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Evaluating energy dispersive X-ray analysis (EDXA) as a diagnostic tool in acanthocephalan taxonomy as evidenced in Palaeacanthocephala and Archiacanthocephala

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Abstract We have examined the anterior and middle hooks of many specimens of 3 species of acanthocephalans from Ukraine including (1) adults of *Acanthocephalus ranae* (Schrank, 1788) Lühe, 1911 from 4 species of frogs in 6 geographical locations, (2) adults of *Southwellina hispida* Van Cleave, (1925) Witenberg, 1932 from 3 species of birds in 2 geographical locations, and (3) adults and cystacanths of *Sphaerirostris picae* (Rudolphi, 1819) Golvan, 1956 from 1 species of birds, 1 species of lizards, and 1 species of mammals in 2 geographical locations**,** to analyze their Ca, S, and P spectra using Energy dispersive x_ray analysis (EDXA), and account for their intraspecific variabilities. Adults of each of *A. ranae* from frogs and adults of *S. hispida* from birds each showed comparable metal spectra irrespective of host species and geography, especially when metal weight percent figures are averaged. In *S. picae*, 5 adult specimens from birds had comparable spectra but the cystacanth from hedgehog, a mammal, had particularly dissimilar pattern with much lower levels of P and Ca. We have also studied the EDXA patterns in anterior, middle and posterior hooks of cystacanths, juveniles, and adults of *Moniliformis kalahariensis* Meyer, 1931 much lower levels of P and Ca. in South Africa, Botswana, and India collected from their invertebrate intermediate and vertebrate definitive hosts to examine the EDXA profiles. Our conclusions were comparable to those reached from the Ukrainian material with the additional emphasis on the importance of using the same developmental stage in comparing interspecific EDXA profiles. The importance of the stability of the levels of S in various developmental stages across the host species barrier is exemplified by its relationship to the genetically based protein synthesis, which gives credibility to the usage of EDXA for diagnostic purposes. Our findings show that EDXA is a useful tool to characterize the taxonomic identity of species of Acanthocephala providing that (1) a population of a number of individual specimens are analyzed and average weight percent figures of metals are used for comparisons, and (2) comparisons are made using specimens of the same developmental stage and from hosts of the same class of vertebrate. When these conditions are met, the

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metal spectra for each species will prove valid for diagnostic purposes.

Introduction

Our studies of acanthocephalan worms have usually involved Energy Dispersive x-ray analysis (EDXA) of FIB-sectioned hooks and spines with special reference to the hardened components, e.g., calcium (Ca), sulfur (S), and phosphorus (P) (Heckmann 2006, Heckmann et al. 2007, 2012a, b, Standing and Heckmann 2014). Large hooks play a major role for host tissue attachment. The taxonomic identity of species is deep-seated at the genetic level, which is expressed by the organism's morphology and biochemistry as revealed, in part, by its elemental spectra. Populations of an acanthocephalan species will consistently have similar EDXA spectra irrespective of host species or geography. We have previously reached those conclusions based on the examination of 1 or 2 individuals of each species at a time (Amin et al. 2017, 2018, 2019b, 2020a, b, c, among others). In the present studies, however, we have examined many specimens of 3 species of acanthocephalans from hosts collected in various geographical locations of Ukraine and cystacanths, juveniles and adults of 1 other species, *Moniliformis kalahariensis* Meyer 1931 (re-described by Amin et al., 2014) from South Africa, Botswana, and India, to evaluate the EDXA pattern of populations of developmental stages for chemical ions (S, Ca, P) and evaluated their elemental identity. Ultimately, the diagnostic utility of EDXA profiles in the taxonomic literature is assessed.

Materials and methods

Collections

Twenty-nine specimens of 3 species of acanthocephalans were collected from a variety of host species in various geographical regions in Ukraine since 1984, but mostly more recently (Table 1). Adults of *Acanthocephalus ranae* (Schrank 1788) Lühe 1911, of *Southwellina hispida* Van Cleave, (1925) Witenberg 1932, and of *Sphaerirostris picae* (Rudolphi 1819) Golvan 1956 were collected from 4 species of frogs, 3 and 1 species of birds, respectively, in various localities. Cystacanths of *S. picae* were also examined (Table 1). Specimens were fixed and transported to our Scottsdale, Arizona and Provo, Utah facilities in 70% ethanol.

Additionally, we collected juveniles and adults of *M. kalahariensis* from 2 individuals of the South African hedgehog, *Atelerix frontalis* Smith (Erinaceidae), 1 from the University of Limpopo grounds, Turfloop, Polokwane, Limpopo Province (23°53’35″S; 29°44’12.9″E) on 17 September 2012, and 40 specimens from the other hedgehog in Mohlonong Village, Mashashane, Limpopo Province, South Africa (23°53’49.9″S; 29°07’56.6″E) on 1 November 2012. Some cystacanths from the German cockroach *Blattella* (*Phyllodromia*) *germanica* Linn. (Blattidae Karny) in Bombay, India, as well as adults from *A. frontalis* and from the Namaqua sandgrouse, *Pterocles namaqua* (Gmelin) (Pteroclididae) in Botswana from the Meyer's (1931) collection published in Hartwich et al., 1998) were obtained courtesy of Dr. Birger Neuhaus, Curator, Museum für Naturkunde, Berlin; see Amin et al. (2014).

Methods

SEM (Scanning Electron Microscopy)

Specimens that had been fixed and stored in 70% ethanol were processed for SEM following standard methods (Lee, 1992). These included critical point drying (CPD) in sample baskets and mounting on SEM sample mounts (stubs) using conductive double-sided carbon tape. Samples were coated with gold and palladium for 3 minutes using a Polaron #3500 sputter coater [Quorum (Q150 TES) www.quorumtech.com] establishing an approximate thickness of 20 nm. Samples were placed and observed in a FEI Helios Dual Beam Nanolab 600 (FEI, Hillsboro, Oregon) Scanning Electron Microscope with digital images obtained in the Nanolab software system (FEI, Hillsboro, Oregon) and then transferred to a USB for future reference. Samples were received under low vacuum conditions using 10 KV, spot size 2, 0.7 Torr using a GSE detector (HTK, Hamburg, Germany).

Table 1. Acanthocephalans from Ukraine collected for this study and from India and South Africa.

No.	Host species	Geographical location	Date	Specimens
Acanthocephalus ranae (Schrank, 1788) Lühe, 1911, Class Palaeacanthocephala Meyer, 1931				
I	*Pelophylax ridibundus* (Pallas) (marsh frog)	Cherson region, Askania-Nova City	12.10.2012	1 adult
		Vinnickiy region	06.04.2013	3 adults.
II	*Bombina bombina* (Linn.) (fire-bellied frog)	Vinnickiy region	29.06.2018	1 adult
III	*Pelophylax esculentus* (Linn.) (edible frog)	Vinnickiy region	05.07.2018	2 adults
		near Kyiv	15.10.2018	1 adult
		Kyiv region, Desna River	01.06.2012	1 adult
IV	*Rana arvalis* Nilsson (moor frog)	Zhitomir region	02.07.2012	2 adults.
		Volynskyj region, Polissia District	29.06.2011	2 adults
Southwellina hispida Van Cleave, (1925) Witenberg, 1932, Class Palaeacanthocephala Meyer, 1931				
I	*Ardea cinerea* Linn. (gray heron)	Cherson region, near Gopry City	24.08.2011	4 adults
II	*Ardea alba* Linn. (great egret)	Cherson region, near Zheleznyj Port	17.08.2011	3 adults
III	*Phalacrocorax carbo* (Linn.) (great cormorant)	Cherson region, near Gopry City	24.08.2011	1 adult
Sphaerirostris picae (Rudolphi, 1819) Golvan, 1956, Class Palaeacanthocephala Meyer, 1931				
I	*Pica pica* (Linn.) (Eurasian magpie)	Cherson Region, Askania-Nova City	13.06.1984	5 adults
II	*Lacerta agilis* Linn. (sand lizard)	Odessa region, Vilkovo City	04.05.2010	2 cystacanths
III	*Erinaceus europaeus* Linn. (common European hedgehog)	Odessa region, Vilkovo City	01.05.2010	1 cystacanth
Moniliformis kalahariensis Meyer, 1931, Class Archiacanthocephala Meyer, 1931				
IV	*Atelerix frontalis* Smith (South African hedgehog)	Limpopo, South Africa	17.09.2012	4 adults
	Atelerix frontalis Smith (South African hedgehog) and *Pterocles Namaqua* (Gmelin) (Namaqua sandgrouse)	Botswana; based on Meyer (1931), Hartwich et al. (1998) from Museum für Naturkunde, Berlin	Pre 1931	4 adults
	Atelerix frontalis Smith South African Hedgehog)	Limpopo, South Africa	17.09.2012	4 juveniles
	Blatella (*Phyllodromia*) *germanica* Linn. (German cockroach)	Bombay, India; based on Meyer ((1931), Hartwich et al., 1998; from Museum für Naturkunde, Berlin	Pre 1931	5 cystacanths

X-ray microanalysis (XEDs), EDAX (Energy Dispersive Analysis for X-Ray)

Standard methods were used for preparation similar to the SEM procedure. Specimens were examined and positioned with the above SEM instrument which was equipped with a Phoenix energy-dispersive x-ray analyzer; the source for the acronym EDXA (FEI, Hillsboro, Oregon). The scope is equipped with software so we use TEAM (Texture and Elemental Analytical Microscopy) when we do the EDXA. For cutting, we use a Gallium ion beam, which can be regulated with beam current depending on what is being cut. X-ray spot analysis and live scan analysis were performed on an anterior and middle hooks of each specimen at 16 Kv with a spot size of 5 and results were recorded on charts and stored with digital imaging software attached to a computer. Hooks examined were marked on the SEM scans of each specimen. The TEAM *(Texture and Elemental

Analytical Microscopy) software system (FEI, Hillsboro, Oregon) was used to profile elements of hooks and spines.

Weight percent (wt%) of each chemical element detected was recorded. Heckmann et al., (2007) recorded wt% peak weights in 5 species of acanthocephalans for comparison. The computer program calculates weight percent using ZAF corrections and depicts peak heights. In spectroscopic analysis of characteristic X-rays, the "ZAF correction method" is used for quantitative analysis of target elements and takes into account atomic number (Z) effect, absorption (A) effect, and fluorescence excitation (F) effect. Weight percent for each species of Acanthocephala is analyzed for probability using statistical analysis (SAS) with P and M values recorded. The wt% data are normalized; they are not absolute numbers and are used for comparing data from different species (Heckmann et al., 2012a). The data includes weight percent and atom percent of the detected elements following correction factors. For example, data used to generate the EDXA spectrum of the first specimens of *Acanthocephalus ranae* (Fig. 1 and Table 3) is shown in Table 2. Only % weight of Ca, P, and S, the primary elements in hook hardening, are used for the purpose for this study, in each of the specimens of the 4 acanthocephalan species studied (Table 3-6) However, Figs. 1-3, 4-6, 7-9 also depict more of the other elements that are sufficiently visually detectable. Data were stored in a USB for future analysis.

Ion sectioning of hooks

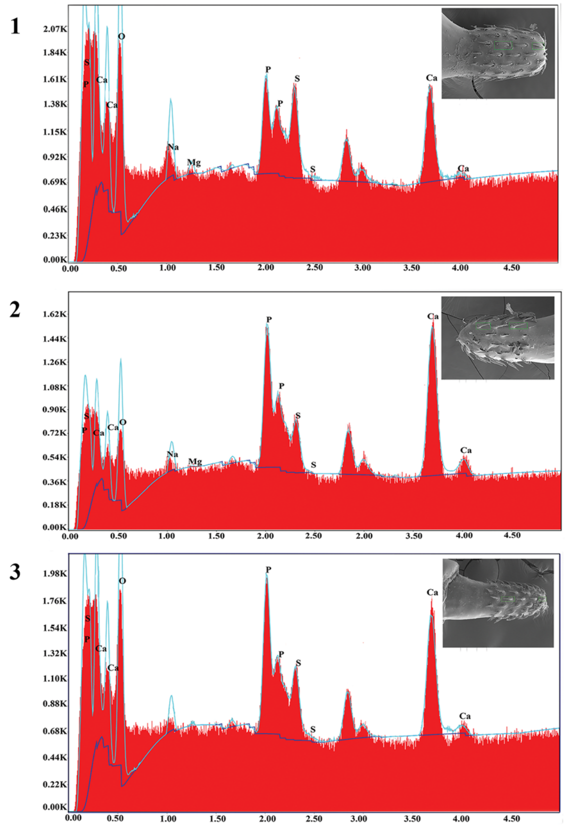
A dual-beam SEM with a Gallium (Ga) ion source (GIS) is used for the Liquid Ion Metal Source (LIMS) part of the process. The hooks of the acanthocephalans were centered on the SEM stage and cross sectioned using a probe current between 0.2 nA and 2.1 nA according to the rate at which the area is cut. The time of cutting is based on the nature and sensitivity of the tissue. Following the initial cut, the sample also goes through a milling process to obtain a smooth surface. The cut was normally analyzed with X-ray at the tip, middle, and base of hooks for chemical ions with an electron beam (Tungsten) to obtain an X-ray spectrum. Results were stored with the attached imaging software then transferred to a USB for future use. The intensity of the GIS was variable according to the nature of the material being cut.

Results

This project was designed to examine the EDXA patterns for a large number of acanthocephalans of different species and to verify pattern consistency in each species irrespective of host species and geography to assess its utility as a diagnostic tool in acanthocephalan taxonomy. It should be noted that EDXA is a qualitative measurement of the chemical ions in a living organism not a quantitative measurement subject to statistical analysis. The scans and spectra provide an understanding of the level but not the specific quantities of the chemicals present. It provides a good comparison of the chemical composition of the organism that lies between its external morphological and anatomical topography and its internal molecular profile. The Xray scans of the gallium cuts help explain the finer morphological nature of studied structures and identify the species unique "personality." Different chemical components are indicators of biophysical qualities of certain organs. Table 1 summarizes data of 26 adults and 3 cystacanths collected from 10 host species in various localities in Ukraine and of developmental stages of another species from South Africa, Botswana, and India. SEM images and EDXA of the anterior and middle hooks of each specimen were completed and the results are presented below.

Table 6 summarizes data of 5 cystacanths from *B. germanica* in India, 4 young unsegmented juveniles and 4 segmented adults from *A. frontalis* in South Africa, and 4 segmented adults from *A. frontalis* and *P. Namaqua* in Botswana. The juveniles and adults from South Africa were all collected from the Limpopo Province in 2012; see the redescription of *M. kalahariensis* (Amin et al., 2014). The cystacanths from India and the segmented adults from Botswana must have been collected sometime before 1931 as they were first reported by Meyer (1931) who gave no date of collection. This makes the age of the Meyer specimens more than 90 years. Meyer (1931) also did not indicate the number of adult specimens collected from each host species and whether his description was based on specimens (n=?) from1 or both host species.

Individual intraspecific variability in percent metal weight was observed for phosphorous (P), sulfur (S), and Calcium (Ca). In all cases, the overall pattern, i.e., population means, was stable and characteristic of



Figs. 1-3 X-ray panels of elemental scans of anterior hooks of *Acanthocephalus ranae* adults showing qualitative EDXA spectra. See Table 3 for % weight of depicted elements. Note the higher cumulative levels of Ca compared to P and S in all cases. Insets include corresponding proboscides with sampled anterior and middle hooks marked in green boxes. **1**, Scan of whole anterior hook of specimen no. 1 from host no. I; **2,** Scan of whole anterior hook of specimen no. 1 of host no. II; **3,** Scan of whole anterior hook of specimen no. 1 of host no. III (Table 3).

each acanthocephalan species irrespective of host species and geographical distribution.

Acanthocephalus ranae adults

The percent weights of P, S, and Ca of anterior and middle hooks 12 adults of *A. ranae* collected from 4 species of frogs in 6 different geographical localities were analyzed (Table 3). Variability in levels of P and S in both hooks was minimal but somewhat higher for Ca. The metal levels were slightly higher in middle hooks than in anterior hooks. The concentration of metals changes along the longitudinal gradient of the proboscis. The mean percent weight of P and S was comparable in anterior (5.67% & 5.46%) and middle hooks (6.13% & 6.47%) but the level of Ca was invariably twice as high in both hooks (11.79% & 12.99%). This appears to be characteristic of *A. ranae*. See also Figs. 1-3.

Southwellina hispida adults

The percent weights of P, S, and Ca of anterior and middle hooks in 8 adults of *S. hispida* collected from 3 species of birds in the same geographical region were analyzed (Table 4). As in *A. ranae*, variability in levels of P and S in both hooks was low but somewhat higher in Ca. The levels of P and Ca were slightly higher in the anterior hooks than in middle hooks and the S levels appears to increase posteriorly as has been found in other studies. In anterior hooks, the mean level of Ca (19.15%) was about twice that of P (9.58%) and the P level was twice that of S (5.19%). This pattern appears to be characteristic of *S. hispida*. This pattern was consistent in the middle hooks and the S level also increased posteriorly (Table 4) as in *A. ranae* (Table 3). These variations are not related to geography since all 8 specimens were collected from the same region (Table 1) and are also not related to host species since, in most parameters, minimum and maximum percent metal weights were found in the same host species; see anterior hooks in specimens nos. 2, 3, 4 from host no. 1 (Table 3).

Sphaerirostris picae adults and cystacanths

The percent weights of P, S, and Ca in anterior and middle hooks of 5 adults of *S. picae* collected from 1 species of birds, *P. pica*, and in 1 geographical region, and those of 3 cystacanths from one other geographical region were analyzed (Table 5). The percent weights of P and S in the anterior hooks of adults (8.04% & 7.38%) and in the middle hooks (7.86% & 7.18%) were almost identical but the Ca levels were more than twice as high in both hooks (19.49% & 17.88%), in the same order (Table 5). The level of the 3 metals followed the same pattern in the sand lizard (host no. II) but at a lower level especially in the anterior hooks. In the European hedgehog (host no. III), a mammal, the levels of P and Ca were diminished

Table 2. EDXA of elements in anterior hook of *Acanthocephalus ranae*.

eZAF Smart Quant Results								
Creation Date	1-14-2022							
Project	Ukraine							
Specimen	*A_ranae*_1_1_12_10_2012_Anterior							
Area								
Sampling Region		Cherson region, Askania-Nova City						
kV	15							
Live Time	100							
Amp Time	7.68							
Takeoff Angle	35.12							
Resolution	127.1664							
Element	Weight %	Atomic %	Error %	Net Int.	K Ratio	Z	A	F
C K	16.78	26.13	99.99	187.11	0.0609	1.1165	0.3251	1
N K	20.88	27.87	11.1	155.47	0.0381	1.0892	0.1673	1
O K	26.22	30.64	10.48	311.78	0.0502	1.0653	0.1797	1
Na K	3.7	3.01	9.52	102.11	0.017	0.9643	0.4747	1.0005
Mg K	0.54	0.42	20.86	22.95	0.0032	0.9799	0.6065	1.001
Al K	0.19	0.13	59.55	9.17	0.0013	0.9429	0.7316	1.0019
P K	4.54	2.74	6.58	195.31	0.0378	0.9241	0.8962	1.0047
Au M	6.31	0.6	9.98	116.57	0.0519	0.6061	1.2903	1.0526
S K	4.33	2.53	5.24	177.55	0.0364	0.9415	0.8912	1.0001
Pd L	6.06	1.07	9.79	103.37	0.0441	0.6998	1.037	1.0016
K K	0.14	0.07	61.19	3.99	0.0012	0.8893	0.9389	1.002
Ca K	10.3	4.81	6.54	244.07	0.0885	0.9048	0.9507	0.9983

almost to negligible levels. This collection of *S. picae* provided a unique opportunity to compare the percent weight variations of metals between adults from the intestines of birds and cystacanths from the body cavity of lizards and hedgehogs; 2 other biological host systems belonging to 2 distinct classes of vertebrates, reptiles and mammals. Anterior and middle hooks of the cystacanths from the body cavity of the sand lizard, a reptile, had a lower but closer level of metals to adults from magpies (Table 5).

Moniliformis kalahariensis cystacanths

Hooks of the cystacanths were undeveloped and lacked the lateral grooves (Fig. 10) characteristic of the mature adults. The percent weights of P, S, and Ca in anterior, middle and posterior hooks of 5 cystacnths of *M. kalahariensis* collected from the German cockroach in 1 geographical region in Bombay, collected before 1931, were comparable within each element with higher means for Ca in all hooks (1.08-1.41) and considerably lower and similar means for S and P (0.35-0.56) (Table 6). This collection of *M. kalahariensis* provided an opportunity to compare the percent weight variations of metals between cystacanths from the body cavity of cockroaches and adults in the gut of 2 other biological host systems belonging to 2 distinct classes of vertebrates, mammals and birds, that had considerably higher levels of metals.

Moniliformis kalahariensis juveniles and adults from Limpopo, South Africa

Hooks of the juveniles are more developed than those of the cystacanths and some begin to show lateral grooves (Fig. 11) normally seen in the adults (Fig. 12, arrow). The young unsegmented juveniles and the older segmented adults collected from the intestines of

Table 3. Composition of metals in anterior and middle proboscis hooks of 12 *Acanthocephalus ranae* adults collected from 4 species of amphibian hosts (I-IV, Table 1) in various geographical regions of Ukraine between 2011 and 2018.

Host no.	Specimen	Anterior hooks			Middle hooks		
		Phosphorous	Sulfur	Calcium	Phosphorous	Sulfur	Calcium
I	1	4.54%	4.33%	10.30%	5.20%	6.52%	11.67%
	2	5.01%	5.23%	9.03%	7.14%	7.46%	13.12%
	3	(3.34%)*	7.91%	(6.48%)	?**	?	?
	4	(8.82%)	5.99%	17.49%	(9.01%)	7.89%	(17.92%)
II	1	8.01%	3.36%	(21.20%)	6.56%	5.31%	16.75%
III***	1	6.85%	(3.33%)	12.61%	7.75%	(3.82%)	14.41%
	2	5.00%	5.00%	9.02%	5.20%	5.64%	9.44%
	4	5.15%	4.53%	10.44%	5.83%	3.96%	11.49%
IV	1	4.23%	6.79%	8.52%	5.09%	(10.46%)	12.39%
	2	4.05%	5.16%	8.61	5.38%	4.75%	11.69%
	3	?**	?	?	6.85%	4.45%	15.31%
	4	6.77%	(8.46%)	15.95%	(3.59%)	10.95%	(8.72%)
Mean	(n=11)	5.67%	5.46%	11.79%	6.13%	6.47%	12.99%
Ratio of means		1	1	2	1	1	2

*Lowest and highest percent weight values are in parentheses.

**Specimens missing posterior or anterior hooks.

***Specimen III-3 near Kyiv missing proboscis.

the same 2 individuals of hedgehogs in Limpopo Province had comparable profiles of Ca, S, and P in anterior, middle and posterior hooks with the anterior and middle hooks having similar highest levels of Ca and P and the posterior hooks with the highest level of S. This pattern is comparable to that of the cystacanths that, however, have much lower levels of Ca and P and slightly lower levels of S (Table 6). In other words, the level of S remains stable from the cystacanth to the adult stage but the levels of P and Ca increase sharply. Throughout this progression, the intraspecific variability in elemental levels for each developmental stage appears rather small especially considering the fact that EDXA values are qualitative in nature.

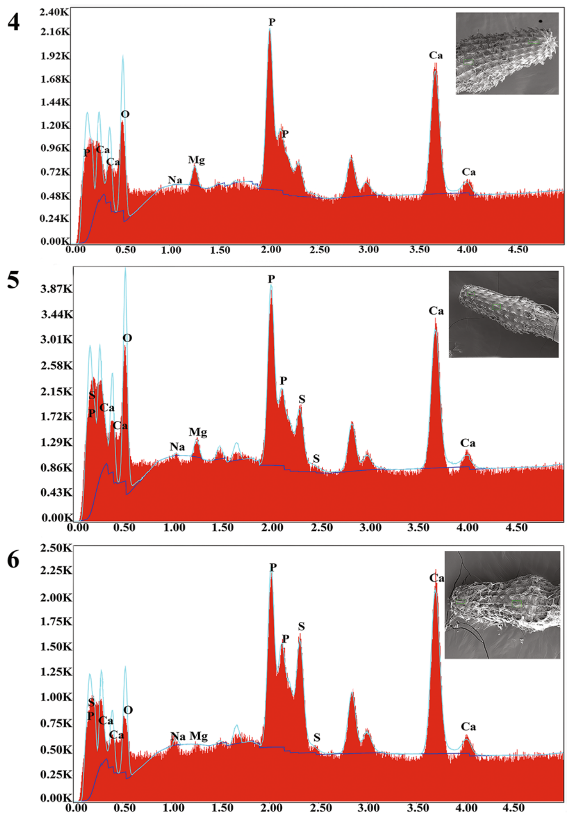
Moniliformis kalahariensis adults from Botswana

Hooks of the Meyer adults were similar to those of the adults from South Africa except that many had breaks and disruptions (Fig. 13). These adults were collected from the groundhog *A. frontalis* (a mammal) and the Namaqua sandgrouse, *P. Namaqua* (a bird) collected over 90 years ago. Meyer (1931) did not indicate which of the 2 definitive hosts he collected most of his specimens from. The level of P in anterior, middle and posterior hooks (7.40%, 5.86%, 1.30%, respectively) was similar to those in the juveniles and adults from South Africa (Table 6). The levels of Ca and S were, however, somewhat higher.

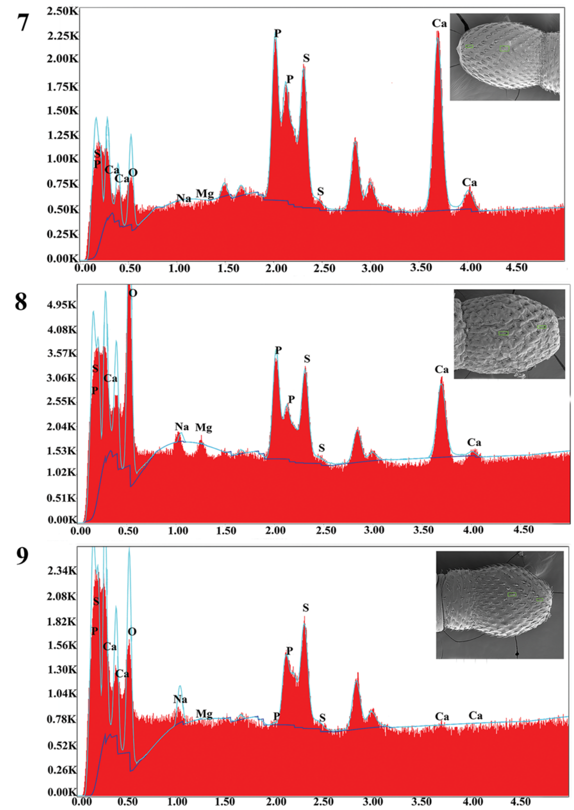
Discussion

The concepts of Energy Dispersive X-ray Analysis (EDXA)

Energy-dispersive X-ray analysis is a qualitative (not a quantitative) measurement of the chemical ions in a living organism that describes the levels but not the specific quantities of chemicals present based on 100% (100% of weight and 100% of ions). The x-ray scans of the gallium cuts help explain the finer morphological nature of studied structures and identify species unique “personality.” The uniqueness of the metal analysis, expressed by x-ray scans, appears to be species-specific and can be regarded as a fingerprint of key diagnostic value that is just as



Figs. 4-6 X-ray panels of elemental scans of anterior hooks of *Southwellina hispida* adults showing qualitative EDXA spectra. See Table 4 for % weight of depicted elements. Note the higher cumulative levels of Ca and phosphorous compared to S in all cases. Insets include corresponding proboscides with sampled anterior and middle hooks marked in green boxes; **4,** Scan of whole anterior hook of specimen no. 1 from host no. I; **5,** Scan of whole anterior hook of specimen no. 1 of host no. II; **6,** Scan of whole anterior hook of specimen no. 1 of host no. III (Table 4).



Figs. 7-9 X-ray panels of elemental scans of anterior hooks of *Sphaerirostris picae* adults and cystacanths showing qualitative EDXA spectra. See Table 5 for % weight of depicted elements. Insets include corresponding proboscides with sampled anterior and middle hooks marked in green boxes. **7,** Scan of whole anterior hook of adult specimen no. 1 from host no. I; **8,** Scan of whole anterior hook of first cystacanth from host no. II; **9,** Scan of whole anterior hook of the cystacanth of host no. III (Table 5). Note the exceptionally high peak of sulfur.

important as molecular analysis (Amin et al. 2019a, b, c, Heckmann et al. 2012a, b). Studies by other observers of other biological systems, ex. Radwan et al. (2012) studies of tegumental spines of four species of digeneans demonstrate species specific differential distribution of Ca, S, and P supporting our conclusions. In our research, *Moniliformis cryptosaudi* Amin, Heckmann, Sharifdini, Albayati 2019 from Iraq is morphologically identical to *Moniliformis saudi* Amin, Heckmann, Mohammed, Evans, 2016 from Saudi Arabia, and it was erected based primarily on its distinctly different EDXA pattern (Amin et al. 2019d) as a cryptic species. Similarly, 2 closely related but molecularly distinct species of *Pallisentis* Van Cleave, 1928, *Pallisentis nandai* Sarkar, 1953 and *Pallisentis paranandai* Amin, Chaudhary, Heckmann, Rubtsova, Singh, 2021were also distinguished by very different patterns of EDXA (Amin et al. 2021, table 3, 4, page 14). The EDXA provides an understanding of the chemical composition of an organism that lies between its external morphological and anatomical topography and its internal molecular profile. Different chemical components are indicators of biophysical qualities of certain organs, for instance, hardness of certain attachment structures. Calcium normally indicates an extreme hardness of the structure, while sulfur and phosphorus indicate a flexibility and toughness (Heckmann et al. 2012a, b; Rubtsova and Heckmann, 2020). EDXA provides a good comparison of the chemical composition of hard structures (hooks) and

Table 4. Composition of metals in anterior and middle hooks of 8 *Southwellina hispida* adults collected from 3 species of seabirds (hosts I-III, Table 1) collected from the Cherson Region, Ukraine between 2011 and 2017.

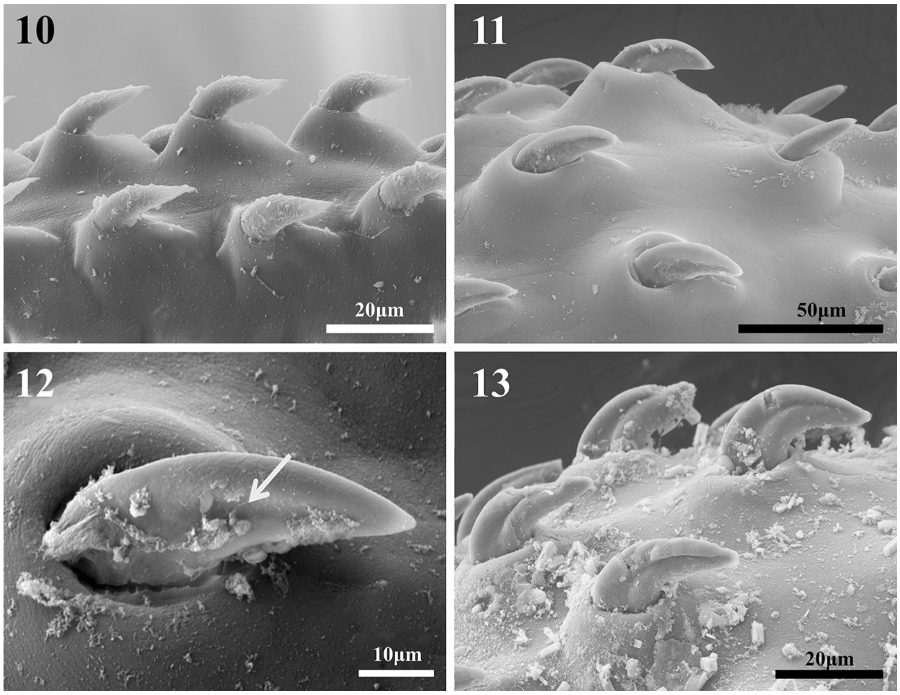
Host no.	Specimen	Anterior hooks			Middle hooks		
		Phosphorous	Sulfur	Calcium	Phosphorous	Sulfur	Calcium
I	1	9.07%	3.73%	15.84%	(12.02%)*	(3.04%)	(22.73%)
	2	(6.27%)	(9.37%)	(14.32%)	(5.75%)	9.59%	(9.12%)
	3	10.46%	(2.29%)	19.24%	9.42%	5.48%	15.71%
	4	(12.73%)	3.09%	(25.50%)	9.13%	8.95%	17.15%
II	1	9.37%	4.40%	16.83%	8.68%	3.30%	15.69%
	2	10.60%	4.10%	19.72%	8.21%	5.49%	14.82%
	3	8.44%	7.57%	19.81%	8.16%	7.20%	15.11%
III	1	9.71%	6.96%	21.93%	7.70%	(13.70%)	17.97%
Mean	(n=8)	9.58%	5.19%	19.15%	8.63%	7.09%	16.04%
Ratio of means		2	1	4	ca 1	ca 1	ca 2

*Lowest and highest percent weight values are in parentheses.

has been valuable for characterizing and diagnosing acanthocephalan species. Our findings are not to be confused with water pollution studies attempting to demonstrate the accumulation of other metals, usually heavy metals, in soft tissues of invertebrates and acanthocephalans and other helminths infecting fishes inhabiting polluted rivers and streams as repeatedly shown by the Sures' team eco-toxicological studies; see for example Zimmermann et al. (1999) and Sures (2003). Our studies demonstrate considerable stability in metal levels in hooks of acanthocephalan species collected from different bio-ecological environments.

Our EDXA-based studies

We have studied many species of acanthocephalans using X-ray scans (EDXA) of FIB-sectioned hooks and spines for metal composition (Heckmann 2006, Heckmann et al. 2007, 2012a, b, Standing and Heckmann 2014). The biological significance of EDXA as a diagnostic tool is exemplified by the observation that populations of an acanthocephalan species will consistently have similar EDXA spectra irrespective of host species or geography, even though comparative morphometrics of different populations of the same species usually vary with host species and geography; see for example Amin and Redlin (1980) and Amin and Dailey (1998). Results of the X-ray analysis of the FIB-sectioned hooks (dual beam SEM) show differential composition and distribution of metals in different hook parts characteristic of the species being examined. Metal analysis of hooks has become the diagnostic standard since hooks have the highest level of elements compared to the mid- and posterior-trunk regions of the acanthocephalan body (Heckmann et al. 2012a). X-ray scans analyses provide insight into the hardened components, e.g., calcium (Ca), sulfur (S), and phosphorus (P), of acanthocephalan hooks. Large hooks play a major role for host tissue attachment. The EDXA spectra and % weight of metals in individuals of many species of acanthocephalans have been documented from many geographical regions of the world by Amin et al. (2017, 2018, 2019b, 2020a, b, c, among others). Sulfur was usually seen at outer edge of large hooks and calcium and phosphorus are major ions in the base and middle of hooks where tension and strength are paramount for hook function. Sulfur levels appear to increase in more posterior hooks especially in hook tips and edges (Amin et al., 2018). Amin et al. (2022a) discussed in detail the biological significance of EDXA as a diagnostic tool exemplified by the observation that populations of an acanthocephalan species will consistently have similar EDXA spectra irrespective of host species or geography. For instance, consider the case of *M. cryptosaudi* discussed above (Amin et al. 2019d). The taxonomic identity of species is deep-seated at the genetic level, which is expressed by the organism's morphology and biochemistry as revealed, in part, by its elemental spectra (Amin et al.,



Figs. 10-13 The middle hooks of the developmental stages of *Moniliformis kalahariensis*. **10,** Underdeveloped hooks of Cystacanths from the body cavity of *Blattella* (*Phyllodromia*) *germanica* in Bombay, India; **11,** Developing hooks of juveniles from the intestine of *Atelerix frontalis* collected in Limpopo Province, South Africa; **12,** Higher magnification of a fully developed hook (arrow points to lateral groove) of an adult from the intestine of *Atelerix frontalis* collected in Limpopo Province, South Africa**; 13,** Developed and somewhat damaged hooks of an adult from the intestine of *Atelerix frontalis* or *Pterocles namaqua* collected in Botswana and reported by Meyer (1931).

2022a). Extremes of metal levels were noted in individual specimens of various species. For instance, *Cavisoma magnum* (Southwell, 1927) Van Cleave, 1931 from *Mugil cephalus* in the Arabian Sea, had sulfur levels in hook tips (43.51 wt. %) and edges (27.46 wt. %) (Amin et al. 2018). This element (Sulfur) is part of the prominent outer layer of most acanthocephalan hooks and is a major contributor of the hardening process of this attachment structure. Our results are comparable to those of mammalian teeth enamel. The center and base of hooks of the same worms had negligible sulfur levels and contained mostly phosphorus and calcium, the two other essential elements for hook structure (Amin et al. 2018).

The above quoted research has invariably dealt with research on various acanthocephalan species using usually 1, or occasionally 2, individual worms representing each species. This data was diagnostically useful in differentiating species apart using the same hook and hook part in various species for comparisons. However, 1 or 2 individuals do not appear to account for intraspecific variability of individuals within the same population or species of acanthocephalan. We have, thus, designed a comparative study of many individuals of specific species of acanthocephalans collected from the same and closely related genera of hosts obtained from various geographical areas to also account for the possible impact of locality on EDXA patterns. Anterior and middle hooks of 29 specimens of 3 species of acanthocephalans from 10 species of hosts (Figs. 1-9; tables 3-5) in various geographical locations of Ukraine were evaluated for chemical ions with S, Ca and P being the prominent elements.

In *A. ranae*, 13 specimens from 4 species and 3 related genera of frogs (Table 1) showed individual intraspecific variations in percent weight values of P, S, and Ca. The range of variations was expected. This variability would not have been detected if only 1 or 2 specimens were analyzed using EDXA. In the anterior hooks, the mean values of the percent weights for P (5.67%) and S (5.46%) were similar and half that for Ca (11.79%), with a ratio of 1:1:2 (Table 3). The

Table 5. Composition of metals in anterior and middle hooks of 5 *Sphaerirostris picae* adults collected from Eurasian magpie (host I, Table 1) in the Cherson Region, 2 cystacanths collected from sand lizard (host II, Table 1), and 1 cystacanth from the European hedgehog (host III, Table 1) collected in the Odessa Region, Ukraine between 1984 and 2010.

Host no.	Specimen	Anterior hooks			Middle hooks		
		Phosphorous	Sulfur	Calcium	Phosphorous	Sulfur	Calcium
I	1	8.50%	8.18%	(21.91%)*	(9.19%)	(5.15%)	(21.06%)
	2	8.18%	(8.41%)	19.69%	7.29%	6.70%	16.09%
	3	(9.20%)	7.06%	22.93%	7.28%	(9.07%)	17.63%
	4	(5.70%)	(5.69)	(13.01%)	(7.10%)	8.49%	(15.15%)
	5	8.64%	5.85%	19.83%	8.45%	6.48%	19.47%
Mean	(n=5)	8.04%	7.38%	19.49%	7.86%	7.18%	17.88%
Ratio of means		1	1	2.5	1	1	2.5
II	Cystacanth	5.79%	5.44%	9.61%	6.75%	6.51%	11.04%
	Cystacanth	6.03%	4.41%	10.83%	6.77%	4.96%	11.35%
Mean	(n=2)	5.91%	4.92%	10.22%	6.76%	5.74%	11.19%
Ratio of means		1	1	2	1	1	2
III	Cystacanth	0.13%	6.84%	0.29%	0.30%	9.65%	0.56%
Ratio		1	53	2	1	32	2

[*] Lowest and highest percent weight values are in parentheses.

pattern in middle hooks was comparable. These values are apparently population patterns not affected by host species or geographical location as maximum and minimum values of one or more metals are found in in acanthocephalans collected from the same host species and in the same geographical region. For example, in anterior hooks, the percent P in specimens nos. 3 and 4 from *P. ridibundus* in the Vinnickiy region were 3.34% (minimum) and 8.82% (maximum) (Table 3). Other examples of extremes of weight values of this and other metals are found in other specimens from the same host species and the same collection site throughout table 3.

In *S. hispida*, 8 specimens from 2 species and 2 related genera of birds (Table 1) in the same locality showed individual intraspecific variations in percent weight values of P, S, and Ca. The range of variations was also normal and expected. This variability would not have been detected if no more than 1 or 2 specimens were analyzed using EDXA. For the anterior hooks, the ratio of mean percent weight of P, S, and Ca. was 2:1:4 (Table 4) which appears to be characteristic of *S. hispida*. The ratio for middle hooks was somewhat comparable. The slight differences between anterior and middle hook values are normal as have been noted in other species of acanthocephalans depending on the position of hooks along the longitudinal gradient of the proboscis. These values are apparently population patterns not affected by host species or geographical location as maximum and minimum values of all 3 metals are found in acanthocephalans collected from the same host species and in the same geographical region. For instance, the minimum and maximum percent weight of all metals (P, S, Ca) were found in acanthocephalan specimens nos. 2, 3, 4 that were collected from the same species of host, *A. cinerea*, from the same locality, Cherson region, near Gopry City (Table 1).

In cystacanths of *S. hispida* from the body cavity of the paratenic host *Gillichthys mirabilis* Cooper (Gobiidae) in California, Amin et al. (2022b, Table 3) found whole anterior and middle hooks with high levels of S of 4.41% and 7.83% while the levels of P and Ca were 0.18%, 0.06%, and 0.19%, 0.22%, in the same order. The level of S was similar to the mean value of 5.19% (2.29-9.37%) of S in anterior hooks in our Ukrainian study adults (Table 4) which is apparently related to the genetically based protein synthesis of *S. hispida* as has been noted for *S. picae* below. The scarce level of P and Ca is comparable to that in *S. picae* cystacanths from hedgehogs in our Ukrainian study (Table 5). The biochemical environment of the

Table 6. Composition of metals (weight %) in anterior, middle and posterior proboscis hooks of adults and cystacanths of *Moniliformis kalahariensis* collected from various hosts in Africa and India.

Specimen no.	Phosphorous %			Sulfur %			Calcium %		
	Ant. hook	Mid. hook	Post. hook	Ant. hook	Mid. hook	Post. hook	Ant. hook	Mid- hook	Post. hook
Cystacanths from *Blatella germanica* in India from Meyer's (1931) material									
1	—	—	—	0.41	0.42	0.48	1.88	1.01	0.98
2	0.39	0.61	0.38	0.42	0.42	0.47	1.88	1.22	1.05
3	0.44	0.55	0.36	0.30	0.47	0.35	1.07	1.02	1.14
4	o.52	0.58	0.35	0.37	0.48	0.62	1.65	0.98	1.09
5	o.31	0.49	0.42	0.26	0.32	0.55	1.43	1.75	1.14
Mean	0.41	0.56	0.38	0.35	0.42	0.49	1.41	1.20	1.08
Juveniles from *Atelerix frontalis* in Limpopo, South Africa collected in 2012									
1	10.18	8.45	1.61	0.25	0.37	0.77	18.50	15.76	3.27
2	7.58	5.82	1.41	0.44	0.56	0.94	12.65	9.99	3.11
3	6.82	7.36	1.26	0.25	0.56	0.92	11.29	13.54	2.62
4	4.41	6.74	1.34	0.42	0.60	0.83	7.67	11.82	2.59
Mean	7.25	7.09	1.40	0.34	0.52	0.86	12.53	12.78	2.90
Adults from *Atelerix frontalis* in Limpopo, South Africa collected in 2012									
1	8.28	7.88	1.09	0.47	0.47	0.81	13.33	13.24	1.56
2	4.26	6.14	1.64	0.54	0.47	0.87	6.42	10.58	2.47
3	5.79	4.68	1.07	0.54	0.54	0.61	8.71	7.51	1.55
4	7.07	6.05	0.89	0.42	0.30	0.60	10.67	10.01	1.63
Mean	6.35	6.19	1.17	0.49	0.44	0.72	9.78	10.33	1.80
Adults from *Atelerix frontalis* & *Pterocles namaqua* in Botswana from Meyer's (1931) material									
1	7.80	6.25	1.06	1.16	0.97	1.06	14.05	11.37	2.47
2	7.18	4.38	1.47	1.34	1.02	1.57	16.18	12.41	3.93
3	6.80	6.84	1.01	1.17	0.89	1.50	12.54	12.66	3.41
4	7.84	5.99	1.68	1.03	1.00	1.59	14.06	13.18	5.06
Mean	7.40	5.86	1.30	1.17	0.97	1.43	14.21	12.40	3.72

body cavity of the paratenic host has clearly impacted the scarce levels of P and Ca.

In *S. picae*, 5 adults were examined from *P. pica* in the same locality at Cherson region, Askania-Nova City (Table 1) showing individual intraspecific variations in percent weight values of P, S, and Ca as have been demonstrated for *A. ranae* and *S. hispida*. The range of variations was narrow for P and S and slightly wider for Ca in both hooks. The ratio of mean percent weight was 1:1:2.5 in both anterior and middle hooks. The cystacanths of *S. picae* present a different pattern. Those from the body cavity of the sand lizard, a reptile, present with lower percent weight values than the adults and their ratio of means (1:1:2) was somewhat different from that of the adults (1:1:2.5). The one cystacanth from the hedgehog, a mammal, presented with drastically lower levels of P and Ca in both hooks but the level of S was high, comparable to that in the adults with the ratio of P:S:Ca of 1:53:2 and 1:32:2 in anterior and middle hooks, respectively. Clearly, the values of P and Ca were affected by the biochemical environment of the body cavity of a mammal while the value of S remained stable. The data from *S. picae* provides a unique opportunity to establish the principal that, for diagnostic purposes, comparisons among acanthocephalan species should be confined to adults collected from the intestine of definitive hosts in the same class of vertebrates, or to cystacanths collected from the body cavity of paratenic hosts of the same class. The importance of the stability of the levels of S in various developmental stages of acanthocephalans across the host species barrier is exemplified by its relationship to the genetically based protein synthesis, explained below, which gives credibility to the usage of EDXA for diagnostic purposes.

Moniliformis kalahariensis

The above arguments for the cystacanths of *S. hispida* and for *S. picae* apply perfectly to our data on *M. kalahariensis* (Table 6) showing greatly reduced levels of P and Ca but similar levels of S compared to juveniles and adults. The similar patterns in juveniles and adults from the same host species in South Africa, *A. frontalis*, suggest that maturity and sexual development do not affect the metal composition of hooks in acanthocephalans and therefore, are adequate for diagnostic comparisons. The similarities in level of P in anterior, middle and posterior hooks of the over 90 years-old Meyer (1931) Botswana material to the levels in the juveniles and adults from South Africa collected in 2012 (Table 6) suggests that age of specimens does not alter the chemistry of hooks. The levels of Ca and S in the Meyer specimens were, however, higher. The hooks in these specimens were partially broken (Fig. 13) and we do not know the history of fixation or preservation of the Meyer adult specimens that may have affected the Ca and S readings. The readings of the adults from South Africa would be more representative of *M. kalahariensis*.

Supporting studies

Erman and Korkut (2011) measured concentrations of 49 different inorganic elements by EDXRF (Energy Dispersive x-ray Fluorescence spectrometry) "in two species of *Agabus* (*A. nebulosus*, *A. conspersus*) … (Dytiscidae), collected from the same locality (Adana Province, Turkey). Mn concentration is shown to be significantly different between the two species." Because the two species were collected in the same locality, Erman and Korkut (2011) concluded that "it is unlikely that these differences are due to physiochemical parameters in their habitats (but) instead …. elemental differences may be driven by genetic and biochemical characteristics between the species." Specifically, the sulfur content in the proboscis hooks is paramount in the composition of disulfide bonds in the thiol groups for cysteine and cystine of the polymerized protein molecules (Stegman 2005). Protein synthesis occurs in two stages, transcription and translation by transferring of genetic instructions in the nuclear DNA to mRNA in the ribosomes followed by post-translational events such as protein folding and proteolysis (Stegman 2005). The formed disulfide bonds are direct by-products of the DNA-based process of protein synthesis which makes up the identity of a biological species (Amin et al., 2022a). Accordingly, the level of sulfur in our EDXA profiles will indicate the number of sulfur bonds, that along with the levels of calcium phosphates, will characterize the identity of a species based on its nuclear DNA personality. Differences in chemical compositions probably indicate differences in allele expression (Amin et al. 2022a). The DNA generated sulphide bonds, evident in our EDXA profiles, have an important role in the stability and rigid nature of the protein accounting for the high sulfur content of the proboscis (Heckmann et al. 2012a). The above processes explain the observed species-specific nature of EDXA profiles noted in our many findings.

Conclusions

This study (1) establishes the concept of variability in the percent weight values of metals in adult acanthocephalans and cystacanths, and documents the range of variations for each of P, S, and Ca in hooks of *A. ranae*, *S. hispida*, *S. picae*, and *M. kalahariensis* studied as models for other species of acanthocephalans. We also (2) document that percent weight values are stable for adults of each acanthocephalan species irrespective of host species and geography. Additionally, we (3) document the stability of S and the differences in Ca and P levels between the EDXA profiles in cystacanths retrieved from the body cavity of the intermediate host and those of the adults from the totally different biochemical environment of the definitive hosts' intestinal environment. We emphasize (4) the importance of performing EDXA for a number of individuals of the same species to obtain a mean value representative of the species in question. (5) The pattern for each species should be compared with others collected from hosts in the same class of vertebrates and from the same organ system. We also (6) document the biological and genetic significance of these findings to confirm the diagnostic value of EDXA in taxonomic literature and that it is species specific. We consider that, under such conditions, the diagnostic significance of the EDXA findings is of comparable value to that of molecular analyses which also considers the genetic divergence among used isolates. Both methodologies emerge from the same genetic foundation. (7) The stability of the levels of S as byproducts of the DNA-based protein synthesis are particularly important in recognizing the specific identity of species. (8) Percent weight values of metals from cystacanths are not comparable to those of adults from a different class of hosts.

EDXA is in its incipient stages of development as an accepted science and much more data utilizing larger sample sizes are needed to build on the foundation that we have laid down. To date, only our teams in Scottsdale Arizona and Provo Utah have pioneered EDXA research in acanthocephalan taxonomy. We are the first to publish research on parasites using EDXA and the Gallium beam. It is commonly used for materials' research. We have just planted a seed.

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Author contributions Amin analyzed the data and wrote the manuscript. Lisitsyna provided the Ukrainian specimens. Heckmann provided the EDXA data for *Moniliformis kalahariensis*.

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Data availability All presented and related data are available by contacting the senior author.

Declarations

Conflict of interest The authors declare no conflicts of interest or competing interests.

Ethical approval The authors declare that they have observed all applicable ethical standards.

Consent to participate All authors consented to participate with noted specimens and analyses.

Consent for publication Authors consented to publish after having pre-reviewed rough drafts.

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