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REFERENCE INTERVALS FOR PLASMA BIOCHEMISTRY OF HEMOLYMPH IN SUBADULT CHILEAN ROSE TARANTULA (*GRAMMASTOLA ROSEA*) UNDER CHEMICAL RESTRAINT

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Abstract: Tarantulas are a commonly kept species that are occasionally presented to veterinarians in exotic practice. A recent study on *Grammastola rosea* hemolymph biochemistry has been performed with nonanesthetized adult theraphosids. The objective of this study was to produce reference intervals for biochemistry biomarkers in hemolymph of chemically restrained *G. rosea* for use diagnostically by exotic veterinarians. Cardiac hemolymph collection was performed on 20 subadult tarantulas under general anesthesia with isoflurane. Samples were processed by a commercial laboratory. Statistics performed on the data include outlier exclusion, descriptive statistics, normality tests, and Pearson correlations. Reference intervals were made for total protein, creatine kinase (CK), aspartate aminotransferase (AST), glucose, uric acid (UA), calcium, and phosphorus. No cortisol was detected. The majority of the intervals produced were normally distributed with the exceptions of UA, phosphorus, and CK. Pearson correlation tests found several significant ($P = <0.05$) correlations between variables. The majority of the data displayed a normal distribution, unlike the previous study, with a greater number of replicates. The total protein, glucose, UA, calcium, and AST values generated were similar to those reported in the previous study. Conversely several variables such as phosphorus, CK, and albumin were not consistent with those previously reported. Evidence is presented for a lack of albumin, CK, and AST in Arachnida and thus previous data for these proteins is likely to be artifactual.

Keywords: Biochemistry, grammastola, hemolymph, intervals, reference, rosea.

INTRODUCTION

Tarantulas (family Theraphosidae), professionally known as theraphosids, are commonly kept as pets and as part of exhibitions in zoological collections. They have an important place in educating the public about biology and conservation of invertebrates.[30,33,52] The British Tarantula Society (BTS) has over four hundred members, with its annual exhibition attracting over 2,000 patrons, illustrating the size of the tarantula-keeping community in the United Kingdom. Similar popularity is seen in Europe and America.

The understanding of pain and welfare of invertebrates is still limited, especially with regard to the number and variability of invertebrate species.[20] Theraphosid spiders display avoidance and aggressive behaviors in response to negative stimuli[9,43] and respond positively to vivarium enrichment,[3] thus it is rational to consider that they can experience pain and distress, though the extent of this needs to be elucidated through study.[20]

In recent years the veterinary profession has expanded its knowledge within invertebrate medicine, with an increasing demand for veterinary expertise in invertebrate care.[7,10] The number of diagnostic tools currently available is limited.

Serum and plasma biochemistry is an invaluable tool to veterinarians, thus extending this modality to tarantula species could provide similar benefits. Reference intervals could be used diagnostically to determine husbandry deficits as it has in other species.[37]

Chilean rose tarantulas (*Grammastola rosea*) are a widely kept theraphosid species in the United Kingdom, being imported and sold regularly by exotic pet suppliers. Their docile nature and availability makes them easy and safe to work with.

There are several studies on composition of hemolymph in arachnids and theraphosids,[14,31,40,47] however little has been published on their clinical pathology. One recent study has been performed on *Grammastola rosea* (*G. rosea*) without chemical restraint.[19] This study has a statistically valid sample size but has limitations as only five variables generated normally distributed data. Manual restraint is a viable restraint technique but requires a confident handler; this study will use chemical restraint as this method will likely be utilized by veterinarians in practice. Another

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relevant study is of *G. rosea* and *Theraphosa blondi*.[56] Drawbacks of this study are that it has a small sample size (with a maximum of 12 individuals) and the majority of the data does not show a normal distribution.

Pericardial hemolymph collection is established as being relatively safe in theraphosids with all animals surviving 8 wk post procedure.[56] Other established hemolymph collection methods have limited utility for this study; collection from the limb is unable to produce enough hemolymph considering the size of the animals used.[14] Iatrogenic autotomy of a limb has also been used but was deemed unnecessarily damaging. Destruction and extraction involves euthanasia and thus wouldn't be applicable to this study.[47] None of the established studies have involved fluid replacement following hemolymph collection though guidelines for blood collection often include fluid replacement if more than 10% of blood volume is collected.[32]

Several methods of anesthesia for tarantulas have been reported.[13] Isoflurane has been established as safe with no mortality in *G. rosea* and other theraphosids,[56] with a significant decrease in induction and recovery time relative to CO_2.[18]

The primary aim of the study is to produce reference intervals for biochemical markers in hemolymph of *G. rosea*. This would provide an additional diagnostic resource to the exotic veterinarians in practice, as well as to academics who keep tarantulas for conservation or research purposes. The biochemical values used to create reference intervals in this study were total protein, calcium, uric acid (UA), phosphorus, creatine kinase (CK), aspartate aminotransferase (AST), and glucose.

Secondary aims of the study will be to evaluate what amount of hemolymph can be practically collected and to evaluate the effect of fluid replacement on recovery following anesthesia and blood collection. Cortisol will also be measured from a pooled sample to assess its use.

MATERIALS AND METHODS

Twenty subadult Chilean rose tarantulas were purchased from an established invertebrate supplier (www.martingoss.co.uk, Martin Goss, 51 Spring Gardens, Elm Park, Hornchurch, Essex, RM12 5BG, United Kingdom). As the spiders were subadult it was not possible to reliably confirm the gender of individual animals without exuvium examination. The spiders all had an abdominal length greater than 45 mm with weights ranging from 5.4 to 10.8 g. The sample size of 20 is the minimum number of animals required to produce a reasonably statistically valid reference interval as advised by the quality assurance and laboratory standards committee of the American Society of Veterinary Clinical Pathology (ASVCP).[21]

Husbandry and enrichment

The husbandry of the spiders during the study was based on established methods.[3,12,56] They were kept in individual 5-L plastic vivaria with a potting soil substrate containing vermiculite. The temperature and humidity were kept within a range of 24.5–28.6°C and 29.7–55.9% respectively within a 12-hr light cycle. Half a plastic plant pot were provided for a hide. Water was provided using a shallow dish and sponge that was topped up through a hole at the top of the vivarium. Locusts were offered to animals every 72 hr and were removed if uneaten within 30 min. Tarantulas were acclimatized for 7 days presampling after veterinary examination to ensure they were grossly healthy. Animals were not fed locusts for 72 hr post procedure. Enrichment was provided in the form of artificial plant material that has been associated with decreased aggression.[3] Animals were handled minimally and kept in a quiet environment.

Anesthesia and restraint

Initially a pilot study was used to determine how the procedure and anesthesia methods could be best achieved within the laboratory environment available. Animals were initially gas induced, with 1–2 L oxygen/min at 5% isoflurane in a mouse induction chamber, however this did not anesthetize the animals reliably enough for the procedure to be safe.

A modified technique was used to induce using higher concentrations of isoflurane. Tarantulas were placed into a perforated 250-ml container. Cotton wool was impregnated with 15–20 ml of isoflurane and laid on the bottom of a 600-ml container. The 250-ml container was placed into the 600-ml container after which the entire setup was then placed into an induction chamber at 1 L/min of oxygen to provide isoflurane scavenging. This method provided the concentrations of isoflurane required to sufficiently anesthetize the animals for the procedure.

Procedure

The hemolymph sampling involved collection from the pericardial sac using a 30-g 0.3-ml

insulin syringe. The needle was inserted through the dorsal midline of the opisthosoma at a 45° angle and advanced into the pericardial sac from which up to 2 ml/100 g of hemolymph was collected. Pressure was applied to the collection site following sampling to ensure minimal hemolymph leakage. After sampling, a Vetivex 11 "ringers lactate fluid replacement" (Dechra, West Pavilion, Sansaw Business Park, Shrewsbury, SY4 4AS, United Kingdom) fluid bolus of two and one-half times that of hemolymph volume taken was given into the pericardial sac with a 29-ga 1-ml insulin syringe using the same method as described above. The collection site was then sealed with tissue glue[35,48,55,56] (GLUture, Zoetis, 10 Sylvan Way, Parsippany, NJ 07054, USA). Following hemolymph collection, animals were monitored as they recovered from anesthesia and then frequently observed for 2 days post procedure. Signs of stress, discomfort, pain, or damage would be observed as anorexia and gradual lethargy progressing to a huddled posture.[25,34] The spiders were further monitored for 8 wk by the supplier. Close contact was maintained with the supplier during this period to ensure the health and survival of the spiders. Rescue protocols were put in place as informed by the literature.[17] One sample from each animal was collected into 1-ml lithium-heparin microtubes (Sarstedt AG & Co. Sarstedtstraße 1, 51588 Nümbrecht, Germany). Previous studies have established that higher levels of heparin than required for anticoagulation will not adversely change final biochemical analysis.[23,24] The microtubes were stored on ice immediately following collection. The five largest samples were used to produce hemolymph smears. The samples were packaged securely and sent to a commercial pathology laboratory (Pinmoore Animal Laboratory Services Ltd, The Coach House, Town House Barn, Cotton, Cheshire, CW6 0EG, United Kingdom). The samples were in transit for approximately 48 hr within an insulated ice box. They were then centrifuged and the supernatant used for analysis. Biochemical analysis was completed on an adapted Prestige 24i analyzer (Tokyo Boeki Group, 28F Kyobashi Edogrand, 2-2-1, Kyobashi, Chuo-ku, Tokyo, 104-0031, Japan) using the recommended Prestige reagents and adapted methods. The reagent in the albumin assay used in the commercial lab was bromocresol green. Cortisol measurements were carried out on two pooled samples, each involving five individuals, and were determined using a Siemens Immunolyte 1000 machine (Siemens Healthcare Ltd, Sir William Siemens Square, Frimley, Camberley, Surrey, GU16 8QD, United Kingdom).

Nuclear magnetic resonance analysis

A hemolymph sample was taken by Venomtech Ltd concurrently using the procedure described in this study under carbon dioxide anesthesia. This sample was sent to the University of Kent for nuclear magnetic resonance analysis. The Topspin 3.2 Chenomx Suite (4232-10230 Jasper Avenue, Edmonton, Alberta T5J 4P6, Canada) was used to analyze the data.

Statistics

Statistics were calculated using Excel Office 365 (Microsoft Corporation, 1 Microsoft Way, Redmond, WA 98052, USA) and a noncommercial software package (PSPP, Free Software Foundation, 51 Franklin Street, Fifth Floor, Boston, MA 02110, USA) on a Windows 8 operating system (Microsoft Corporation). Outliers were detected using the difference range ratio with a cutoff of less than 0.33. Individual data points identified as outliers were not included in subsequent statistical analysis; all other data points from the same animal were still used.[21] A Shapiro-Wilk test was performed along with skewness and kurtosis tests to determine normality of data with a cutoff *P*-value of 0.05 for determining whether the data was normally distributed. A two-tailed Pearson correlation with a significance cutoff of 0.05 was carried out between all variables obtained. Values attained were compared qualitatively with data reported in the existing literature.

Procedure and methods were reviewed and approved by the Royal Veterinary College Ethics and Welfare Committee, Reference URN 2015 1377 (2015/T325).

RESULTS

All spiders survived the procedure with no problems observed during recovery. All animals in the study fed at least twice in the 7-day acclimatization period, as well as undergoing a health inspection, and were deemed to be healthy. Hemolymph smears were produced from the five largest samples with minimal hemacytes identified (less than two per slide).

Normal distributions were identified for total protein, calcium, AST, glucose, and weight. Nonnormal distributions were identified for UA, phosphorus, and CK. The albumin assay data was also normally distributed but it is unknown what component of the hemolymph this assay is

Table 1. Variables with normal distributions.

	Mean	SD	Min–max	95% CI	Sample size
Albumin (Bromocresol Green Assay) (g/L)	11.75	4.2	2–20	9.91–13.59	20
Total Protein (g/L)	32.65	13.58	4–55	26.7–38.6	20
AST (IU/L)	15.63	13.83	0–88	9.41–21.85	19
Calcium (mM/L)	3.34	0.43	0.81–4.07	2.91–3.53	19
Glucose (mM/L)	0.23	0.16	0–0.6	0.16–0.3	20
Weight (g)	8.5	1.52	5.3–10.8	7.83–9.17	20

binding. Reference ranges are expressed in Tables 1 and 2. Histograms of normally distributed reference ranges are expressed in Figure 1.

Statistically significant two-tailed Pearson positive correlations were found between total protein, glucose, calcium and phosphorus. These values were also collated with data produced by the albumin assay. The correlations are expressed in Table 3.

Induction and recovery times during anesthesia showed normal distributions and are recorded in Table 4.

The NMR analysis determined a glucose concentration of 0.87 mM and trehalose concentration ranges from 0.25 to 0.75 mM.

DISCUSSION

Raw biochemical data was successfully generated with minimal outliers detected. For the majority of variables a sample number of 20 was achieved, thus complying with the recommendations of the ASVCP. All animals survived the procedure and were alive 8 wk post procedure. One of the animals died 10 wk post procedure due to dysecdysis. This is not unexpected as juveniles are more susceptible to mortality secondary to dysecdysis.

This study shows that a greater volume of hemolymph can be safely collected from *G. rosea* than previously defined in the literature. Six animals had 0.2 ml taken with no effect on survival despite being of similar sizes to those of previous studies.[56] In vertebrates it is recommended that no more than 1 ml per 100 g be collected without fluid therapy,[16] however the average taken in this study was 1.98 ml per 100 g.

Some of the individuals sampled from were significantly smaller than in previous studies. One of the individuals in the study was 5.4 g with the equivalent of 2.8 ml per 100 g body weight taken with no observed ill effect or influence on survival. A previous study established hemolymph volume to be 19% of body weight in a similar tarantula species.[46] If *G. rosea* is similar, then an average of 10.5% of the hemolymph volume was taken in this study without issue. This is in line with recommendations for vertebrates.[16]

This study indicates that these animals are more tolerant of this procedure than previously established though fluid replacement likely contributed to the animal's capacity to tolerate the procedure despite the additional injury of another needle penetration.

Biochemical data

The assays used in the biochemical analysis by the commercial laboratory (Pinmoore Animal Laboratory Services Ltd) have not been validated for *G. rosea* and further validation would be needed to ensure the accuracy of the values generated.

There are no albumin, CK, or AST proteins deposited in the UniProt (www.uniprot.org) database and using NCBI BLASTP to find similar sequences in the nonredundant nucleotide database with cat, cow, or cobra serum albumin, CK, and glutamic-oxaloacetic transaminase (GOT1), the gene for AST, revealed no similar sequences with the taxon limited to Arachnida (TaxID: 6854). Further to this, the NCBI nBLAST tool could not identify any analogue sequences within

Table 2. Variables with nonnormal distributions.

	Median	10%–90% Quantiles	Min–max	Range	Sample size
Uric acid (μM/L)	0	0–2.1	0–8	8	20
Creatine kinase (IU/L)	1	0–3.3	0–18	18	19
Phosphorus (mM/L)	0.97	0.45–3.1	0.15–4.75	4.6	20

Table 3. Pearson correlations with positive correlations with coefficient values. Bracketed values represent *P*-values.

	Albumin	Total protein	Calcium	Phosphorus
Total protein	0.930 (<0.001)			
Calcium	0.920 (<0.001)	0.970 (<0.001)		
Phosphorus	0.690 (<0.001)	0.740 (<0.001)	0.680 (<0.001)	
Glucose	0.580 (<0.001)	0.570 (<0.001)	0.480 (<0.001)	0.630 (<0.001)

the draft assembly of *Acanthoscurria geniculata* or *Parasteatoda tepidariorum* genomes.[39,42] This demonstrates the limitations of interpreting biochemical data in new taxa without genomic data confirming the presence of certain proteins. Values for albumin, CK, and AST have been generated through as yet unidentified biochemical reactions that may prove useful when understood fully.

Total protein values in this study were less than previous studies of both *G. rosea* and other tarantula species.[56] There were higher levels attributed to albumin found than previously reported, however this data is likely to be artifactual. The difference is likely due to the avian-reptile rotor used in previous studies that uses a bromocresol purple assay.[19,56] Additionally to the lack of evidence presented in this study, albumin has not been found in papers where mass spectrometry has been used to analyze hemolymph.[44] The albumin data either represents the bromocresol green assay binding to an unknown protein or to some other component of the hemolymph.

An average of 62% of the total protein in hemolymph did not bind to the albumin assay. Hemocyanin is expected to be the most abundant protein within the hemolymph[44] but is not known to bind to bromocresol green. Hemocyanin is a multimodal globulin in many invertebrates and is established as being present free in plasma rather than within dedicated cells; it has oxygen carrying capacities as well as a role in theraphosid immunity.[11,45] It could be speculated that if total protein could be indicative of an anemia-like clinical state in theraphosid species if lower than the reference range. Protein gel electrophoresis could be used in the future to determine more about the identity and role of the nonalbumin assay binding protein fraction.[27]

Table 4. Induction and recovery times for isoflurane anesthesia.

	Induction time (min:sec)	Recovery time (hr:min:sec)
Mean	27:24	1:13:08
Range	30:00	1:30:00
Min–max	12:00–42:00	24:00–1:54:00
Sample size	15	15

UA levels are consistent with those previously reported by Zachariach et al.[56] Nitrogen has been reported to be principally excreted as guanine in tarantulas and other arachnids, so low levels of UA are consistent with current understanding of arachnid physiology.[38,41,53] Guanine and other nitrogen-containing compounds could be assessed in future work as potential determinants of nitrogen metabolism and excretion.

CK is generated as a response to muscle damage in vertebrates.[6] Tarantulas have striated skeletal muscle that is used for most movement, although the righting capacity is dependent on hydrostatic pressure,[58] so a similar process could occur. The values found in this study were lower than previously reported by Zachariach et al or Eichelmann et al.[19,56] Efforts were made to ensure animals were minimally handled, which could explain the lower CK values relative to those found in the previous studies. The decision to use chemical restraint instead of manual restraint could also explain the lower reference range reported in this study. The CK assay is dependent on a chemical reaction; though a direct theraphosid CK analogue isn't present, arginine kinase is present and this likely performs a similar role and may facilitate the same chemical reaction used by the assay.[4]

AST can be produced in response to muscle or liver tissue damage in vertebrates.[6] The AST assay is dependent on the ability of AST to facilitate a chemical reaction, though it is unlikely that GOT1 is present in theraphosid spiders in the same way as vertebrate species, it could be speculated that a GOT1 equivalent is being measured by the AST assay, especially as GOT1-like genes are reported in other invertebrate species.[50] The AST data showed a greater range and were higher than values previously reported.[19,56] There was no correlation between AST and CK found in the

data, which is expected as neither represents known proteins within arachnids.

Calcium values were consistent with previous studies of both *G. rosea* and other tarantula species. Phosphate was high relative to previously reported data of *G. rosea* and other tarantula species.[19,22,56] Both were correlated with total protein, which suggests that they could be protein bound in a similar way to vertebrates.

A positive correlation was found between phosphate and calcium. This correlation is also present in mammals and is associated with bone remodelling with the involvement of vitamin D.[49] In some invertebrates, calcium and phosphorus are important components in chitin growth and formation, though the primary composition of invertebrate chitin is predominately N-acetylglucosamine.[5,54] Chitin remodelling in other invertebrate species has also been associated with ecdysis.[2,28] It could be speculated that chitin remodelling can be used to maintain levels of calcium and phosphorus in hemolymph in a similar way to vertebrates utilizing bone, especially in light of a recent study showing presence of Vitamin D precursors in another tarantula species.[55] Further study into the composition of tarantula chitin and a study into growth deficits in malnourished animals would be useful to elucidate this further.

The glucose values found are consistent with Zachariah et al but are lower than those reported by Eichelmann et al.[19,56] The Eichelmann et al study involved manual restraint with a different feed source, which could explain the variance between the two data sets. Manual restraint can be associated with stress hyperglycemia in mammalian species and a similar process may occur in theraphosids.[36] The low level of glucose relative to mammals is consistent with the current biological understanding of spider carbohydrate transport. Carbohydrate is primarily transported as the disaccharide trehalose in previously reported studies of other tarantula species.[8,51] This is a plausible explanation for the relatively low values found for glucose in this study. The positive correlation between glucose and the assumed protein bound to the albumin assay could suggest that glucose is protein bound (as yet unknown carrier) as is the case with humans and other vertebrate species.[1,29]

Analysis of the of three *G. rosea* hemolymph samples by nuclear magnetic resonance (NMR) identified the presence of glucose monosaccharides and trehalose disaccharides. The concentration of these sugars seem relatively low compared with other components of the hemolymph. There are indications of the presence of inositol in the samples, however their assignment is ambiguous. Because inositol is a sugar alcohol it resonates with other hemolymph components and therefore could not be positively identified.

Cortisol levels were below the sensitivity threshold for the biochemistry analyzer used in this study. A more sensitive cortisol detection method such as enzyme-linked immunosorbent assay (ELISA) may be required to assess cortisol as a biochemical measure.

Induction and recovery times

The isoflurane technique was modified so that animals could be anesthetized more effectively. There was no change in the survival rate compared with previous studies though induction and recovery time were longer.[18,57] There were no correlations between induction, recovery times, or the biochemical variables. The data collected on anesthesia method within this study indicates that the anesthetic modifications made are safe and could be applied again in future studies.

Hematology

Hemolymph slides showed low cellularity. The oxygen carrying capacity of hemolymph is achieved by hemocyanin, which is free in the plasma rather than cell bound and thus the low cellularity is expected.[26,44] The 30-ga needle used for collection could explain the low cellularity on the hemolymph smears due to shear stress hemolysis of cells in the sample.[15] A previous study of hematology of arachnid and theraphosid hemolymph using 27-ga needles (a larger bore needle) had a higher cellularity of samples consistent with lower shear force.[26,44]

CONCLUSIONS

The total protein, UA, calcium, and AST intervals in the study were consistent with previous studies and phosphorus, glucose, and CK were not consistent with previous studies. The authors conclude that the genomic and mass spectrometry data confirm the absence of albumin in spiders and thus the albumin data generated is artifactual. The genomic data concludes that AST and CK do not have direct analogues but likely have proteins that perform a similar function.

The authors conclude that larger volumes of hemolymph can be collected from *G. rosea* without issue. Fluid therapy following blood

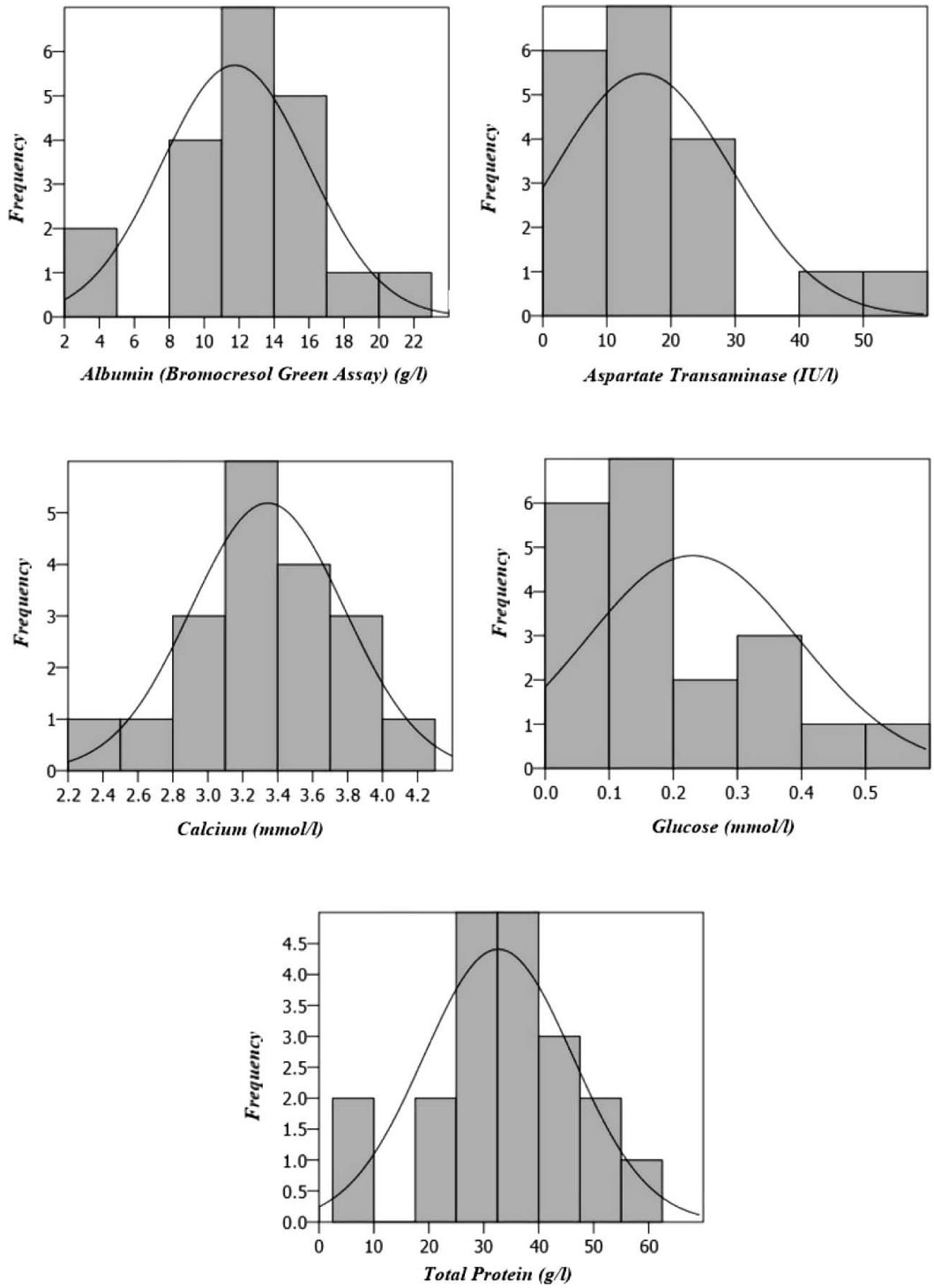

Figure 1. Histograms of variables with normal distributions.

collection can prove useful for facilitating recovery following blood collection.

There are some limitations in the study. Only one sample was taken from each of the animals, meaning that the chronicity of the data isn't fully appreciated. The size of this study gives a good overview for the species. Although the biochemical assays haven't been validated for arachnids, they are standard techniques for exotic species and thus future work will help to confirm the translation of these techniques to Theraphosidae. This study could be repeated in older animals to determine if the reference intervals vary with age. Data from sick animals is required to determine clinical significance of the reference intervals particularly with regards to nitrogen metabolism and UA.

Further work is required to assess the effect of different restraint methods on glycemic control and other hemolymph parameters in theraphosids in order to propose a standard technique for hemolymph collection as a diagnostic tool.

Reference intervals have been generated during this study, thus enabling the potential of hemolymph plasma biochemistry to be used diagnostically in the future to improve the level of veterinary care that can be achieved in theraphosid spiders.

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