HEMOLYMPH CHEMISTRY REFERENCE RANGES OF THE CHILEAN ROSE TARANTULA *GRAMMOSTOLA ROSEA* (WALKENAER, 1837) USING THE VETSCAN BIOCHEMISTRY ANALYZER BASED ON IFCC-CLSI C28-A_3

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HEMOLYMPH CHEMISTRY REFERENCE RANGES OF THE CHILEAN ROSE TARANTULA *GRAMMOSTOLA ROSEA* (WALKENAER, 1837) USING THE VETSCAN BIOCHEMISTRY ANALYZER BASED ON IFCC-CLSI C28-A_3

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Abstract: The use of invertebrate hemolymph chemistry analysis has the potential to become a major diagnostic tool. The goal of this study was to generate statistically sound hemolymph reference ranges from healthy tarantulas. Hemolymph was drawn from wild caught, acclimatized, and apparently healthy female Chilean rose tarantulas *Grammostola rosea* (Walkenaer, 1837) ($n = 43$) using a modified technique. Hemolymph samples were separately analyzed using the Avian-Reptilian Profile Plus reagent rotor for VetScan® for the following chemistries: aspartate aminotransferase, bile acids, creatine kinase, uric acid, glucose, total calcium, phosphorus, total protein, albumin, potassium, and sodium. With this method the authors were able to establish statistically sound reference ranges for aspartate aminotransferase, creatine kinase, glucose, phosphorus, and total protein. Further in situ studies will determine the practical usability of these values in the evaluation of tarantula health.

Key words: Arachnida, *Grammostola rosea*, hemolymph chemistry, spider, Theraphosidae, VetScan.

INTRODUCTION

Like many species of exotic pets, invertebrates can provide the clinician with diagnostic challenges. Utilizing tested and proven clinical pathology methods provides for consistent and reliable data that can be used to assist in clinical case management. Due to the small size and fragility of many arachnid patients, the volume of hemolymph that can be obtained is necessarily small. Thus, in some cases dilution is necessary in order to meet the requirements of different chemistry analyzers. One advantage of the method used in this study is the low required sample size of 0.09–0.12 ml, making it possible to extract hemolymph from animals with a very low body weight without dilution. This study was designed to investigate the development of a practical method of tarantula hemolymph collection and examination.

MATERIALS AND METHODS

Forty-three female adult Chilean rose tarantulas *Grammostola rosea* (Walkenaer, 1837) were captured in Chile and shipped to the author's laboratory in Germany (Höhle M, pers. comm.) from March to April. On the day of the arrival each tarantula appeared healthy based on gross examination. Quarantine for the spiders lasted for 2 wk under the following husbandry parameters: temperature: 22 ± 2°C (71.6 ± 3.6°F) during the day and 20 ± 2°C (68 ± 3.6°F) at night; humidity: 80%; feeding: after 2 wk house crickets (*Acheta domesticus*) once and then weekly with adult dubia roaches (*Blaptica dubia*) from the same source; water: cotton buds, which were replenished every 4 days.

The spiders were restrained with a modified technique. For this inverted pinching method, the animals were approached cranially. Thumb and middle finger were placed in the intercoxal spaces between legs II and III while applying gentle pressure with the index finger on the carapace. Hemolymph withdrawal was achieved by intracardiac puncture in which the needle was positioned with the tip pointing upwards and the bevel towards the puncture site (Figs. 1, 2). When indicated, hemolymph stasis was achieved with sterile cotton swabs and n-Butyl cyanoacrylate tissue glue (Surgibond Gewebekleber, SMI AG, St. Vith, 4780, Belgium).

The body weight of the animals ranged from 5 to 13 g (mean 7.6, median 7.0, standard deviation 2.4). Physiologic hemolymph volume is estimated at 20% of the body weight in one species of tarantula.[35] For the purpose of this study, the

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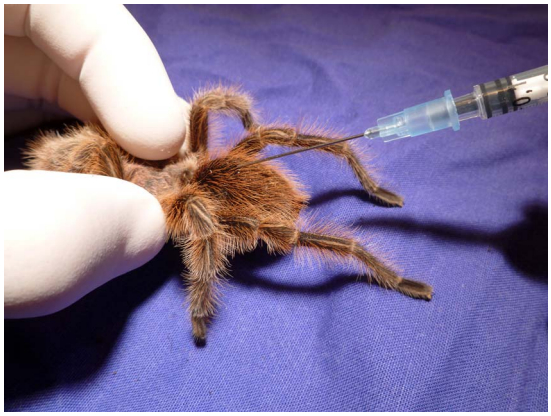

Figure 1. Intracardiac hemolymph withdrawal technique from an adult female *Grammostola rosea*, macroscopically.

maximum hemolymph volume in ml was chosen as 10% of the physiologic hemolymph volume. The amount of hemolymph required by the analyzer was 0.09 ml, just below the 0.10 ml maximum for the smallest animal. Hemolymph samples were obtained using heparinized (Heparin-Natrium-25000-ratiopharm®, 5000 UI/ml) 1-ml syringes fitted with 23-ga needles (BD Microlance™ 23 G 1-inch No. 16 0.6–25 mm, Henry Schein, Langen, Hessen, 63225, Germany). These samples were directly transferred to a 1-ml lithium heparin tube (Multivette® 600 LH, Sarstedt AG & Co., Nümbrecht, Nordrhein-Westfalen, 51588, Germany) and centrifuged with an EBA 20 centrifuge (Andreas Hettich GmbH & Co. KG, Tuttlingen Baden-Württemberg, 78532, Germany) at 3.46 x g for 10 min. Hemolymph supernatant was extracted and examined by a VetScan® Point-of-Care Blood Analyzer, Model No. 200-1000 (Abaxis Inc, Union City, CA 94587, USA) using Avian-Reptilian Profile Plus reagent rotors.

International recommendations for determination of reference intervals continuously updated by the International Federation of Clinical Chemistry (IFCC) and the Clinical and Laboratory Standards Institute (CLSI) confirm that a non-parametric method is preferred if the number of reference individuals is between 1 and 120. If the number of specimens is low, the CLSI guideline provides a calculation of reference values based on a robust method. A distribution as close as possible to the Gaussian distribution is preferred. Therefore, a Box-Cox transformation is often used to transform data to normality. The data of this study was processed by Reference Value Advisor v2.1 (Microsoft Office Professional Plus 2010 Version 14.0.4760.1000®, Microsoft Corporation, Redmond, WA 98052, USA) and the computed reference intervals placed in a spreadsheet according to CLSI guidelines.[13,19]

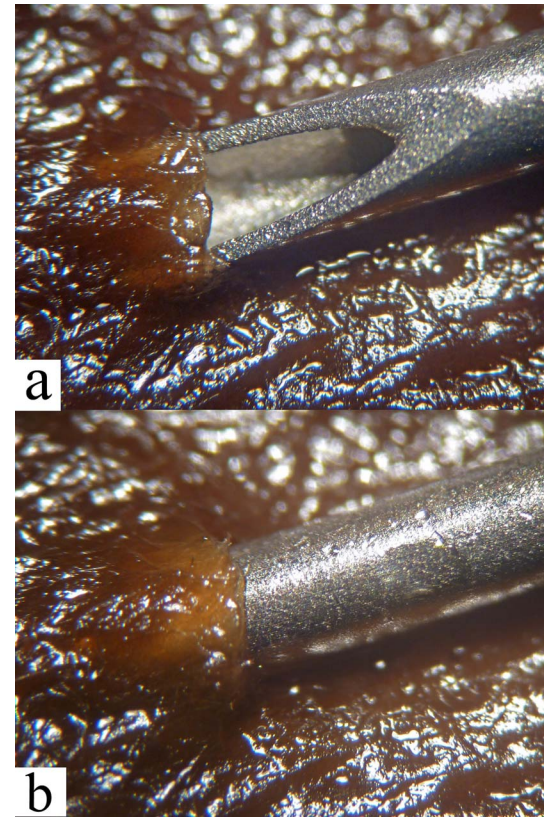

Figure 2. Comparison between the classical hemolymph withdrawal technique (**a**) and the modified method (**b**) from an adult female *Grammostola rosea*, microscopically (×40). Urticating hair were removed from the cutis with a cotton bud and the area was lucubrated with 75% isopropanol prior to the procedure to visualize the puncture site for imaging. The curved rigid cutis and hemolymph pressure leads to a rapid ejection of fluid when the classical punction method is performed. With the modified method, the lesion is sealed much more efficiently with no observed disadvantages.

Following the hemolymph collection, the animals were maintained as during the quarantine period and monitored daily for 6 mo.

RESULTS

Aspartate aminotransferase (AST): After Box-Cox transformation ($\lambda_1 = 0.087$, $\lambda_2 = 0.371$), the Anderson-Darling test indicated a Gaussian dis-

tribution ($P = 0.855$). The standard method for Box-Cox transformed values lead to the reference interval for AST ranging between 1.9 (90% confidence interval = 0.8–3.6 U/L) and 54.7 U/L (90% confidence interval = 44.0–65.7 U/L). One outlier was detected by the Tuckey method (57.9 U/L) and was retained.

Bile acids (BA): The results for BA in this study were 35 µmol/L for each specimen, which is the lower limit for the dynamic range of the rotor. Computing reference intervals was not possible.

Creatine kinase (CK): The results for CK were quite similar to those for AST. Values transformed via Box-Cox ($\lambda_1 = 2.33$, $\lambda_2 = 0.431$) showed a suspected Gaussian distribution ($P = 0.843$). Values calculated lead to a reference interval from 1.2 (90% confidence interval = –0.5 to 3.4 U/L) to 50.3 U/L (90% confidence interval = 41.6–59.1 U/L). The Tukey method detected only one outlier, but the box and whiskers diagram showed that at least one more extreme value might be an outlier. If the reference values for CK presented in this study are used in further examinations, the upper limit should be interpreted with caution.

Uric acid (UA): With the exception of six outliers, all results for UA were 0.3 mg/dl. As with BA (see above), this is exactly the lower limit of the dynamic range of the reagent rotor and computing of reference ranges was not possible.

Glucose (GLU): The Anderson-Darling test indicated a Gaussian distribution ($P = 0.606$), but visualization via Q-Q plot and tighter 90% confidence intervals (8.2–11.1 mg/dl and 30.2–36.7 mg/dl) suggested that a Box-Cox transformed ($\lambda_1 = -6.318$, $\lambda_2 = 0.649$) robust method is more reliable where reference intervals range between 9.5 and 33.5 mg/dl.

Total calcium (CA^{++}): Eighty-six percent of the measured values exceeded the precise measurement limits (>12 mg/dl). After examining error reports, even >30% of the values exceeded the upper dynamic range limit (>16 mg/dl), thus, computing reference ranges for Ca^{++} was not possible (Fig. 2).

Phosphorus (PHOS): The null hypothesis for Anderson-Darling test could not be rejected for Box-Cox transformed ($\lambda_1 = -0.115$, $\lambda_2 = 0.037$) data ($P = 0.410$), but results were not as convincing as previous reference values. Presumably, this was due to the narrower ranges of data in this case. The box and whiskers diagram revealed a high density within the lower third of the reference range. The reference interval lay between 0.5 (90% confidence interval 0.5–0.6 mg/dl) and 1.8 mg/dl (90% confidence interval 1.5–2.0 mg/dl).

Total protein (TP): The results indicated a high possibility ($P = 0.788$ and visualization via Q-Q plot) for a Gaussian distribution of untransformed data. Computed with the standard method, the reference interval ranged between 1.7 and 9.0 g/dl (90% confidence interval, 1.0–2.5 g/dl and 8.2–9.8 g/dl).

Albumin (ALB), Globulin (GLOB): Precision of the results for ALB was low because values are concentrated in the area of the rotors lower detection limit (1 g/dl). The amount of GLOB is directly dependent on ALB because it is calculated from ALB and TP. Therefore, it was not possible to compute the reference interval for ALB and GLOB.

Potassium (K^+): Data distribution of K^+ was very compact around the lower limit of the rotors dynamic range (37% of the values: 25.5% at 1.5 and 12.5% at 0 mM/L). Computing for K^+ was not possible (Fig. 2).

Sodium (Na^+): The results for Na^+ exceeded the upper range of the detection limit (180 mM/L) for each specimen.

After hemolymph withdrawals, the leakage at almost all injection sites was small enough to be sealed with the use of a cotton swab. Two animals showed excitement during the procedure and required additional sealing with tissue glue. Four mortalities unrelated to hemolymph withdrawal occurred during this poststudy period. One death was due to mismolting and three to a severe outbreak of phorid flies (*Megaseila scalaris*), which was introduced into the group with an infested food box of dubia roaches. All other animals molted normally with complete wound healing.

DISCUSSION

A main goal of this study was to determine if hemolymph can be routinely analyzed by laboratory equipment commonly used in exotic pet medicine. Another goal was to develop useful, statistically sound reference ranges for selected chemistries in one species of tarantula.

Although previous studies have presented hemolymph values, practical reference values are lacking established guidelines such as ASVCP Reference Interval Guideline or IFCC-CLSI C28-A_3 (Table 1).[3,8,9,12,17,24,29–33,35,37,38,40,41] In order to form a solid foundation to fulfill these requirements, and to minimize external influence factors, it was very important to standardize patient population, husbandry, and nutrition.

The Abaxis Inc VetScan point-of-care system was chosen because it is widely available to exotic animal veterinarians, requires very small sample

Table 1. Overview of published hemolymph chemistry values of different mygalomorph species.

	AST (U/L)		CK (U/L)		UA (mg/dl)		Glucose (mg/dl)		Calcium (mg/dl)		PHOS (mg/dl)		TP (g/dl)		Albumin (g/dl)		Potassium (mM/L)		Sodium (mM/L)		Caseload (n =)	Source (citation No.)
	Min	Max	Min	Max	Min	Max	Min	Max	Min	Max	Min	Max	Min	Max	Min	Max	Min	Max	Min	Max		
Grammostola rosea	1.9	54.7	1.2	50.3	ND	ND	9.5	33.5	ND	ND	0.5	1.8	1.7	9.0	ND	ND	ND	ND	ND	ND	0.43.0	This study
Theraphosa blondi	3.3	5.4	5.0	82.2	0.1	0.3	17.1	20.1	0.6	13.2	1.6	2.4	0.8	7.4	0.0	0.2	2.3	2.6	178.7	200.7	6.5.0	40
Grammostola rosea	2.1	34.7	20.9	30.5	0.0	0.3	13.9	27.8	15.7	18.1	1.0	1.5	3.7	7.2	0.0	0.2	1.7	2.4	219.8	262.4	0.0.12	40
Aphonopelma steindachneri	—	—	—	—	—	—	12.0	12.0	15.3	16.3	—	—	5.4	5.9	—	—	1.9	1.9	184.7	196.9	—	33
Aphonopelma hentzi	—	—	—	—	—	—	0.0	11.0	—	—	—	—	7.4	7.4	—	—	—	—	—	—	—	35
Aphonopelma hentzi	—	—	—	—	—	—	—	—	16.0	16.0	—	—	—	—	—	—	5.0	5.0	220.0	220.0	—	32
Aphonopelma echinum	—	—	—	—	—	—	—	—	3.9	3.9	9.2	9.2	5.3	5.3	—	—	1.4	1.4	188.3	188.3	—	31
Bothriocyrtum californicum	—	—	—	—	—	—	—	—	3.8	3.8	9.4	9.4	4.0	4.0	—	—	1.5	1.5	190.0	190.0	—	31
Euagrus comstocki	—	—	—	—	—	—	—	—	4.0	4.0	9.6	9.6	5.0	5.0	—	—	1.4	1.4	191.0	191.4	—	31
Sphodros rufipes	—	—	—	—	—	—	—	—	3.8	3.8	9.3	9.3	5.8	5.8	—	—	1.6	1.6	201.1	201.1	—	31

AST, aspartate aminotransferase; CK, Creatine kinase; UA, uric acid; PHOS, phosphorous; TP, total protein; ND, not detectable.

volumes, and provides accurate results (although to our knowledge this system has not been validated specifically for arachnids).

Several techniques of hemolymph withdrawal have been described. Intracardiac puncture is the preferred method in Mygalomorphae.[3,18,23,34,35,38,40] Femoral segment puncture has also been used.[3] The techniques described for Araneomorphae such as puncture of the lateral abdomen,[8,29,30] joint membrane,[17,24] or leg removal[9] were not considered for this study.

Prior to our study, intracardiac puncture for hemolymph withdrawal or intravenous injection in medical cases showed that intracoelomic pressure and firm consistency of the dermis often led to a rapid discharge of hemolymph alongside the needle when the classical puncture method (tip of the needle down towards the injection side, bevel end up) is performed.[38] The risk of hemolymph loss can be reduced significantly if the needle is turned 180 degrees as described (Fig. 2). No disadvantages were observed using this method.

Manual restraint was sufficient for our purpose, but less docile spiders, like members of the Stromatopelminae or Poecilotherinae, might require sedation. The most efficient methods in this case are aeration with CO_2,[4,8,24,38] isoflurane in oxygen (3–6%),[28,40] or sevoflurane in oxygen (5%).[33] Hypothermia has also been used for minimally invasive manipulation like heart puncture.[16] Different hemostatic methods following hemolymph withdrawal have been used including beeswax,[35] candle wax,[38] n-Butyl cyanoacrylate,[40] and cotton.[32] A cotton ball with optional use of n-Butyl cyanoacrylate worked well in our study as evidenced by a lack of dehiscence, dysecdysis, or other pathological consequences in our cohort of tarantulas. Needle punctures healed without complication.

Bile acids are produced in the liver and play a significant role in lipid digestion.[10] They have been used as an indicator for liver disease in birds but the diagnostic value for reptiles is up for debate.[12] Each value for BA in this study lays exactly on the lower limit of the rotors dynamic range. Either tarantula BA differ enough from birds or reptiles that they are undetected by the rotor, or, physiological levels are too low to be detected. The latter appears more likely because spiders are known to produce BA, which are utilized as color pigments in the dermis.[13] Further studies may reveal the usability of BA in spider medicine but the determination of a reference value in this study was not possible.

Arachnids use similar strategies as vertebrates to transform end products of protein metabolism in order to reduce toxicity and water consumption.[12,20] In spider excreta, xanthine derivatives, amino acids, or urea can only be found in trace amounts. Spiders are termed guaninothelic because the end product of their nitrogen metabolism is primarily guanine. One study found 74–88% guanine and 10–25% UA in arachnid excreta. However, it was assumed that nitrogen compounds other than guanine were either found due to contamination with unabsorbed material or silk in the cloaca.[2] Measuring UA under the conditions of this study was not successful.

Unlike vertebrate species where calcium metabolism is well known,[12,20] there is still a lack of knowledge on the exact distribution of calcium in spiders. Mineral congregations in the digestive and secretory cells of the midgut gland in the intestinal tract seem to act as a calcium reservoir (spherites of calcium mineral).[19] The values for calcium found in this study were significantly higher than in vertebrates, which is consistent with the literature.[32,33,40] Data distribution in the box and whiskers diagram assumes that the lower reference value lies within the dynamic range of the rotor and might be clinically significant, but a statistically sound reference range according to IFCC-CLSI C28-A_3 cannot be established.

Electrolyte concentrations have to be kept in narrow ranges to maintain basic body function.[10] Previously published data found that the physiological concentration of K^+ in spider hemolymph is significantly lower than in vertebrates.[32,33,40] This is presumably the reason for K^+ exceeding the lower dynamic range of the rotor, making a computation impossible. Conversely, Na^+ is mainly found in the extracellular fluid and exceeded the upper range for the rotor. With this method, it was not possible to establish reference values for Na^+. However, shifts into the dynamic range might be clinically valuable.

The TP of vertebrate blood plasma consists primarily of ALB and GLOB. In clinical conditions, measuring TP, ALB, and (calculated) GLOB can give valuable information about clinical states like hydration, oncotic pressure, body condition, or inflammation.[2] In spiders, the respiratory protein hemocyanin, with a molecular weight of approximately 75 kDa, is not incorporated in cells, comprising 80–82 % of TP in the hemolymph plasma, whereas 18–20% belongs to a second nonrespiratory protein unrelated to hemocyanin.[6,15,21,22,26,37] Therefore, interpretation of TP might substitute analyzing hemoglobin for theraphosids. Despite different measuring methods, the reference interval computed for TP in this study (1.7–9.0 mg/dl) is roughly congruent with previously published values (Table 1).[40]

In this study, values for ALB indicate that the rotor was not able to detect this protein in hemolymph samples. One study assumes that there is no need for a protein sustaining oncotic pressure in spiders.[40] Liposoluble hormones such as ecdysone can be transported by hemocyanin.[14] Nutritional functions are performed by a network of intestinal diverticula, reaching into all parts of the body including the prosoma and legs.[11]

Aspartate aminotransferase is an enzyme present in both cytoplasm and mitochondria of different vertebrate tissues. Creatine kinase occurs mainly in the cytoplasm of tissues with a high metabolic rate such as heart or muscle. If any of those enzymes leak into the bloodstream, an elevation can be measured.[10,12] The presence of AST and CK has already been confirmed in invertebrate species: Western honey bee (*Apis mellifera*), silkworm (*Bombyx mori*), several *Drosophila* spp., European lobster (*Homarus gammarus*), and lumbricus (*Lumbricus terrestris*).[1,5,7,25,36] The source and clinical value of both enzymes in arachnids remains unknown and should be examined in further studies.

Glucose is involved in several major metabolic pathways of vertebrates and invertebrates.[12,27] Glucose in the bloodstream is influenced by many factors (hormonal, neural, humoral, and nutritional). Hence, there are quite a few pathologic or physiologic conditions that can lead to changes in serum GLU and might be important for the diagnosis of theraphosid diseases. Some of these factors have been described for invertebrates such as the juvenile hormone methyl farnesoate in the portly spider crab (*Libinia emarginata*) and insulin-like peptide hormones and receptors in insects.[16,39]

In vertebrate species, phosphate is an important ligand for metabolic pathways. It is absorbed by the intestinal tract and distributed widely in the body, with the main storage in bone tissue and excretion via the kidneys. Phosphorous homeostasis is sustained by the same hormones that affect calcium. Changes in serum PHOS can be caused by renal or nutritional diseases, fluid imbalances, or changes in pH.[10,12] Not much is known about metabolism of phosphate in invertebrates. However, spiders share many of the metabolic pathways with vertebrates, such as pentose phosphate pathway and citrate cycle.[15] There is a high concentration of phosphorus in

Table 2. Hemolymph[a] reference values obtained in this study.[b]

AST (U/L)	Min	1.9
	Max	54.7
CK (U/L)	Min	1.2
	Max	50.3
UA (mg/dl)	Min	ND
	Max	ND
Glucose (mg/dl)	Min	9.5
	Max	33.5
Calcium (mg/dl)	Min	ND
	Max	ND
PHOS (mg/dl)	Min	0.5
	Max	1.8
TP (g/dl)	Min	1.7
	Max	9.0
Albumin (g/dl)	Min	ND
	Max	ND
Potassium (mM/L)	Min	ND
	Max	ND
Sodium (mM/L)	Min	ND
	Max	ND

[a] The hemolymph was withdrawn from 43 healthy adult female *Grammostola rosea* during March–April.

[b] AST, aspartate aminotransferase; CK, Creatine kinase; UA, uric acid; PHOS, phosphorous; TP, total protein; ND, not detectable.

spherite structures of the digestive and secretory tissue in the midgut gland.[19]

CONCLUSIONS

This study showed that with a modified technique it is possible to withdraw theraphosid hemolymph less invasively than in previous studies, and that not all tests of the Avian-Reptilian Profile Plus reagent rotor are equally useful for the examination of tarantula hemolymph (Tables 1, 2). Calculation of reference values following approved guidelines has been established for the first time, but further research, such as comparison between tissue and hemolymph content and longer duration studies, is necessary to determine clinical usefulness. Devices such as Samsung PT10V Clinical chemistry analyzer (scil animal care company GmbH, Viernheim, Baden-Württemberg, 68519, Germany) or i-STAT® (Abaxis Inc) could play a significant role here.

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