills play an important role to regulate of ionic balance (Alcorlo et al. 2006). High metal concentrations in the hepatopancreas can be interpreted as the active transport of metals to detoxification.

Hepatopancreas is important tissue for the crustaceans due to it is main region of metal sequestration. Metallothioneins are an important proteins due to metal sequestration function and they plays an important role in the protection against metal toxicity. The metallothionines, capable of binding metal ions selectively, exist in all invertebrates and are rich in cysteine (Ahearn et al. 2004). The main targets of metallothionines are IB and IIB metals such as Zn, Cd and Cu etc. (Pourang & Dennis 2005). Their most important function is to neutralize metals and prevent them from reacting. They are also responsible for transporting any unnecessary metals or excess amounts of metals. In this way, they prevent metal toxicity. Correlation of +2 (As, Cu, Ni, Zn, Mn) valence metals was not observed in the hepatopancreas in this study as expected. It may be related to the fact that hepatopancreas is the place where detoxification of harmful metals occurs. This detoxification is due to the high metallothionein concentration in hepatopancreas.

Many proteins facilitate the transport of divalent metals in vertebrates such as DMT1 (DCT1 or Nramp1) Ahearn et al. 2004). There are homologues of DMT1 in invertebrates (Smyth et al. 2006) and it is mainly an iron transporter (Bai et al. 2014) but it transports not only Fe, but also Zn, Mn, Cu, Co, Ni, Cd and Pb (Kwong & Niyogi 2009). The strong correlations between +2 valence metals recommend that the existence of a +2 valence metal transporter may be influential in +2 valence metals sequestration. It is yet unknown that Ni is an essential or nonessential for crayfish (Tunca et al. 2013b) but Cd is a nonessential metal. Ni was indicated an interesting trend and showed a strong correlation with Cd in the hepatopancreas, gills and muscles (r= 1.000). PCA of individual tissues place Cd-Ni in the same component and also the metals are placed in the same cluster in all tissues except exoskeleton. A strong correlation between Ni and Cd was reported on crayfish (*A. leptodactylus)* by Tunca et al. (2013b). Cr^{+2} and Cr^{+6} are essential metals for some oxidation states but its other forms are toxic for organisms (Bankar et al. 2009).

As and Pb are highly toxic, carcinogenic and nonessential metals. As can cause inhibition of glycolytic energy metabolism and also mitochondrial degradation (Fattorini et al. 2006). Exoskeletal As concentrations were found to correlate strongly with Pb. As

was found to be correlate with Pb perfectly in the gills, hepatopancreas and muscle. These metals are placed in the same component (PCA) and cluster in all tissues. The greatest As and Pb accumulations were in gills, followed by the hepatopancreas. There was a negative correlation between As and Cr. Cr and As take place in metabolisms by using phosphate transport pathways (Tripathi et al. 2007). Therefore, a strong correlation between As and Cr was expected. Negative correlation might be observed since Cr metal can also use sulphate pathways. In this study, however, correlation of As with other metals was not found. Arsenic correlates positively only with Pb in the tissues. It may be depend on its low binding ability to transport proteins. Hemocyanin is an important metalloprotein for invertebrates. As it transports Cu-linked oxygen, Cu is an important metal for invertebrates.

Iron is also an important metal because it is a part of pigments and enzymes of decapod crustaceans. The bioavailability of iron depends on the valence of the metal. For instance, Fe^{+2} is essential for most animals while Fe^{+3} is nonessential (Sanders et al. 1998). Strong correlation was found between Cu and Fe in the all tissues. PCA placed Cu and Fe in the same components for exoskeleton and hepatopancreas. Transferrin is an important protein that transports iron (Chua et al. 2007) and it is also involved in the transport of Cu^{2+}, Mn^{2+}, Al^{3+}, Cr^{3+}, Zn^{2+}, Ni^{2+}, Ga^{3+} and Ti^{4+} (Quarles et al. 2011). It has been reported by Quarles et al. (2011) that transferrin shows different preferences for different metals depending on the concentration. In this study, the correlations between Fe-Cu in the exoskeleton and muscle, Al-Zn in the exoskeleton, muscle and hepatopancreas, Fe-Cu in the gills, hepatopancreas and muscles were observed. Al is a bioavailable metal and it can be toxic to crayfish (Ward et al. 2006).

Gills were determined as important tissues for the accumulation of Al. Moreover, it was observed that Al was the most accumulated metal among the tested metals. Previous studies also supported our result (Tunca et al. 2013a, Fikirdeşici-Ergen et al. 2015). Mn is one of the essential metal and it was shown that crayfish has potential to gathered high amounts of Mn (Tunca et al. 2012) Al-Mn corellations were found perfectly in the hepatopancreas and the muscle (r=1.000). It was reported that Mn–Al correlation coefficient is between + 0.74-0.79 in the muscle tissue of crayfish (*A. leptodactylus)* (Kurun et al. 2010). In the present study, correlation coefficient of Mn–Al in the muscle tissue was detected as 1.000. Zn has an important mission as a cofactor in enzymatic systems and it can be used as an active core in metalloenzymes. Al-Zn correlations were found perfectly in the hepatopancreas and the muscle (r=1.000). Al-Zn and Al-Mn correlations were found very strong in the gills (r= 0.997). Trace metals such as Fe, Al, Mn, Zn etc. are transported by transferrin and this transportation was kept limited with chordates until recently. But it has been reported from Huebers et al. (1982) for *Cancer magister* and Liang et al. (1997) for *Pacifastacus leniusculus*.

CONCLUSION

As, Cu, Ni, Zn, Mn, Pb, Fe, Al, Cd and Cr metals were evaluated in four crayfish tissues. The amount of the accumulation orders of the tissues were determined as gills> hepatopancreas> exoskeleton> muscle. All correlations in the tissues of hepatopancreas, exoskeleton and gills were detected as positive. This might suggest that these metals show a trend in a synergic manner. This result was expected due to gills and exoskeleton function as accumulation centres and the hepatopancreas tissue functions

as a detoxification centre for various metals. Negative correlations were detected between Cr-As and Cr-Pb in the muscle tissue. This result showed that there is antagonism between these metals during absorption. Nonessential metals incline to gather in tissues using the metabolic pathways of the essential metals, thus, the common way of metal uptake can lead to metal-metal(loid) interactions in the tissues. Ni-Cd, As-Pb showed perfect correlations in the hepatopancreas, gills and muscles (r=1.000). Metal–metal(loid) interactions have been determined to play an important role in the profile of the metal accumulation in the crayfish tissues.

This study shows that uncovering the effects of metal-metal interactions and their tendency to accumulate in the tissue is a requirement to improve our understanding of bioaccumulation, bio-monitoring studies.

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The author conceived of the study and carried out the fieldwork and performed the analyses and wrote the manuscript.