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Serum protein electrophoresis in European mink (*Mustela lutreola*): reference intervals and comparison of agarose gel electrophoresis and capillary zone electrophoresis

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ABSTRACT

Background: Knowledge of reference intervals for blood analytes, including serum protein fractions, is of great importance for the identification of infectious and inflammatory diseases and is often lacking in wild animal species.

Material and methods: Serum samples were obtained from European minks enrolled in the breeding program (n = 55). Agarose gel electrophoresis (AGE) and capillary zone electrophoresis (CZE) were used to separate and identify protein fractions. Albumin, α1, α2, β, and γ-globulins fractions were identified in all mink sera by both electrophoresis methods. Reference intervals (90% CI) were determined following the 2008 guidelines of the Clinical Laboratory Standard Institute. The methods were compared using Passing-Bablok regression, Bland–Altman analysis, and Lin's concordance correlation.

Results: A significant bias was found between methods for α1, α2, and γ-globulin. Lin's concordance correlation was considered unacceptable for α1, α2, and β-globulins. Differences for gender between methods were found for albumin and α2-globuins, which were higher for males than females. γ-globulins were higher for adults than young minks using both methods; however, α1 and α2-globulins were lower.

Conclusion: Both methods are adequate for identifying serum protein disorders, but the AGE and CZE methods are not equivalent. Therefore, reference intervals for each technique are required.

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AGE; age; concordance analysis; CZE; electrophoresis; European mink; serum protein gender; reference intervals

1. Introduction

In wildlife, it is important to generate clinical laboratory data on captive non-domestic species, especially critically endangered species, to provide objective parameters that enable the evaluation of animal health (Kophamel et al. 2022). The European mink (*Mustela lutreola*) is a small, semi-aquatic, carnivorous mammal considered to be the most endangered mammal in Europe (Maran et al. 2016). The European mink is currently listed in appendix II and IV of the Habitats Directive and appendix II of the Bern Convention, enforcing the status of 'strictly protected fauna species' (Bern Convention 82/72/EEC). Habitat loss and degradation, anthropogenic interactions and mortality, interactions with feral American minks (*Neogale vison*), and infectious diseases are among the main causes of population decline. Several infectious diseases have been identified in European minks, which may have an impact on the survival of the species.

Similar to other species, European minks rarely manifest clinical signs until late onset of the disease. In captive animal populations, potential health issues and diseases can be detected through periodic health examinations (Peterson and Ferro 2012; Barrows et al. 2017; Kershaw et al. 2020).

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This article has been corrected with minor changes. These changes do not impact the academic content of the article.

This arises not only from the inaccessibility of individuals, particularly in the wild, but also from the lack of reliable reference intervals that truly represent healthy animals. The health status of European minks is commonly evaluated using blood and biochemistry tests that extrapolate reference intervals from closely related species such as the American mink (Farid and Rupasinghe 2022) or pet ferrets (Ravich et al. 2015).

Serum protein electrophoresis is useful to determine the albumin and globulin fractions of blood proteins, which are indicative of specific patterns of infection or inflammation. Unfortunately, very few studies have used protein electrophoresis in European minks (Sánchez-Migallón Guzmán et al. 2008), and none have provided specific reference intervals. Protein electrophoresis is an easy and economical method for separating serum proteins based on their net charge, size, and shape. Fractions of albumin, α1-globulin, α2-globulin, β1-globulin, β2-globulin, and γ-globulin are separated, identified, and quantified, providing both qualitative and quantitative data (Kaneko et al. 2008). This technique is particularly useful for determining acute-phase responses and serum protein disorders such as polyclonal gammopathy, hypoalbuminaemia, and hypoglobulinemia (Moore and Avery 2019) and is widely used in pet clinical practice by veterinarians.

In domestic animals such as dogs and cats, the detection of serum protein alterations is the first step in diagnostic algorithms. In these animals, alterations in electrophoretic patterns can be observed in different diseases, and detection of a specific electrophoretic pattern can be attributed to a particular disease. This is the case for leishmaniosis, which can affect dogs (Solano-Gallego et al. 2011), cats (Alcover et al. 2021), ferrets (Villanueva-Saz et al. 2021), and American and European minks in Europe (Giner et al. 2022).

Traditionally, two techniques were available, Cellulose Acetate Electrophoresis (CAE) and Agarose Gel Electrophoresis (AGE). CAE has been replaced by AGE with a novel technique known as Capillary Zone Electrophoresis (CZE) which provides a number of advantages for protein analysis, including high separation efficiency, reduced assay times, and a diverse range of protein applications (Giordano and Paltrinieri 2010; Paltrinieri et al. 2016). Both techniques have been used in clinical practice (Giordano and Paltrinieri 2010; Jeffries et al. 2021) and in wild species such as green turtles (*Chelonia mydas*) (Toonder et al. 2020), bald eagles (*Haliaeetus leucocephalus*) (da Fonseca et al. 2023), and bottlenose dolphins (*Tursiops truncatus*) (Zaias et al. 2021).

The advantages of CZE over AGE and CAE include a higher resolution, lower cost, and complete automation. Studies using serum or plasma from green sea turtles (*Chelonia mydas*) (Toonder et al. 2020), dogs (*Canis lupus familaris*) (Giordano and Paltrinieri 2010; Jeffries et al. 2021), mice (*Mus musculus*) (Crivellente et al. 2008), and humans (*Homo sapiens*) (Lissoir et al. 2003) have further shown that CZE results in better resolution of the protein fractions; however, because profiles differ from those obtained *via* traditional techniques, species-specific assessment is needed for accurate interpretation (Crivellente et al. 2008; Giordano and Paltrinieri 2010; Toonder et al. 2020). However, in two studies conducted on green turtles and dolphins, it was concluded that the two techniques were not equivalent (Zaias et al. 2021; da Fonseca et al. 2023), thus, reference intervals specific for each technique are required.

Another common problem encountered while using blood parameters in many wildlife species is the lack of reliable reference intervals. It is often difficult to obtain adequate sample sizes from healthy animals to establish reference intervals. Additionally, factors such as stress, diet, and captivity can influence certain parameters (Weber et al. 2002; Cattet et al. 2003; Harvey 2012; Martínez-Pérez et al. 2020).

The goals of this study were to present European mink serum protein electrophoresis values using both CZE and AGE techniques, and the reference intervals of the serum protein fractions of the European mink. Reference intervals were established according to the 2008 Clinical Laboratory Standards Institute (CLSI) guidelines. Another objective was to establish whether the CZE and AGE electrophoresis methods are interchangeable for mink sera. These data will serve as preliminary baseline values for practitioners and biologists who manage European minks medically.

2. Material and methods

2.1. Sampling

Blood samples were collected from 55 captive European minks from 2019 to 2022 from the nacional breeding program. This samples were collected in Foundation for Research in Ethology and Biodiversity (FIEB), located in Casarrubios del Monte (Toledo, Spain). The health status of these animals was monitored because they were visually checked daily, dewormed both externally and internally on a regular basis, and fed a controlled diet by certified suppliers. Since almost all specimens were born in captivity, the exact age can be known. In the case of wild specimens captured as part of the ex-situ conservation program, they are estimated at the time of capture to be generally juveniles or one-year-old adults. However, because all specimens captured for monitoring in the field are identified with a microchip, this helps estimate the age in cases of recapture of adult specimens. Young (<1 year) and adult (>1 year) animals were examined. These samples were collected during routine annual check-ups that assessed the clinical state of the animal by monitoring and recording the temperature, heart rate, respiratory frequency, length of the animal (snout to vent), dentition, upper and lower canines, body condition, weight, and size of testes in males, and abdominal exploratory ultrasound with measurement of both kidneys. Only

samples obtained from healthy animals during these check-ups were included in this study. Additional information, such as age, microchip identification, and sex, was recorded.

Blood was extracted under isoflurane inhalation anaesthesia. Sampling consisted of collecting 1 mL of blood by cranial cava venipuncture using aseptic technique. The collected volume was divided equally between a sterile blood collection tube to obtain serum for biochemistry and a second tube containing ethylenediaminetetraacetic acid (EDTA) anticoagulant for complete blood cell count (CBC). Different parameters were determined in serum samples, including amylase, lipase, fructosamine, cholesterol, bilirubin, alkaline phosphatase, gamma glutamyl transferase, alanine aminotransferase, aspartate aminotransferase, creatine kinase, creatinine, blood urea nitrogen, inorganic phosphorus, magnesium, calcium, potassium, sodium, iron, triglyceride, and glucose levels. The CBC and biochemical parameters of these animals were not considered altered because they were within established internal reference intervals. In addition, serological techniques have been used to detect the presence of antibodies against SARS-CoV-2, *Leishmania infantum*, *Toxoplasma gondii*, and *Dirofilaria immitis* as described previously (Giner et al. 2022; Villanueva-Saz et al. 2022a, 2022b, 2023) were performed. All animals were classified as seronegative.

The FIEB is a registered zoo centre covered by the Conserjeria de Agricultura y Servicios Periféricos de Castilla la Mancha with registration code: ES450410000053. This registration carries the implications of housing and handling animals according to animal welfare criteria. Furthermore, FIEB is a participating centre in the Ex-situ Conservation Program for European mink, acting as a breeding and research centre promoted by the Ministerio para la Transición Ecológica y Reto Demográfico of Spain. The care and use of animals was carried out according to the Spanish Policy for Animal Protection (RD 53/2013), which meets the European Union Directive 2010/63 on the protection of animals used for experimental and other scientific purposes. Ethical review and approval were waived due to the use of residual samples (serum) for diagnostic purposes.

2.2. Serum protein electrophoresis

Samples were collected during routine annual check-ups. After collection of whole blood, the blood was allowed to clot by leaving it undisturbed at room temperature for 30–40 min. Finally, the clot was removed by centrifugation at 1,500 × *g* for 10 min. The samples were maintained at 2–8 °C while handling. The samples were not frozen before testing. Haemolytic and lipemic specimens were excluded from the study. Serum protein electrophoresis was performed 48–72 h after collection and total protein was measured using the biuret method on an automated analyser.

Serum protein electrophoresis was performed by agarose gel electrophoresis (AGE) (the Hydragel Kit 1-2 (Sebia, Issy-les-Moulineaux, France). Serum was electrophoresed for 21 min at 92 V and stained with diluted amidoschwarz dye at pH 2 (4 g/L amidoschwarz dye and 6.7% ethylene glycol). The AGE procedure was conducted according to the manufacturer's instructions and commercial human serum was used as a control (normal control serum; Sebia, Evry, France). The electrophoretic curve for each sample was displayed and read with a GELSCAN TM densitometry system (Sebia, Issy-les-Moulineaux, France). The electrophoretic curve for each sample was assessed using the Phoresis software. Protein fractions were determined as a percentage of optical absorbance, and the absolute concentration g/L was automatically calculated from the total serum protein concentration using a spectrophotometer. Albumin-to-globulin (A:G) ratios were also calculated. The same operator analysed all samples.

Capillary zone electrophoresis was performed in a private laboratory, following routine protocol (Crivellente et al. 2008). The analysis was performed using Capillary Protein (E) 6 (Sebia, Issy-les-Moulineaux, France) with a Capillary System. Serum proteins were separated based on their electrophoretic mobility at a specific pH in an alkaline buffer (pH 9.9). CAPILLARYS automatically analyzes all sequences to obtain a protein profile for qualitative or quantitative analysis. Proteins separated in the silica capillaries were directly detected at an absorbance of 200 nm. Electropherograms can be visually interpreted to screen for pattern abnormalities. Direct detection provides accurate relative quantification of individual protein fractions.

2.3. Statistical analysis

Statistical analysis was performed using MedCalc® Statistical Software version 20.118 (Ostend, Belgium; https://www.medcalc.org; 2022). Reference intervals (RI) were determined using the robust method because the sample size was between 40 and 120 individuals and the non-Gaussian distribution of the data followed the recommendations of the Clinical Laboratory and Standards Institute (CLSI) (Friedrichs et al. 2012). Before setting the RI, outlier data were identified using Tukey's test and these values were excluded from the analysis. The 90% confidence intervals (CI) were calculated according to the CLSI guidelines (Friedrichs et al. 2012). The distribution of the RI was assessed by examining the histogram and confirmed using the Shapiro-Wilk test.

A non-parametric test was used because a non-Gaussian distribution was detected for all total proteins and protein fractions analysed using both CZE and AGE methods. To determine statistically significant differences between the capillary and agarose electrophoresis methods, a Wilcoxon signed-rank test was performed. Differences between females and males and between adults and young

individuals were analysed using the non-parametric Mann-Whitney test.

Concordance correlation analysis, Passing-Bablok regression analysis, and the Bland–Altman method were used to determine agreement between the two methods. Lin's concordance correlation was used as an indicator of the strength of the concordance between measurements. The limits were defined as good over 0.75, moderate between 0.6 and 0.75, poor between 0.5 and 0.6, and inacceptable under 0.5 (Lenoir et al. 2017). The regression equation reflects constant bias, the slope reflects proportional bias, and the residual standard deviation (RSD) reflects random bias. Confidence intervals reveal whether these values differ from 0 for the intercept and 1 for the slope only by chance. A cusum test for linearity was performed for each comparison, and a value of $p < .05$ indicates that there was no linear

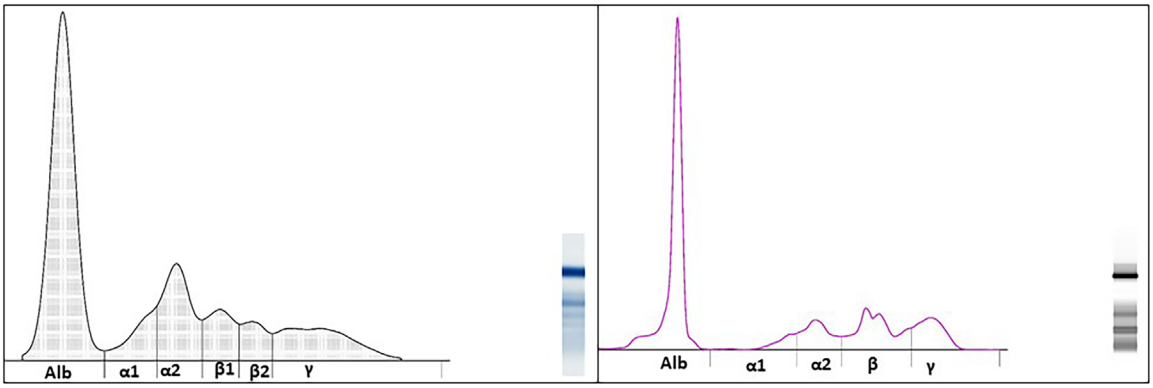

Figure 1. Comparison of the same mink electrophoretic tracings produced using AGE (left) and CZE (right) methods.

Table 1. Reference intervals of protein fractions (in percentage and g/L) obtained by CZE electrophoresis in mink serum.

Analyte	n	Mean	SD	Median	CV	Min	Max	RI	LRL 90% CI	URL 90% CI	*p* Value
Total protein (g/L)	55	54.9	5.8	55.0	10.5	43	70	44.7–64.5	42.8–46.7	61.4–66.5	.636
Albumin (%)	52	52.5	4.3	52.6	8.2	40.9	63.4	45.2–59.9	43.6–46.9	58.4–61.4	.947
Albumin (g/L)	52	28	2.6	28.4	9.3	22.9	33.2	23.7–32.7	22.6–24.9	31.9–33.6	.11
α1-globulins (%)	53	5.1	1.4	4.9	27.4	1.7	8.6	2.7–7.5	2.3–3.2	7.0–8.0	.984
α1-globulins (g/L)	53	2.8	0.8	2.9	29	0.77	4.7	1.4–4.2	1.2–1.7	3.9–4.5	.835
α2-globulins (%)	52	10.8	1.2	10.6	11.1	7.9	13.2	8.8–12.8	8.4–9.3	12.4–13.2	.458
α2-globulins (g/L)	54	5.9	0.8	6.0	13.6	4.2	7.6	4.6–7.3	4.3–4.9	7.1–7.6	.758
β-globulins (%)	54	17.5	3.2	17.1	18.3	9.9	24.1	12.1–22.9	10.7–13.1	21.6–24.0	.314
β-globulins (g/L)	54	9.6	2.2	9.8	22.9	5.6	15.4	5.8–13.2	5.1–6.6	12.5–14.1	.389
γ-globulins (%)	52	12.7	3.9	12.7	30.7	5.5	23.8	6.2–19.5	4.9–7.6	18–21.0	.455
γ-globulins (g/L)	54	7.7	3.0	7.2	38.9	2.5	16.1	2.1–12.4	0.9–3.2	11.1–13.7	.008
A:G ratio	52	1.1	0.19	1.09	17.2	0.69	1.51	0.78–1.42	0.73–0.85	1.35–1.48	.817

Notes: Total protein (g/L) were obtained by biuret method. CV: coefficient of variation; RI: reference intervals; SD: standard deviation; LRL 90% CI: lower reference limits, 90% confidence interval; URL 90% CI: upper reference limits, 90% confidence interval.

Table 2. Reference intervals of protein fractions (in percentage and g/L) obtained by AGE electrophoresis in mink serum.

Analyte	n	Mean	SD	Median	CV	Min	Max	RI	LRL 90% CI	URL 90% CI	*p* Value
Albumin (%)	53	50.8	4.6	50.5	9.1	42.5	60.5	42.9–58.8	41.4–44.7	57.3–60.4	.163
Albumin (g/L)	54	27.4	2.8	27.3	10.2	21.4	32.9	22.7–32.7	21.8–23.7	31.1–33.0	.71
α1-globulins (%)	55	6.64	1.4	6.4	21	3.8	9.7	4.3–8.9	3.8–4.8	8.4–9.4	.576
α1-globulins (g/L)	54	3.6	0.8	3.4	22.2	2.0	5.8	2.1–5.1	1.8–2.4	4.7–5.3	.27
α2-globulins (%)	54	15.1	1.8	14.6	11.9	1.8	19.4	11.6–18.1	11.1–12.3	17.3–18.9	.196
α2-globulins (g/L)	55	8.3	1.4	8.1	16.8	6.3	11.9	5.7–10.5	5.2–6.1	9.9–11.2	.01
β1-globulins (%)	55	8.8	1.5	8.3	16.9	6.7	12.1	5.6–10.9	5.2–6.3	10.4–11.8	.001
β2-globulins (%)	55	8.2	1.9	8.1	23.4	5.1	13.5	4.8–11.3	4.2–5.4	10.5–11.9	.042
β-globulins (%)	55	17.0	2.6	17.0	15.2	11.8	24.2	12.5–21.3	11.6–13.3	20.4–22.3	.638
β1-globulins (g/L)	55	4.9	1.1	4.5	20.9	3.2	7.4	2.6–6.5	2.3–3	6–7.1	.004
β2-globulins g/L)	55	4.5	1.1	4.1	22.7	2.6	7.4	2.3–6.3	2–2.8	5.8–6.7	.039
β-globulins (g/L)	55	9.4	2.0	9.5	21.2	6.3	14.5	5.9–12.6	5.1–6.5	11.9–13.4	.065
γ-globulins (%)	53	9.9	3.0	9.5	30	4.9	15.8	4.6–15.0	3.7–5.5	13.7–16.0	.062
γ-globulins (g/L)	52	5.4	1.9	5.0	35.1	2.6	9.3	2.1–8.6	1.5–2.7	7.8–9.3	.038
A:G ratio	55	1.03	0.21	1.01	20.3	0.43	1.38	0.67–1.4	0.6–0.75	1.33–1.48	.832

Notes: The β-globulins fraction is shown separately into β1 and β2, and the sum of the two fractions (β-globulins). CV: coefficient of variation; RI: reference intervals; SD: standard deviation; LRL 90% CI: lower reference limits, 90% confidence interval; URL 90% CI: upper reference limits, 90% confidence interval.

relationship between the two measurements. The limits of agreement (Bland–Altman plots) were used to assess the extent of agreement between the two methods. If the 95% confidence interval of the bias contained a value of 0, then no significant systematic bias between methods was assumed (Bland and Altman 1999). Statistical significance was set at $p < .05$.

3. Results

3.1. Reference intervals of total protein fractions in European minks

In all sera analysed, albumin, α1, α2, β, and γ-globulin fractions were detected in both CZE or AGE (Figure 1). The reference intervals for total protein are shown in Table 1. The reference intervals for the protein fractions of minks (in percentage and concentration g/L) for the CZE (Table 1) and AGE method (Table 2) are shown. Table 2 shows the β1 and β2-globulin fractions obtained by the AGE method, and the sum of the betas (β-globulins). All the peaks were narrower in the CZE method than in the AGE profile. CZE profile showed more subpeaks in the α and β regions, making identification of inflection points difficult to assess. However, software of the techniques for both methods distinguished five fractions: albumin, α1, α2, β, and γ-globulins.

Table 3. Comparison of the protein fractions (percentage and concentration) of electrophoresis according to the method used in mink serum.

	CZE	AGE	*p* Value
Analyte			
Albumin (%)	52.3 ± 4.6	50.7 ± 4.6	.005
Albumin (g/L)	26.4 ± 2.6	27.2 ± 2.6	.006
α1-globulins (%)	5.1 ± 1.4	6.6 ± 1.3	.000
α1-globulins (g/L)	2.82 ± 0.81	3.61 ± 0.86	.000
α2-globulins (%)	10.7 ± 1.16	15.3 ± 1.9	.000
α2-globulins (g/L)	5.9 ± 0.78	8.3 ± 1.4	.000
β-globulins (%)	17.4 ± 3.1	17.04 ± 2.7	.465
β-globulins (g/L)	9.61 ± 2.2	9.33 ± 2	.513
γ-globulins (%)	13.1 ± 3.9	9.78 ± 2.9	.000
γ-globulins (g/L)	7.3 ± 2.6	5.44 ± 1.9	.000
A:G ratio	1.1 ± 0.19	1.04 ± 0.19	.008

Notes: A Wilcoxon signed rank to paired test was performed. Data are mean ± SD.

3.2. Comparison between capillary and agarose electrophoresis methods

3.2.1. Protein fractions

Table 3 presents a comparison of the CZE and AGE methods. Mean values obtained by the two methods were similar. Using the AGE method, the albumin, γ-globulins, and A:G ratio were lower ($p < .05$) and the α1 and α2-globulin values were higher. The β-globulin fraction did not show statistically significant differences between both methods ($p > .05$).

3.2.2. Differences between sex

Table 4 shows the differences between males and females. For the CZE method, only albumin and α2-globulin concentrations differed between sexes, with both levels being higher for males than females ($p < .05$). However, for the AGE method, we found that males had a higher level than females for albumin, α1-globulins, and A:G ratio ($p < .05$), while it was lower for α2-globulins and β-globulins ($p < .05$). The γ-globulin fraction was similar between males and females ($p > .05$). There were not statistically significant differences ($p > .05$) in TP levels between sexes.

3.2.3. Differences between young and adult minks

The differences between young and adult individuals for both the CZE and AGE methods are shown in Table 5. The total protein concentration was higher in adult minks ($p < .001$). Variations in the percentage or concentration values were found for α1-globulins, with α2-globulins being lower in adult minks. However, γ-globulin levels were higher in adult minks ($p < .01$). Using the AGE method for protein electrophoresis, higher values in adult than in young minks were observed only for the γ-globulin fraction ($p < .05$).

3.3. Analysis of concordance

Tables 6 and 7 show the results of Passing-Bablok regression analysis and Bland–Altman plots of the comparison between CZE and AGE electrophoresis methods for mink serum proteins. A graphical representation of the Bland–Altman plot analysis for both

Table 4. Differences between mink's female and male for total protein and protein fractions for capillary and agarose electrophoresis in mink serum.

	CZE			AGE		
Analyte	Female	Male	*p* Value	Female	Male	*p* Value
Total protein (g/L)	53.7 ± 4.7	55.8 ± 6.5	.187	–	–	–
Albumin (%)	51.2 ± 3.8	53.2 ± 5	.121	48.2 ± 3.3	52.9 ± 4.5	.001
Albumin (g/L)	26.8 ± 2.4	29 ± 2.4	.002	26.5 ± 2.1	28 ± 3	.049
α1-globulins (%)	5.3 ± 1.6	5 ± 1.2	.478	7.3 ± 1.1	6.1 ± 1.3	.001
α1-globulins (g/L)	2.8 ± 0.82	2.83 ± 0.82	.815	3.33 ± 0.73	3.8 ± 0.87	.024
α2-globulins (%)	11 ± 1.5	10.6 ± 1.16	.284	16.2 ± 1.8	14.3 ± 1.5	.001
α2-globulins (g/L)	5.6 ± 0.76	6.2 ± 0.78	.020	7.9 ± 1.2	8.5 ± 1.5	.116
β-globulins (%)	17.9 ± 2.8	17.2 ± 3.4	.388	18.4 ± 2.5	16.1 ± 2.5	.001
β-globulins (g/L)	10 ± 2.2	9.2 ± 2.1	.182	9.2 ± 1.9	9.6 ± 2	.425
γ-globulins (%)	13.8 ± 3.6	13 ± 4.6	.471	9.8 ± 3.2	10 ± 2.9	.835
γ-globulins (g/L)	7.9 ± 3.6	7.5 ± 2.5	.635	5.44 ± 1.7	5.44 ± 1.9	.998
A:G ratio	1.06 ± 0.16	1.14 ± 0.2	.153	0.95 ± 0.15	1.1 ± 0.2	.007

Notes: A non-parametric Mann–Whitney unpaired test was performed. Data are mean ± SD. Females = 24, males = 31.

Table 5. Differences between adult and juvenile mink for total proteins and protein fractions for capillary and agarose electrophoresis methods in mink serum.

	CZE			AGE		
Analyte	Adult	Young	*p* Value	Adult	Young	*p* Value
Total protein (g/L)	55.8 ± 0.5	51.1 ± 0.44	.001	–	–	–
Albumin (%)	51.5 ± 4.4	53.7 ± 4.7	.095	50.1 ± 4.7	52.1 ± 4.4	.138
Albumin (g/L)	28.4 ± 2.7	27.3 ± 2.4	.163	27.7 ± 2.6	26.7 ± 3	.172
α1-globulins (%)	4.8 ± 1.6	5.7 ± 1.7	.038	6.4 ± 1.3	7.1 ± 1.3	.066
α1-globulins (g/L)	2.8 ± 0.7	2.9 ± 0.9	.438	3.6 ± 0.9	3.7 ± 0.9	.788
α2-globulins (%)	10.4 ± 1.06	11.5 ± 1.1	.003	14.9 ± 1.7	14.4 ± 2.1	.338
α2-globulins (g/L)	5.9 ± 0.9	5.9 ± 0.9	.980	8.5 ± 1.5	7.9 ± 1.3	.135
β-globulins (%)	17.8 ± 3.1	16.9 ± 3.3	.339	17.2 ± 3	17.1 ± 2.3	.896
β-globulins (g/L)	9.7 ± 2.4	9.3 ± 1.7	.471	9.8 ± 2.1	8.8 ± 1.5	.066
γ-globulins (%)	14.4 ± 3.9	11.5 ± 4	.012	10.2 ± 3	8.3 ± 2.5	.005
γ-globulins (g/L)	8.7 ± 3	5.9 ± 2.2	.001	6.1 ± 1.8	4.3 ± 1.5	.001
A:G ratio	1.08 ± 0.2	1.15 ± 0.18	.202	1 ± 0.22	1.1 ± 0.19	.096

Notes: A non-parametric Mann–Whitney unpaired test was performed. Data are mean ± SD. Adult= 36, young= 19.

Table 6. Intercept, slope and residual standard deviation of Passing-Bablok regression as well as bias, lower and upper limit of agreement from de Bland–Altman plots and their 95% confidence intervals (95% CI) from the comparisons between the CZE and AGE electrophoresis, when data are expressed in percentage.

	Passing-Bablok regression			Bland–Altman plot		
Fraction	Intercept (95% CI)	Slope (95% CI)	Residual standard deviation (95% CI)	Bias (95% CI)	Lower limit (95% CI)	Upper limit (95% CI)
Albumin	−1.26 (−13.75 to 8.13)	0.99 (0.8 to 1.22)	2.74 (−5.37 to 5.37) Cusum test = 0.49	−1.64 (−2.68 to 0.6)	−9.17 (−10.9 to −7.39)	5.88 (4.09 to 7.66)
α1-globulin	2.2 (0.07 to 3.82)	0.866 (0.55 to 1.28)	1.20 (−2.35 to 2.35) Cusum test = 1	1.3 (0.84 to 1.77)	−2.05 (2.84 to −1.25)	4.66 (3.87 to 5.46)
α2-globulin	−11.33 (−58.5 to 3)	2.38 (1.1 to 6.7)	1.71 (−3.35 to 3.35) Cusum test = 0.01	3.98 (3.25 to 4.71)	−1.3 (−2.56 to −0.05)	9.28 (8.02 to 10.54)
β-globulin	2.21 (−6.64 to 7.43)	0.85 (0.55 to 1.38)	2.63 (−5.15 to 5.15) Cusum test = 0.72	−0.34 (−1.34 to 0.65)	−7.61 (−9.33 to −5.89)	6.92 (5.19 to 8.64)
γ-globulin	−1.17 (−3.8 to 1.7)	0.83 (0.64 to 1)	2.08 (−4.08 to 4.08) Cusum test = 0.92	−3.46 (−4.28 to −2.63)	−9.44 (−10.86 to −8.02)	2.52 (1.1 to 3.94)
A:G ratio	−0.01 (−0.23 to 0.17)	0.94 (0.76 to 1.15)	0.11 (−0.23 to 0.23) Cusum test = 0.72	−0.07 (−0.11 to 0.025)	−0.4 (−0.47 to −0.32)	0.25 (0.18 to 0.33)

Table 7. Intercept, slope and residual standard deviation of Passing-Bablok regression as well as bias, lower and upper limit of agreement from de Bland–Altman plots and their 95% confidence intervals (95% CI) from the comparisons between the CZE and AGE electrophoresis, when data are expressed in g/L.

	Passing-Bablok regression			Bland–Altman plot		
Fraction	Intercept (95% CI)	Slope (95% CI)	Residual standard deviation (95% CI)	Bias (95% CI)	Lower limit (95% CI)	Upper limit (95% CI)
Albumin	−1.32 (−6.35 to 5.15)	0.97 (0.78 to 1.19)	1.50 (−2.94 to 2.94) Cusum test = 0.92	−0.90 (−1.47 to 0.33)	−5.04 (−6.02 to −4.06)	3.23 (2.25 to 4.21)
α1-globulin	0.86 (0.30 to 1.64)	1.007 (0.69 to 1.41)	0.65 (−1.28 to 1.28) Cusum test = 0.09	0.75 (0.51 to 1)	−1.01 (−1.44 to −0.59)	2.53 (2.1 to 2.59)
α2-globulin	−5.47 (−15.51 to 0.15)	2.31 (1.40 to 4.06)	0.80 (−1.56 to 1.56) Cusum test = 0.08	2.35 (1.95 to 2.71)	−0.43 (−1.1 to 0.24)	5.13 (4.45 to 5.8)
β-globulin	3.09 (−2.01 to 5.73)	0.62 (0.33 to 1.12)	2.11 (−0.15 to 4.15) Cusum test = 0.17	−0.45 (−1.31 to 0.4)	−6.61 (−8.08 to −5.13)	5.69 (4.21 to 7.16)
γ-globulin	−0.44 (−1.77 to 0.94)	0.81 (0.64 to 0.95)	1.13 (−2.21 to 2.21) Cusum test = 0.92	−1.92 (−2.37 to −1.47)	−5.16 (−5.93 to −4.4)	1.32 (0.55 to 2.08)
A:G ratio	0.263 (−0.006 to 0.48)	0.38 (0.26 to 0.53)	0.18 (−0.36 to 0.36) Cusum test = 0.17	−0.79 (−0.89 to −0.69)	−0.15 (−1.68 to −1.34)	−0.07 (−0.024 to 0.1)

methods is shown in Figure 2. The Passing-Bablok regression analysis shows that a significant negative bias ($p < .0001$) was found between AGE and CZE methods for α1-globulin, α2-globulin, and γ-globulin when data were expressed in percentage or concentration (g/L). The Bland–Altman plot analysis shows, for α1 and α2-globulins, a significant positive systematic bias between the CZE and AGE method ($p < .0001$) when data are expressed in percentage and concentration. For the γ-globulin fraction, a significant negative systematic bias between both methods was found ($p < .0001$) when data are expressed in percentage and concentration. In addition, a significant negative systematic bias was observed in the A:G ratio ($p < .0001$).

Table 8 shows Lin's concordance correlation for both CZE and AGE methods. The concordance between methods was considered moderate for albumin, γ-globulin, and A:G ratio. The concordance was considered unacceptable (<0.5) for α1-globulin, α2-globulin, and β-globulin.

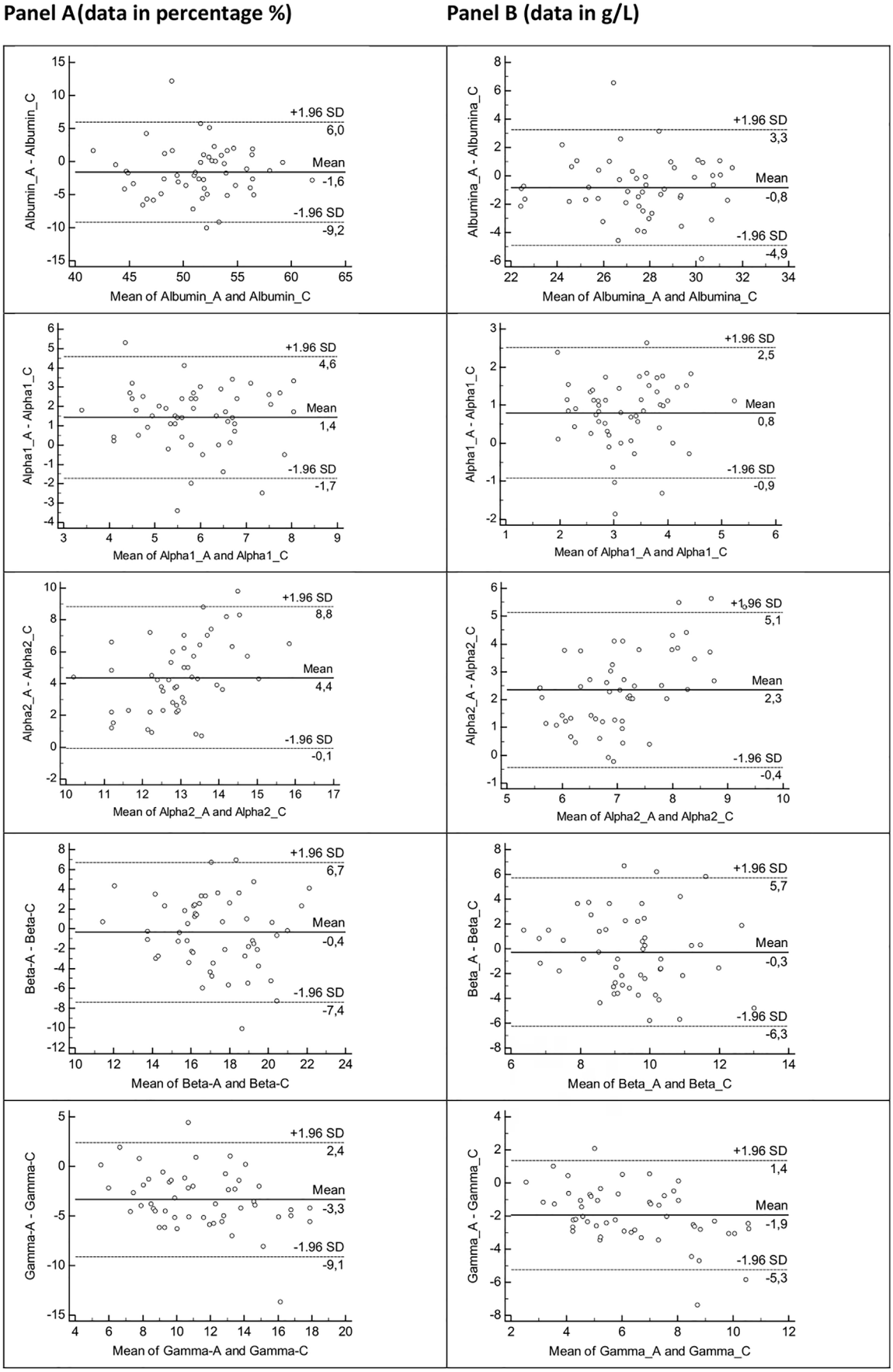

Figure 2. Bland–Altman plot comparison of CZE versus AGE for serum protein electrophoresis from mink. Panel a shows results as a percentage and panel B as concentration (g/L). The dashed lines mark the upper and lower limits of agreement; the solid line reflects the mean difference (bias).

4. Discussion

Serum protein electrophoresis is an invaluable tool for veterinary practitioners in domestic animal species when combined with haematology and serum biochemistry assays. This information will contribute to the identification, prognosis, and monitoring of various diseases. Similar advantages are desirable when evaluating the health status of wildlife, especially because inflammation and infection are important factors implicated in the survival and success of conservation strategies for endangered species (O'Brien and Evermann 1988).

The advantages of using samples from breeding centres or other controlled environments, such as those used in the present study, include the guarantee of using healthy animals and the availability of sufficient samples to establish de novo reference intervals. The American Society for Veterinary Clinical Pathology recommends that the de novo reference interval for veterinarian species should be calculated using sample sizes larger than 20 animals (Friedrichs et al. 2012). The 55 European minks used in this study had a complete and thoroughly monitored clinical history, which was sufficient to provide reference intervals.

Determination of TP is a simple method that provides valuable information for understanding the immune, health, and nutritional status of animals (Kaneko et al. 2008). In this sense, Farid and Rupasinghe (2022) found that infection with Aleutian mink disease virus (AMDV) increased the levels of TP and γ-globulins in American mink, which could help in the diagnosis of diseases and the identification of tolerant minks. The TP concentrations in this study were similar to those reported by Farid and Rupasinghe (2022) and Han et al. (2022) in a nutritional study of male minks. These authors did not find differences between sex and other variables, such as time and degree of tolerance (Farid and Rupasinghe 2022), or the level of protein or metabolic energy in the diet (Han et al. 2022). In other similar species such as pet ferrets, slightly higher values of 61 g/L (Ravich et al. 2015) and 63 g/L (Eshar et al. 2021) have been reported.

Table 8. Concordance correlation analysis between CZE and AGE electrophoresis.

Measured variables	Lin's r_{ccc} (95% CI)	Precision p	Accuracy C_b
Albumin (%)	0.727 (0.581 to0.827)	0.761	0.955
Albumin (g/L)	0.747 (0.609 to 0.841)	0.779	0.958
α1-globulin (%)	0.284 (0.097 to 0.452)	0.399	0.713
α1-globulin (g/L)	0.317 (0.129 to 0.453)	0.443	0.716
α2-globulin (%)	−0.017 (−0.093 to 0.059)	−0.06	0.285
α2-globulin (g/L)	0.069 (−0.006 to 0.143)	0.257	0.268
β-globulin (%)	0.375 (0.126 to 0.579)	0.378	0.991
β-globulin (g/L)	0.056 (−0.198 to 0.304)	0.059	0.948
γ-globulin (%)	0.635 (0.495 to 0.743)	0.812	0.782
γ-globulin (g/L)	0.721 (0.601 to 0.809)	0.869	0.824
A:G ratio	0.702 (0.549 to 0.809)	0.741	0.946

Notes: Values of Lin's $r_{ccc} < 0.5$ were considered unacceptable. Lin's concordance correlation coefficients (r_{ccc}) with 95% confidence intervals (95% CI), precision (Pearson p) and accuracy (bias correction factor, C_b) are reported.

In European minks, the serum protein fractions were separated, reference intervals were determined using two different protocols, and the degree of agreement between the two methods was determined. The number of fractions identified was similar between the two techniques; however, the peak sizes were slightly smaller when the CZE method was used. A similar result was obtained by Giordano and Paltrinieri (2010) analysing serum from healthy dogs and cats, for which the CZE method offered narrower and lower peaks than the AGE, and also more subpeaks in the α and β-globulin regions found, which could complicate the differentiation of monoclonal or polyclonal gammopathies.

Protein electrophoresis was previously performed on European minks (Sánchez-Migallón Guzmán et al. 2008). The authors applied protein electrophoresis using the cellulose acetate method to blood samples from 82 free-ranging European minks as part of an overall study evaluating Aleutian disease in free-ranging European minks from Navarra, Spain. Hypergammaglobulinaemia was defined as globulins exceeding 20% of the total plasma protein level; however, the study failed to mention how this threshold was determined. Information and protein fractions specific to healthy European minks are lacking, and laboratories tend to extrapolate values from closely related domestic species, such as ferrets. Using AGE methods in pet ferrets, the same protein fractions have been identified as in minks but with lower levels in the percentage of albumin and higher levels for those of β-globulins (Ravich et al. 2015). The interpretation of the existence of inflammation or the reaction of γ-globulins to infectious diseases in European mink is greatly limited due to this fact.

In this study, all minks were healthy. To determine whether one method or the other is better for detecting gammopathies, it is necessary to carry out a joint study using the two methods and with sick animals, especially those with inflammatory or chronic diseases (Eckersall and Bell 2010; Gori et al. 2022; Pierini et al. 2022). Aleutian disease, caused by a parvovirus and canine distemper, is known to infect European minks (Sánchez-Migallón Guzmán et al. 2008), and can result in alterations in total protein fractions in domestic species such as ferrets and dogs, usually causing hypergammaglobulinaemia. However, information on how these protein fractions are affected in European minks is currently lacking. However, the results and reference intervals established in this study set the basis for further investigations on pathologic diseases in European minks and the use of protein electrophoresis as a diagnostic tool.

Differences were observed in the protein fractions between males and females. Hormonal influences and the state of pregnancy or lactation in females can affect the electrophoretic profile (Kaneko et al. 2008). In pregnant females there is a migration of γ-globulins towards the mammary gland. In this study, no differences in γ-globulins were found, which could be explained because none of the

females was pregnant at the time of sampling. In other wild carnivores, such as genets (*Gennetta genetta*), higher levels of albumin were detected in males (Millán et al. 2015), similar to the results of our study. The differences that were found regarding age are considered normal since adult animals have more total proteins and increased γ-globulin which was observed in both the CZE and AGE methods (Kaneko et al. 2008). Young animals had higher percentages of α1 and α2-globulins detected by the CZE method. Many of the acute phase proteins (APPs) migrate in the α1 and α2-globulin regions and are a common finding in acute inflammatory diseases (Kaneko et al. 2008; Eckersall and Bell 2010). However, these animals were healthy, and no disease was found during the clinical examination; therefore, it can be considered that the differences found were not clinically significant.

CZE and AGE produce similar pherograms to those of other mammals, particularly dogs and cats (Giordano and Paltrinieri 2010; Jeffries et al. 2021). The differences observed in the peaks were due to the use of different software programs to identify the inflection points. However, both the techniques provided diagnostic information. In CZE, the proteins move towards the cathode, which allows for better separation of proteins and generates more subpeaks and narrower peaks. In this study, we have included reference intervals for β1 and β2-globulin fractions with the AGE technique, but these fractions are not used to interpret changes due to clinical diseases, such as in pet ferrets (Ravich et al. 2015) or dogs (Skeldon 2018). Multiple subpeaks can make it difficult to transition and identify between α and β or between β and γ-globulins, which is the major difference between the CZE and AGE method. An advantage of the high-resolution CZE method is its increased ability to identify biclonal gammopathies (Giordano and Patrinieri 2010). Similar observations have been reported in other studies in different species (Toonder et al. 2020; Comolli et al. 2022).

Passing-Bablok regression analyses and Bland–Altman difference plots demonstrated that the CZE and AGE methods for protein electrophoresis were not equivalent. Neither a high correlation coefficient nor a comparison of means as a measure of agreement between methods is appropriate and does not necessarily mean agreement, because a high correlation is no guarantee of good agreement (Bland and Altman 1999; Jensen and Kjelgaard-Hansen 2006). With the CZE method, more subpeaks were obtained, but in this study, the reference interval for CZE have been offered as albumin, α1, α2, β, and γ-globulins as they usually appear in the case of small animals (Crivellente et al. 2008; Paltrinieri et al. 2016; Villanueva-Saz et al. 2021) or pet ferrets (Ravich et al. 2015). Further studies using mink serum and the CZE technique are needed to identify the classes of proteins that are the subpeaks in the CZE profile. The results of the concordance analysis suggest that the methods are not comparable; hence, there is a need for reference intervals for each method. However, the analysis of protein fractions using the two methods provided similar results, but with significant biases and errors that may affect the clinical interpretation of the protein fractions.

A potential limitation of this study was the lack of a positive assessment related to a panel of different infectious diseases to determine the electrophoretogram associated with each infectious disease, and the small number of samples included from one location; therefore, the lack of diversity of habitat and diet could have affected the results. The strengths of this study include the clinical classification of healthy animals based on their clinicopathological characteristics (absence of evident clinical signs detected during physical examination and laboratory abnormalities).

5. Conclusions

Our study compared AGE and CZE techniques using sufficient blood samples from healthy European minks, which enabled the creation of objective reference intervals for each technique. However, our results show that the methods are not equivalent; therefore, the reference intervals must be considered and used accordingly, depending on the method used to separate mink blood proteins. Changes in the serum concentration of proteins indicate the need for a more detailed clinical evaluation of minks. The results obtained in this study serve to establish serum protein reference intervals for European minks and may contribute to the conservation of the species. Additionally, these results serve as a basis for future studies focusing on specific inflammatory diseases or infections in European minks.

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No potential conflict of interest was reported by the author(s).

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Data availability statement

The datasets supporting the conclusions of this study are included in this article. All analysed data are available from the corresponding author upon request.

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