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ORIGINAL RESEARCH ARTICLE

Comparative chemical element analysis using Energy Dispersive X-ray Microanalysis (EDXA) for four species of Acanthocephala

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Abstract. Four species of Acanthocephala: *Rhadinorhynchus ornatus, Acanthocephalus dirus, Neoechinorhynchus idahoensis* and a species of *Sphaerirostiris sp.* from Pakistan were evaluated with X-ray microanalysis (EDX, EDXA) for chemical elements. Acanthocephala were collected from fish inhabiting marine and fresh water habitats while the specimen from Pakistan came from a Myna bird. For the first and second species, both males and females were examined, while only females were available for the third species. An additional specimen from a Myna bird was evaluated for comparison. EDXA scans were completed in triplicate on 3 individual specimens. For each species, three regions of the proboscis (tip, middle, and base), mid body and posterior body, the weight-percent and peak height were registered. Data were statistically analyzed using a SAS computer program. Elements characteristic of living cells (C, H, N, O) were recorded as well as sulphur (S), calcium (Ca) and phosphorus (P). The later three were stressed in this study. The proboscis has the highest concentration of S, Ca, and P followed by the posterior body and the lowest concentration was detected in the mid body. Female Acanthocephala have a statistically higher concentration of the examined elements in the body regions than males. The level of S is high at the proboscis base and gradually decreased towards the tip where both Ca and P, having the same distribution pattern, reach their highest level. Sulfur ions are probably polymerized as complex polypeptides using disulphide bonds represented by the thiol groups of the amino acids cystine and cysteine. Chemical element data for each species are listed with statistical analysis. Calcium and phosphorus were statistically higher (P.01) for acanthocephalon hooks of the avian host versus the piscine host. The proboscis hooks for the marine piscine host have higher levels of the three chemical elements in comparison to the freshwater fish hosts.

Keywords: EDXA; Acanthocephala; Calcium; Phosphorus; Sulphur; Comparisons; Energy Dispersive Analysis for X-ray.

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Introduction

A major advantage of EDXA (Energy Dispersive Analysis for X-Ray), used in conjunction with electron optics is that the data can be obtained while observing the specimen with an electron microscope. It is a non-destructive technique whereby the sample can be analyzed several times and at different locations and magnifications (Sures, 2003; Whallon et al., 1989). The use of micro-analysis of chemical elements based on the spectrum properties of the x-ray beam has been utilized extensively during the last two decades in conjunction with scanning electron microscopes (SEM) (Heckmann et al., 2007). It has been applied to numerous species of animals to further understand the nature of protoplasm and animal organs (Smith and Richards, 1991).

There have been multiple uses of x-ray microanalysis in relation to living organisms. Animal diseases such as fish die-offs can be further understood by using this technique (Heckmann, 1997). Evidence for pathological problems such as prostate cancer have employed EDXA (Ericksen, 1997), while Heckmann in 1997 used the technique for quality control of products.

EDXA has been used in conjunction with electron optics for many years (Whallon et al., 1989; Lee, 1992; Heinrich, 1981). The method is based on x-ray emissions characteristic of a chemical element from the sample which are sorted by energy rather than wavelength using a diffraction crystal. High energy electrons from the SEM cause the excitation of x-rays whose energies and relative abundance depend upon the composition of the sample (Cazaux, 1984; Johnson, 1993; Vaughan, 1989). This technique, EDXA would be an excellent method for detecting relatively high concentrations (>1% wt) of heavy metals in small amounts of living tissue (Sures, 2003).

The goals of this project were to determine differences in chemical element composition for the following a) between species of Acanthocephala, b) between male and female specimens of the same species, and c) between areas of the body from proboscis to the posterior region. Data will be statistically analyzed using an SAS program (ANOVA). The four species selected represented four different families of Acanthocephala and came from different fish hosts (table 1). The fish hosts represented both freshwater and marine habitats. The fourth specimen came from a Myna bird.

Table 1. Hosts and locations for the examined Acanthocephala

Host	Species and family of Acanthocephala	Host location
Skipjack tuna ***Katsuwonuspelamis***	*Rhadinorhynchusornatus* (Rhadinorhynchidae)	Pacific ocean off the west coast of South America
Bridgelip sucker ***Catostomuscolumbianus***	*Neoechinorhynchusidahoensis* (Neoechinorhynchidae)	Salmon River, Idaho, USA
• **Brook trout** ***Salvelinusfontinalis*** • **White sucker** ***Catostomuscommersoni***	*Acanthocephalusdirus* (Echinorhynchidae)	Various rivers, New Hampshire, USA
Myna Bird	*Sphaerirostris sp.* (Centrorhynchidae)	Pakistan

This study centered on the evaluation of three chemical elements: sulphur (S), calcium (Ca), and phosphorus (P) using EDXA due to the unique role of these elements for the attachment structure of the acanthocephalan (Heckmann et al., 2007). All elements detectable with x-ray microanalysis were recorded. EDXA methods can be used to detect most chemical elements on the periodic chart.

Detection of the concentration and pattern of distribution of chemical elements within the Phylum Acanthocephala from different hosts and localities is of great importance. The consistent finding of these elements in

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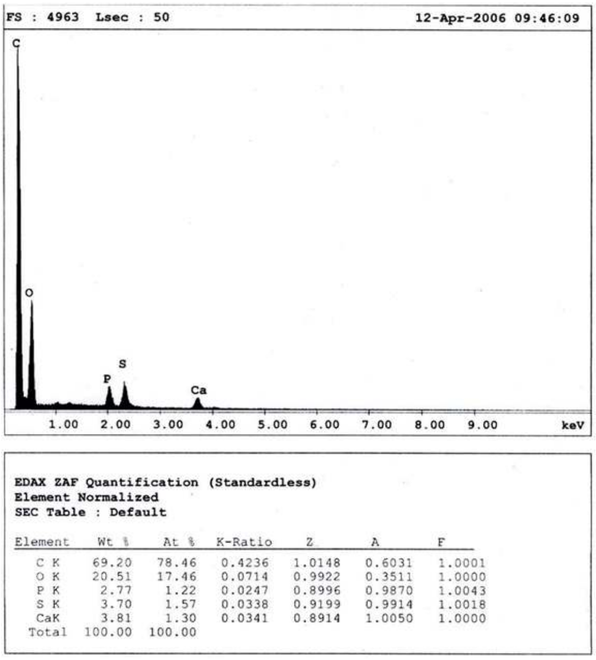
acanthocephalans and other profile organisms may help in understanding the vital role of these elements. Evolutionary ties and similarities in structure composition may also be explained based on the chemical element profiles.

Material and methods

Specimens of *Rhadinorhychus ornatus, Neoechinorhynchus idahoensis* and *Acanthocephalus dirus* were obtained from various hosts and locations (table 1). Each specimen, previously fixed in formalin, was dehydrated in an ascending series of ethanol solutions to 70% and then stored until needed. With the exception of *N. idahoensis* (females only), at least 3 males and 3 females of each species were processed. Standard methods were followed to prepare each specimen for SEM and X-ray microanalysis (Lee, 1992) which included critical point drying (CPD) after being processed from 70% ethanol (ETOH) and mounted on SEM sample mounts using conductive double sided carbon tape. Most of the samples were then gold coated for 3 minutes using a Polaron E5300 sputter coater (approximate gold coating thickness of 20nm). Samples were then placed in a FEI XL30 ESEM FEG (FEI, Hillsboro, Oregon) under low vacuum conditions. Samples were imaged at 10KV, spot size 3 at 0.7 torr using the GSE large field detector. X-ray spot analysis and line scan analysis were performed at 15KV spot size 5 utilizing the EDAX Genesis X-ray analyzer package. EDXA scans were completed at least triplicate for 5 major regions of each acanthocephalon with an average recorded. Spines unique to *R. ornatus* were analyzed and compared to the hooks of the proboscis for the same species. A single specimen of *Sphaerirostris* sp. from a bird (Myna) in Pakistan was prepared for comparison.

Weight percent (wt%) of each chemical element detected was recorded. In a previous paper (Heckmann et al., 2007) we recorded peak height for comparison. The computer will calculate weight percent using ZAF corrections and depict peak height (figure 1). Weight percent (wt%) for each species of Acanthocephala was analyzed for probability using statistical analysis (SAS) with P and M values recorded. The wt% data are normalized and are not absolute numbers which can be used for comparing data from the worms listed.



EDAX ZAF Quantification (Standardless)
Element Normalized
SEC Table : Default

Element	Wt %	At %	K-Ratio	Z	A	F
C K	69.20	78.46	0.4236	1.0148	0.6031	1.0001
O K	20.51	17.46	0.0714	0.9922	0.3511	1.0000
P K	2.77	1.22	0.0247	0.8996	0.9870	1.0043
S K	3.70	1.57	0.0338	0.9199	0.9914	1.0018
CaK	3.81	1.30	0.0341	0.8914	1.0050	1.0000
Total	100.00	100.00				

Figure 1. Typical printout for X-ray Microanalysis showing Chemical Elements at their KeV peak; Note the chart with wt % recorded. Specimen not coated with gold

Data were obtained for 3 areas on the proboscis (Tip, Middle, and Base) and 2 body regions (Middle and Posterior) for both males and females for *R. ornatus* and *A. dirus*. These data were analyzed with SAS for probability. For the other two acanthocephalon species only females were available.

This study emphasized variations in 3 chemical elements; sulfur, calcium, and phosphorus. Common elements consistent for protoplasm were recorded but not included in the analysis (figure 1). Not all samples were gold coated. For the statistical analysis, all comparisons of sulfur, calcium and phosphorus were made by an analysis of variance (ANOVA) using a computer program, SAS Proc Mixed (version 9.1). The variables of interest were gender, species, location, body regions, and hooks, along with their interactions which were considered with resultant p and m values. Reduced models eliminating insignificant interactions were used for the final comparisons.

Results

Results from the SEM, X-ray microanalysis can be represented by peak height or weight percent (figure 1) of each element in relation to other chemical elements. We used wt% for this study of the four different species of Acanthocephalawhereby a statistical analysis was possible. Tables 2, 3, and 4 summarize the results of the chemical element analysis.

Table 2. Weight Percent (wt%) of Sulphur (S), Calcium (Ca), Phosphorus (P) from three species of Acanthocephala

Proboscis (Female) Weight Percent

	A. dirus			*R. ornatus*			*N. idahoensis*		
Area	**S**	**Ca**	**P**	**S**	**Ca**	**P**	**S**	**Ca**	**P**
Tip	4.90	12.07	6.070	13.30	4.50	3.40	5.10	1.15	1.24
Middle	4.71	9.73	5.60	13.30	4.56	3.40	7.84	0.42	0.34
Base	**6.80**	**5.90**	**3.40**	**17.21**	**2.02**	**1.61**	**-**	**-**	**-**

Proboscis (Male) Weight Percent

	A. dirus			*R. ornatus*			*N. idahoensis*
Area	**S**	**Ca**	**P**	**S**	**Ca**	**P**	**No Sample**
Tip	1.54	12.08	6.20	8.80	5.00	2.97	
Middle	2.80	9.90	5.40	11.20	2.52	1.70	
Base	3.70	7.45	4.10	12.80	1.90	1.20	

Body Regions (Female) Weight Percent

	A. dirus			*R. ornatus*			*N. idahoensis*		
	S	**Ca**	**P**	**S**	**Ca**	**P**	**S**	**Ca**	**P**
Middle Body	0.94	0.56	0.31	1.20	0.23	0.36	1.11	0.22	0.29
Posterior Body	1.00	0.47	0.27	1.33	0.29	0.42	0.83	0.39	0.06

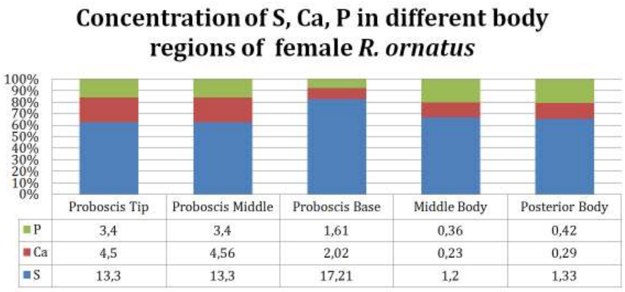
Body Regions (Male) Weight Percent

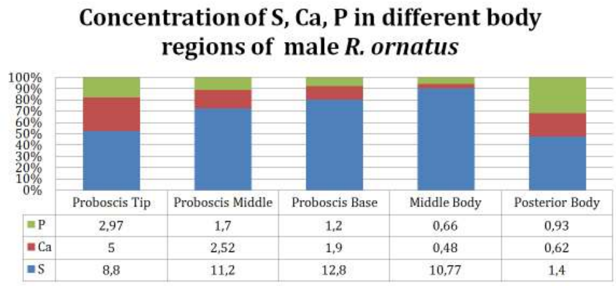
	A. dirus			*R. ornatus*			*N. idahoensis*
	S	**Ca**	**P**	**S**	**Ca**	**P**	**No Sample**
Middle Body	0.98	0.26	0.34	10.77	0.48	0.66	
Posterior Body	0.83	0.21	0.25	1.40	0.62	0.93	

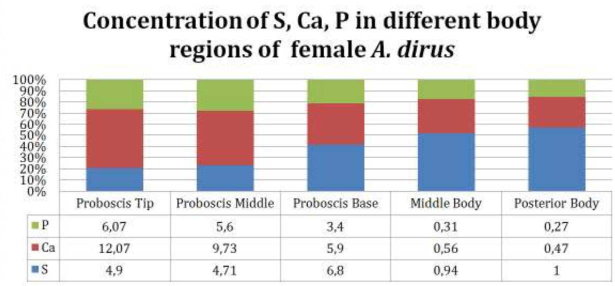
The concentration of sulphur (S), calcium (Ca), and phosphorus (P) for the four examined species of Acanthocephala reached their highest levels in the proboscis due to the presence of the hooks. The mid body showed the lowest concentration for these elements with a slight increase noted in the posterior body of the acanthocephalons. *A. dirus* and *R. ornatus* had higher levels of Ca and S in the middle and posterior body.

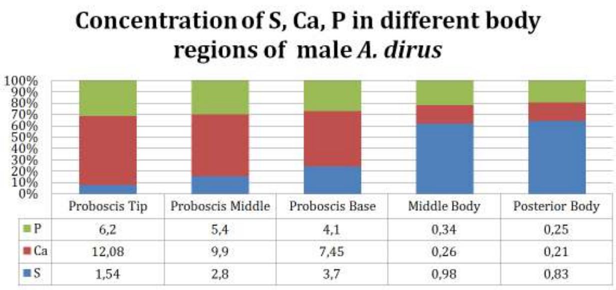
The concentration of sulphur in all examined species gradually increased from the tip of the proboscis towards the base, where it reached its highest level while calcium and phosphorus reversed this trend. The number and maturity of the hooks on the proboscis relates to these data.

The aquatic environment of the piscine host also showed a difference for the chemical elements. In general, body regions of *A. dirius* and *N. idahoensis* (fresh water fish) have lower weight percents of S, Ca, and P than *R. ornatus* (marine fish) (tables 2, 5). The species of a female Acanthocephala in the avian host *Sphaerirostris* has higher element values than those from fresh water fish. The female Acanthocephala had higher concentrations of S, Ca, and P in the different body regions than males for *R. ornatus* and *A. dirus* (table 5).









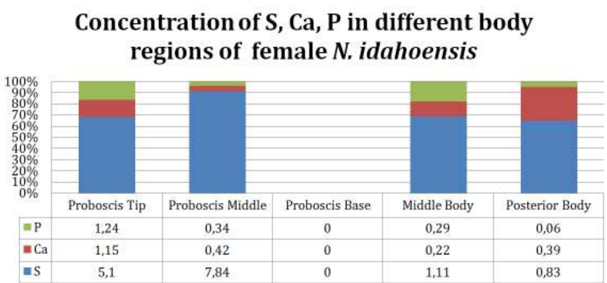


Figure 2. Weight percent (wt%) of Ca, P, and S from Table 2 plotted on graphs for comparison

Table 3. Weight Percent (wt%) of Sulphur (S), Calcium (Ca), Phosphorus (P) from *Sphaerirostris sp.* from a Myna bird from Pakistan

Proboscis (Female) Weight Percent

Area	S	Ca	P
Tip	3.29	13.80	9.10
Middle	3.95	11.50	7.30
Base	5.60	6.25	4.00

Body Regions (Female) Weight Percent

Area	S	Ca	P
Middle Body	1.00	0.40	0.33
Posterior Body	1.30	0.80	0.60

Table 4. Weight Percent (wt %) of Sulphur (S), Calcium (Ca.) and Phosphorous (P) from spines of *R. ornatus* as compared with the proboscis hooks of the same species

R. ornatus

		S	Ca	P
****Spines**	Tip	7.30	0.80	0.82
	Middle	6.20	0.90	0.83
	Base	5.38	1.30	1.40
***Hooks**	Tip	16.50	3.80	14.35
	Middle	20.80	2.45	1.80
	Base	14.90	5.80	3.60

*At posterior end of proboscis, Three levels for the hooks
** Spines located on the body of *R. ornatus*

Table 5. Habitat of Hosts: Body Regions (Females Only)

	Mid Body			Posterior Body		
	S	**Ca**	**P**	**S**	**Ca**	**P**
A. dirus*	0.94	0.56	0.31	1.00	0.47	0.27
N.idahoensis*	1.11	0.22	0.29	0.83	0.39	0.06
R. ornatus**	1.20	0.23	0.36	1.33	0.29	0.42
Sphaerirostris sp. **	1.00	0.40	0.33	1.30	0.80	0.60

Habitat of Hosts: Proboscis (Females Only)

	Tip			Middle			Base		
	S	**Ca**	**P**	**S**	**Ca**	**P**	**S**	**Ca**	**P**
A. dirus*	4.90	12.07	6.70	4.71	9.73	5.60	6.80	5.90	3.40
N.idahoensis*	5.10	1.15	1.24	7.84	0.42	0.34			
R. ornatus**	13.30	4.50	3.40	13.30	4.56	3.40	17.21	2.02	1.61
Sphaeroristris sp. **	3.29	13.80	9.10	3.95	11.50	7.30	5.60	6.25	4.00

*Freshwater fish
**Marine fish
***Terrestrial bird

Spines (figure 4) which are characteristic of both gender of *R. ornatus* had lower S, Ca and P levels than the hooks (figure 3) for the proboscis (table 4). There is a significant difference (P=0.05 and 0.001) between the concentration of the three elements in relation to the body regions and sex of all the examined species (see listed values).

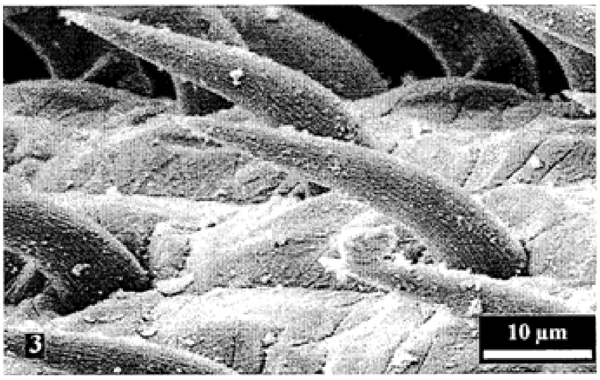


Figure 3. Proboscis hooks

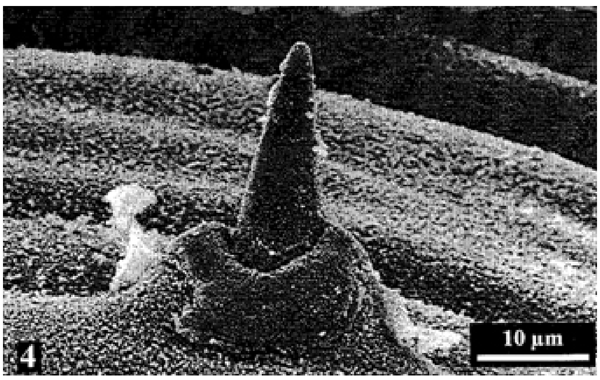


Figure 4. Spines for *R. ornatus*

Tables 6 and 7 represent the SAS analysis of the wt% data for the chemical elements; calcium (Ca), sulphur (S) and phosphorus (P) analyzed for three of the four species of Acanthocephala.

Discussion

Detection of the concentration and pattern of distribution for chemical elements within the phylum Acanthocephala from different hosts and localities is of great importance. The consistent finding of these elements in the acanthocephalons and other organisms may help in understanding the vital role of these elements in helminth parasites. Evolutionary ties and similarities in structure composition may also be explained based on the chemical element profile.

Results of the EDXA study on four species of Acanthocephala, *Rhadinorhynchus ornatus*, *Neoechinorhynchus idahoensis*, *Acanthocephalus dirus* and *Sphaerirostris* sp. resulted in statistically significant differences for 3 chemical elements (sulphur, calcium, and phosphorus) for gender, body regions and proboscis areas (table 7). The study represented fish hosts from marine and fresh water and a Myna bird from Pakistan. For gender, we were able to examine males and females of *R. ornatus* and *A. dirius*. Only females were available for the other 2 species. For *A. dirus*, the female had significantly higher levels of calcium in both the middle and posterior body regions. The sulphur and phosphorous levels were similar. For *R. ornatus* calcium was higher for the female in the middle body region. These calcium levels are probably due to the production of eggs in the female (Anantaram, 1976).

Table 6. Statistical analysis of EDXA data (cut %); ANOVA using a computer program, SAS Proc Mixed (version 9.1). Those with high probability are in bold.

Sulphur

Location of Scan	Gender	A vs. R *	A vs. N*	R vs. N*
Proboscis Tip	Female	**<0.0001**	1.0000	**<0.0001**
Proboscis Middle	Female	**<0.0001**	0.9816	**0.0206**
Proboscis Base	Female	**<0.0001**	1.0000	**<0.0001**
Proboscis Tip	Male	**<0.0001**	No Sample	No Sample
Proboscis Middle	Male	**<0.0001**	No Sample	No Sample
Proboscis Base	Male	**<0.0001**	No Sample	No Sample
Middle Body	Female	1.0000	1.0000	1.0000
Posterior Body	Female	1.0000	1.0000	1.0000
Middle Body	Male	1.0000	No Sample	No Sample
Posterior Body	Male	1.0000	No Sample	No Sample

*A = *Acanthocephalus dirus;* R = *Rhadinorhynchus ornatus;* N = *Neoechinorhynchus idahoensis.*

Phosphorus

Location of Scan	Gender	A vs. R *	A vs. N*	R vs. N*
Proboscis Tip	Female	**<0.001**	**<0.0001**	0.9989
Proboscis Middle	Female	**<0.001**	**<0.0001**	1.0000
Proboscis Base	Female	**0.1864**	**0.1574**	1.0000
Proboscis Tip	Male	**0.1298**	No Sample	No Sample
Proboscis Middle	Male	**0.0077**	No Sample	No Sample
Proboscis Base	Male	**0.1187**	No Sample	No Sample
Middle Body	Female	1.0000	1.0000	1.0000
Posterior Body	Female	1.0000	1.0000	1.0000
Middle Body	Male	1.0000	No Sample	No Sample
Posterior Body	Male	1.0000	No Sample	No Sample

*A = *Acanthocephalus dirus;* R = *Rhadinorhynchus ornatus;* N = *Neoechinorhynchus idahoensis.*

Calcium

Location of Scan	Gender	A vs. R *	A vs. N*	R vs. N*
Proboscis Tip	Female	**<0.0001**	**<0.0001**	1.0000
Proboscis Middle	Female	**<0.0001**	**<0.0001**	1.0000
Proboscis Base	Female	**0.2348**	**0.3845**	1.0000
Proboscis Tip	Male	**0.0476**	No Sample	No Sample
Proboscis Middle	Male	**0.0044**	No Sample	No Sample
Proboscis Base	Male	**0.1091**	No Sample	No Sample
Middle Body	Female	1.0000	1.0000	1.0000
Posterior Body	Female	1.0000	1.0000	1.0000
Middle Body	Male	1.0000	No Sample	No Sample
Posterior Body	Male	1.0000	No Sample	No Sample

*A = *Acanthocephalus dirus;* R = *Rhadinorhynchus ornatus;* N = *Neoechinorhynchus idahoensis.*

Table 7. Statistical analysis of EDXA data by gender, ANOVA using a computer program, SAS Proc. Mixed (version 9.1). Those with high probability are in bold.

Rhabinorhynchus ornatus

Gender Comparison	Sulphur levels Proboscis		
	Tip	Middle	Base
Male vs. Female	<0.0001	<0.0001	<0.0001
Female vs. Male	<0.0001	<0.0001	<0.0001

Acanthocephalus dirus

Gender Comparison	Calcium levels Proboscis		
	Tip	Middle	Base
Male vs. Female	<0.0001	<0.0001	<0.0001
Female vs. Male	<0.0001	<0.0001	<0.0001

For habitat, the spiny heads studied represented marine *(R. ornatus)* and fresh water *(N. idahoensis, A. dirus)* fish hosts and the bird host. For body regions the sulphur level is significantly higher for the marine host habitat versus the fresh water specimens. The data from the regions of the proboscis also show significantly high levels of sulphur for *R. ornatus* versus the fresh water species, *A. dirus* and *N. idahoensis*. Sulphur in the proboscis is probably involved with disulphide bonds such as the thiol groups for cysteine and cystine found in polymerized protein molecules (Stegman, 2005). The disulphide bonds that are formed play an important role in the stability and rigid nature of the protein. The proboscis with hooks represent the reason for high sulphur content for that region of the parasite. There are many more hooks on the proboscis of *R. ornatus* versus the other acanthocephalan species. Taraschewski (2000) has an excellent review on the chemistry and morphology of the Acanthocephala. There are statistical differences between the four species of Acanthocephala (tables 6 and 7). An example is *A. dirus* and *R. ornatus*. The body regions of the female for *R. ornatus* has high sulphur content while calcium content is higher for *A. dirus*. There are recorded differences for the other gender and species of the Acanthocephala studied for this project.

Another interesting observation is the elemental differences between the spines and hooks of *R. ornatus*, a species of acanthocephala with both structures. The hooks which are part of the proboscis play an essential role in host attachment while the spines are located on the body (table 4). For the three regions on both the hooks and spines the hooks have significantly higher content for the three elements.

Conclusion

It has been recorded that S, Ca, and P are basic chemical elements involved in the formation of different body regions of acanthocephalans, and the distribution pattern of the three elements through the proboscis and body regions is the same in each of the examined species, though variations in the comparative concentration between the three elements were detected in different species.

Females have higher concentrations of the elements than males. The high sulfur level may be due to the presence of mature eggs in gravid females. The examination of the acanthocephalan egg revealed the presence of high concentration of sulphur (Heckmann et al., 2007).

The high level of the three examined elements in the proboscis compared to the other body regions may be due to the presence of numerous hooks on the proboscis. Previous X-ray microanalysis of *Rhadinorhynchus ornatus* proboscis hooks showed the involvement of S, Ca, and P in the chemical composition of the hook (Heckmann et al., personal communication).

Higher concentration of the examined elements in the hind body than the mid body, might be related to the presence of acanthocephalan genitalia, in both sexes, in the posterior region. However there is a slight difference between the elements concentrations in the two regions.

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