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Monthly and Pregnancy-Related Concentration of Cu and Zn in Serum of Mares in an Equine Breeding Herd

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Abstract: Copper (Cu) and zinc (Zn) are trace minerals with multiple biological functions, playing roles in fetal development and immune regulation. Despite their known significance, research on Cu and Zn administration and supplementation for pregnant mares is insufficient. This study aims to evaluate Cu and Zn serum concentrations monthly throughout the year and through pregnancy in Polish Konik mares. The study was conducted in 2020 on 36 mares from the Polish state stud farm, with 24 mares in the pregnant group and 12 mares in the non-pregnant group. Monthly blood samples were collected, and serum Cu and Zn concentrations were measured. The total Cu concentration in the serum of all mares was higher ($p < 0.0001$) in August and September (13.98 ± 3.00 µmol/L) than in May, June, July, and November (11.04 ± 2.74 µmol/L). Similarly, the total Zn concentration was higher ($p < 0.0001$) in August, September, October, November, and December (19.80 ± 9.72 µmol/L) than in May, June, and July (14.50 ± 6.94 µmol/L). No evidence of a linear relation between Cu and Zn concentrations was demonstrated (slopes difference $p < 0.001$). No differences in Cu ($p > 0.05$) or Zn ($p > 0.05$) concentrations were found between pregnant (Cu: 12.08 ± 3.01 µmol/L; Zn: 18.02 ± 9.03 µmol/L) and non-pregnant (Cu: 11.23 ± 2.51 µmol/L; Zn: 18.59 ± 10.37 µmol/L) mares in any of the examined months of the year. In conclusion, the month of the year, rather than pregnancy, affects serum Cu and Zn concentrations.

Keywords: trace minerals; zinc; copper; gestation; season of the year; horses; livestock animal

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1. Introduction

Inorganic components of livestock animals' bodies, including those of horses, are a group of substances necessary for the proper functioning of the organism, such as copper (Cu) and zinc (Zn) [1]. The concentrations of Cu and Zn in the serum of pregnant mares have been the subject of recent research. While the effects of these minerals on equine metabolism are well known, their impact on pregnancy remains unclear. Pregnancy is a unique physiological condition marked by ongoing changes that influence the mother's metabolism. The mare's warmblood nutritional needs significantly rise in the third trimester, when most pregnancy-related illnesses are also more likely to manifest [2,3], as well as immediately after giving birth, due to milk production [4]. Moreover, equine hematological and biochemical profiles show various physiological changes taking place throughout pregnancy [3] and in lactation [5]. These changes during pregnancy are significant because stressors can affect the foal's size, cardiovascular and pituitary–adrenal function, carbohydrate levels, etc. [6,7]. The nutrition of the mare during pregnancy can also have a significant impact on the health of the foal [8]. Proper conceptus nutrition during the pregnancy affects the first months of life as well, allowing for the storage of more necessary compounds [8]. As metabolism and nutrition are crucial for fetal development, it is important to understand the role of minerals in the health of pregnant mares and their offspring, including Cu and Zn [1,6–11].

The differences in Cu and Zn concentrations throughout different months of the year in mares may have potential physiological effects. Cu and Zn are essential trace minerals involved in various metabolic processes in the body [11–18]. Cu is involved in plenty of cellular and metabolic processes. It is necessary for the proper myelination of neurons and the production of hemoglobin and keratin. Cu plays a role in enzymatic processes such as the elimination of free radicals, cell respiration, pigmentation, and development of connective tissue, which during gestation is crucial for a developing fetus [1]. The scope of Cu activity also encompasses antibody activation in immune processes, estrogen metabolism, keratin bridge formation in the hoof, and the enabling of iron (Fe) absorption [1,16,18]. Its deficiency may result in anemia, susceptibility to infections, growth retardation, deformation of joints and limbs, and reproductive disorders [1,15–17]. During gestation, Cu not only takes part in proper fetal development, but is also stored in the fetal liver, which is used after parturition, as mares' milk is not a sufficient source [1]. Given Cu's function in the formation of connective tissue, Cu availability may affect the prevalence of osteochondrosis in foals. Numerous experimental studies have provided evidence for a relationship between Cu in the diet and osteochondrosis [9,19].

Zn is an important factor regulating hundreds of enzymes, e.g., superoxide dismutase, liver dehydrogenase, and alkaline phosphatase. It affects a plethora of processes, from the most fundamental, such as DNA and RNA replication in cell division; through tissue repair mechanisms, fetal development and growth, and immune system functioning; to reproduction and providing horn strength and hardness [1,12]. Thus, an insufficient supply can result in lower immunity, laminitis, insulin resistance, fetal maldevelopment, and skin and coat disorders.

The majority of feeding guidelines for pregnant mares have been based on the nutrient deposition estimates in the fetus that Meyer and Ahlswede reported in 1978 [20]. Mineral absorption from eating plants or plant products does not always result in sufficient absorption. Extra supplements are frequently needed [1]. The soil type, plant species, stage of vegetative development, fertilizer, and irrigation all impact the mineral content of hay [21,22]. Because they vary depending on the type of grass, the area, and the time of year, it is impossible to make exact suggestions regarding the requirement for mineral supplementation. Numerous fresh forages will satisfy the mare's macromineral requirements for K, calcium (Ca), and phosphorus (P) during pregnancy, but they may be deficient in sodium (Na) and a few trace elements, such as selenium (Se), Cu, and Zn [20–23]. On the other hand, acidic soils, Fe abundance, and Cu deficiency cause low Cu values in plants. Fe additionally causes a decrease in Cu absorption in the gastrointestinal tract [15,24]. In the case of Zn, alkaline soils with high Ca contents diminish plant absorption and result in lower daily Zn intake in horses. While Ca is supplemented in lactating mares, it can cause lower Zn absorption and deficiency.

According to the sources, the normal ranges of Cu and Zn concentrations in the serum of horses can vary depending on the study and the population of horses being evaluated. However, some studies have reported the following normal ranges: Cu: 0.56–1.77 µg/dL [22,25,26] and Zn: 7.48–40.20 µg/dL [22,25,26]. It is important to note that these ranges may not apply to all populations of horses and that the interpretation of Cu and Zn concentrations should take into account factors such as age, breed, and diet. As Cu and Zn have an antagonistic relationship, it is important to balance their intake, and the ratio of Cu:Zn should be 1:3 or 1:4 [1,27]. According to Lawrence, the Cu requirement in mares (500–600 kg b.w.) in the first eight months of pregnancy is 100–120 mg/day, and in the following months of pregnancy, it is 125–150 mg/day [28]. Wichert et al. reported that the mean Cu concentration in biological substrates of horses was within the normal range expected for horses, with values between 1.04 and 1.77 µg. It has been shown that adult horses tolerate high doses of Zn, even up to 500 mg/kg of dry matter [25]. The digestibility is considered to be around 20%, although the content of other minerals and the digestive capability of an animal may alter this value. The Zn dosage has to be adapted to the individual requirements of an animal. Especially in sport horses, lower serum Zn values indicate increased mineral losses through sweating [14]. The supplementation of this element in more sensitive foals needs additional consideration. Foals receiving a high dose of this element may react with symptoms of Cu deficiency (e.g., tendon contractures, osteochondrosis). Also, when arranging nutritional doses for mares in foal, attention should be paid to high doses of Zn—it is likely to increase in concentration in the fetal liver. Contrary to Cu, toxicity is unlikely to occur, even in the case of oversupply, although the digestion of other minerals may be hampered [1]. The zinc requirement for pregnant mares is 400–800 mg/day throughout pregnancy [28]. Ali et al. suggested that deficiencies in Cu and Zn, as well as other minerals, may be considered among the possible causes of infertility in mares [10].

The Cu and Zn serum concentrations of Polish Konik mares during pregnancy and the subsequent months of the year have not been extensively studied. Studies regarding general seasonal variations in Cu and Zn concentrations are inconsistent; however, it seems that the occurrence of variability in their concentration is dependent mostly on the content in feed, which is commonly lowered in the autumn season [11,13,15,16]. Gaining knowledge about these minerals' concentrations is crucial for providing the highest level of care and medical attention to breeding mares. These findings can serve as valuable guidance for veterinarians and breeders, enabling a better evaluation and significance of these minerals in equine pregnancy and potentially leading to improved nutritional and management practices for both pregnant mares and their offspring. Therefore, this study aims to fill this gap. The main objective of this study was to analyze changes in the concentrations of Cu and Zn in the serum of mares from one equine breeding herd throughout the months of pregnancy and the year.

2. Materials and Methods

2.1. Breeding Mares' Housing

The subjects of the study were mares housed in the equine breeding herd at the Polish state stud farm, Dobrzyniewo. In this breeding herd, the basic herd consisted of 90 mares. All mares represented the Polish Konik breed and were included in the conservation breeding of the Polish Konik horses program. All mares were housed in stalls with the same management in an all-day open stable with daily access to a large area of grass pasture for at least 12 h per day. During the spring, summer, and autumn seasons, the primary source of nutrition for the horses was fresh grass. Additionally, in the spring and autumn, pasture feeding was supplemented with hay, while during the winter, the diet primarily consisted of hay. The hay was produced on-site and sourced from a meadow adjacent to the horse pastures. From the official nutritional documents of the stud farm, the grassy hay Cu and Zn content ranged from 5 to 6 mg Cu/kg and from 35 to 40 mg Zn/kg. According to the estimates of the main horse breeding manager at the stud farm, each mare ate from 2 to 3 kg of hay per day in the spring and autumn, as well as from 4 to 6 kg of hay per day in the winter. Throughout the year, the breeding mares received a consistent dose of oats, adjusted to maintain their optimal and healthy conditions without causing obesity. The oats were also home-produced and obtained from a field neighboring the horse pastures and meadows. The contents of Cu and Zn in grass and oats were not reported. The mares had continuous access to fresh water and mineral licks containing 1000 mg/kg of Cu and 5000 mg/kg of Zn. The mares did not receive any other concentrates or supplements.

2.2. Breeding Mare Grouping

The first inclusion criterion for all mares was the absence of clinical signs of diseases or any abnormal conditions, as determined through a basic clinical examination. The basic clinical examination followed international veterinary standards and assessed internal temperature, heart rate, respiratory rate, mucous membranes, capillary refill time, and lymph nodes. Only mares with no clinical signs of disease were included in the study. Additionally, the mares' overall conditions were assessed by palpation and visually based on a 5-point body condition score (BCS) scale, where a scale of 1 was poor and a scale of 5 was obese. Only mares assessed as 2 or 3 on the BCS scale were included in the study. The second inclusion criterion for mares with no clinical signs of disease was that they were multiparous and that lactation was present.

Out of the 90 mares in the breeding herd, 64 mares met these criteria, as they had no clinical signs of diseases in previous seasons and had successfully given birth to at least one foal, maintaining normal lactation that season.

Out of 64 mares that met the first two criteria, 12 mares were not mated this year due to the breeding assumptions of the stud farm. These mares, aged 4–9 years (mean age 6.42 ± 1.79 years; mean height 143.10 ± 2.09 cm), were included in the non-pregnant group. The remaining 52 mares were naturally mated between January and April. If the 1st mating in January was unsuccessful, the mares were re-mated in February. If the 2nd mating in February was also unsuccessful, the mares underwent another mating attempt in March. If the 3rd mating in March was also unsuccessful, the mares were once again mated in April. However, if the 4th mating in April failed, those mares were excluded from the study. The pregnancy confirmations were carried out by detailed examination of the reproductive tract at 14 and 35 days post-ovulation. The uterus and ovaries were evaluated ultrasonographically following international veterinary standards using MyLabOne ultrasound (ESAOTE, Florence, Italy) and a 5 MHz linear probe (ESAOTE, Florence, Italy). After four matings, pregnancy was confirmed in 24 mares. These mares, aged 4–10 years (mean age 6.29 ± 1.92 years; mean height 142.40 ± 2.12 cm), were included in the pregnant group. The remaining 28 mares that were mated in the following months were excluded from the study. The total group consisted of 36 mares (Figure 1). The sample size was calculated by determining the 95% confidence interval for the expected mean Cu concentration value in a reference range [22,25,26] from 0.56 µg/mL (8.81 µmol/L) to 1.77 µg/mL (27.85 µmol/L).

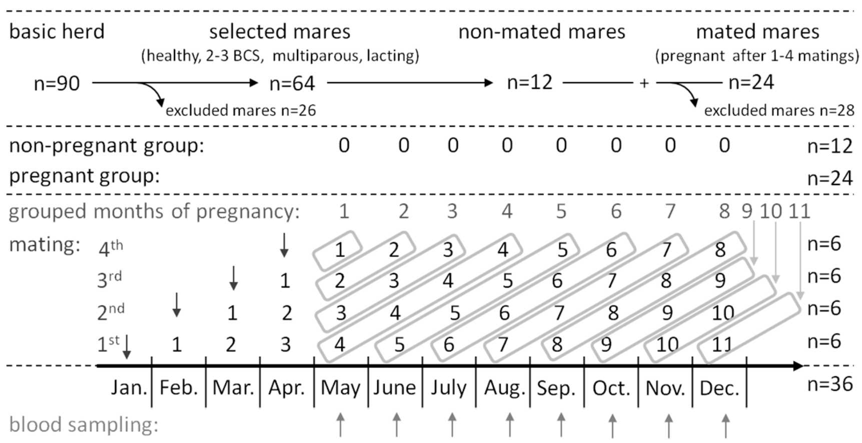

Figure 1. Study design regarding the selection of mares for the non-pregnant (n = 12) and pregnant (n = 24) groups from the basic herd (n = 90), and mares grouped within the pregnant group, throughout the year. The study design includes four matings in January, February, March, and April and eight blood samplings conducted monthly from May to December.

2.3. Sample Collection

Blood samples were collected from all included mares once a month, from May to December, in the morning between 8 a.m. and 10 a.m. Blood samples were not collected from January to April due to coronavirus (COVID-19) restrictions. Blood sample collection was completed after the last mare of the pregnant group had foaled. Blood samples were acquired by a jugular venipuncture using a BD Vacutainer® system with dry tubes (BD Plymouth, Plymouth, UK). Blood samples were cooled (+4 °C) and transported to the laboratory within 5 h. In the laboratory, tubes were centrifuged (2000× *g*, 5 min) and hemolysis-free serum was aspirated. Serum samples were stored (−20 °C) in Eppendorf safe-lock tubes (Bio-Rad Sp. z o.o., Warsaw, Poland) for further Cu and Zn concentration analyses.

2.4. Sample Grouping

Raw numerical data obtained from the Cu and Zn concentration analysis were grouped twice according to the month of the year as well as to the month of the pregnancy in the pregnant group. Data were grouped throughout the months of the year in May to December subgroups for the total group, pregnant group, and non-pregnant group separately. Specific climatic data for this period are summarized in Table 1.

Table 1. Specific climatic data for the Kuyavian-Pomeranian Voivodeship in Poland in the period from May to December 2020, including average air temperature, average rainfall, average humidity, rainy days, and average sunny hours per day (https://pl.climate-data.org/europa/polska/kuyavian-pomeranian-voivodeship/, accessed on 12 December 2023).

Data/Months	May	June	July	August	September	October	November	December
Temperature (°C)	14.1	17.4	19.4	19.2	14.7	9.5	4.9	1.0
Rainfall (mm)	40	61	66	88	64	54	46	43
Humidity (%)	66%	65%	69%	69%	73%	80%	87%	85%
Rainy days (day)	8	8	9	8	7	7	7	8
Sunny hours (h)	10.5	11.0	10.9	10.2	7.5	5.0	3.0	2.3

Data were grouped, based on the month of the pregnancy in the pregnant group, into eleven subgroups from the 1st to 11th month of pregnancy. In the group of pregnant mares, the first mare foaled on 19 December 2021, and the last one on 9 March 2022.

The relevant non-pregnant group was created so that the non-pregnant May group would correspond to the pregnant group for the 1st month, the non-pregnant group of May and June would correspond to the pregnant group for the 2nd month, etc., until December, according to the scheme in Figure 1.

2.5. Cu and Zn Concentrations

The Cu and Zn concentrations in serum were measured using a flame atomic absorption spectrometer with deuterium background correction (Avanta PM, GBC, Melbourne, Australia). The biochemical assays were conducted following the standard protocols. The concentration of Cu was determined by wavelength: 324.8 nm, slit width (nm): 1.3, lamp current (mA): 7.5. The concentration of Zn was determined by wavelength: 213.9 nm, slit width (nm): 1.3, lamp current (mA): 5.0. The calibration curves were prepared based on the standard solutions of Cu and Zn, which were prepared from a concentration of 1000 mg/L (Merck, Darmstadt, Germany). Concentrations of the standard solutions for the Cu calibration curve were 10, 20, and 40 µg/L. Concentrations of the standard solutions for the Zn calibration curve were 0.5, 1.0, and 1.5 mg/L. Serum samples (5 mL) were deproteinized with 1 mol/L Nitric acid (prepared from 69% Suprapur®, Merck, Darmstadt, Germany) and diluted to a 1:5 ratio with 0.15% m/v Triton X-100 (laboratory-grade, Sigma-Aldrich, St. Louis, MO, USA). Then, samples were centrifuged (2000× *g*, 10 min). Each sample was tested in duplicate. The result was presented as the average of two measurements.

The method's reliability was controlled by analyzing the series of samples from a certified reference material, Seronorm Trace Elements Serum L-2 (Sero Inc., Billingstad, Norway). Recoveries between 90 and 110% were accepted to validate the calibration for both elements. The accuracy was 1.3% for Cu and 1.4% for Zn, and the coefficient of variation was 2.5% for Cu and 2.7% for Zn. The detection limit was 0.00058 mg/L for Cu and 0.011 mg/L for Zn.

2.6. Statistical Analyses

The statistical analyses were performed using GraphPad Prism6 software (GraphPad Software Inc., San Diego, CA, USA), starting from Gaussianity testing of the data series. Univariate marginal distributions of the data series were tested for Cu and Zn concentrations in examined mares (total group), pregnant mares (pregnant group), and non-pregnant mares (non-pregnant group), for all months of the year and months of pregnancy independently. The significance level was established as $p < 0.05$. Numerical data are presented on plots as the mean ± SD.

First, the distribution of features was examined to select an appropriate statistical test. For this purpose, a Shapiro–Wilk normality test was used.

Next, to address the research question on changes in the concentrations of Cu and Zn throughout the year, trace mineral concentrations were compared between months of the year. The Cu concentration in the total group was compared using repeated measures one-way ANOVA, followed by the Friedman test due to the normal distribution of all compared data series. The Zn concentration in the total group was compared using the Friedman test, followed by Dunn's multiple comparisons test due to the non-normal distribution of at least one data series. The Cu concentrations in the pregnant and non-pregnant groups were compared using repeated measures one-way ANOVA, followed by the Friedman test due to the normal distribution of all compared data series. The Zn concentrations in the pregnant and non-pregnant groups were compared using the Friedman test, followed by Dunn's multiple comparisons test due to the non-normal distribution of at least one data series.

Next, to address the research question on changes in the concentrations of Cu and Zn throughout the pregnancy, trace mineral concentrations were compared between months of the pregnancy. Cu and Zn concentrations were compared using the Kruskal–Wallis test, followed by Dunn's multiple comparisons test due to the non-normal distribution of at least one data series. Data series representing pregnant and non-pregnant groups were compared using an unpaired t-test with Welch's correction when both data series were normally distributed, and using the Mann–Whitney test when one data series was non-normally distributed.

Finally, to address the question on the relation between trace mineral concentrations, linear regressions between Cu and Zn were calculated throughout the months of the year and the months of pregnancy. Linear regressions were calculated for the following pairs of trace mineral concentrations data series: Cu/Zn in the total group, Cu/Zn in the pregnant group according to the month of the year, Cu/Zn in the non-pregnant group according to the month of the year, Cu/Zn in the pregnant group according to the month of pregnancy, and Cu/Zn in the related non-pregnant group. Numerical data were presented on plots by the regression equation and r square. In the first step, the slopes were compared. Differences between the slopes were significant at $p < 0.05$. In the second step, the intercepts were compared; however, this was only performed when the slopes were not significantly different. For those slopes, one slope was calculated. Differences between the intercepts were significant at $p < 0.05$.

3. Results

The total Cu concentration in the serum of all examined mares differed ($p < 0.0001$) throughout the months of the year. Cu concentrations were higher in August and September than in May, June, July, and November, with no differences between the periods of October and December (Figure 2A). The cumulated Cu concentration (mean ± SD) for August/September was 13.98 ± 3.00 μmol/L, and for May/June/July/November, 11.04 ± 2.74 μmol/L. The total Zn concentration in the serum of all examined mares also differed ($p < 0.0001$) throughout the months of the year; however, in different specific periods. The Zn concentration was higher in August, September, October, November, and December than in May, June, and July (Figure 2B). The cumulated Zn concentration for August/September/October/November/December was 19.80 ± 9.72 μmol/L, and for

May/June/July, 14.50 ± 6.94 µmol/L. However, no evidence of a linear relation between the Cu and Zn concentrations was demonstrated (Figure 2C).

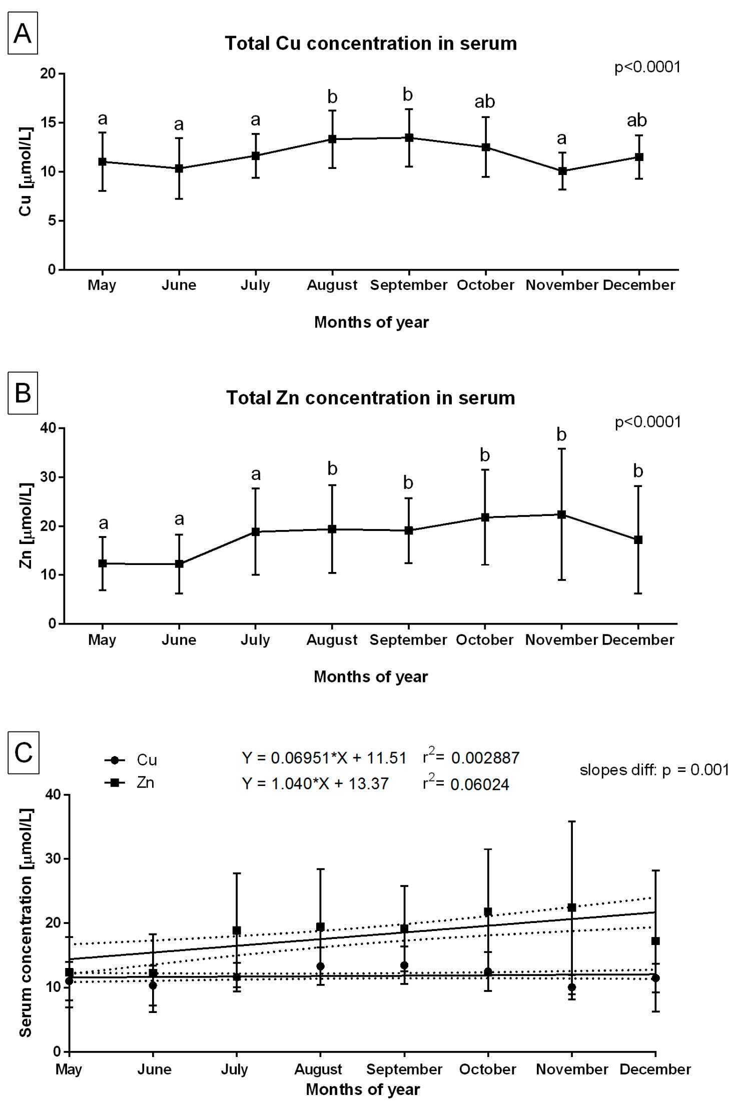

Figure 2. Total Cu (**A**) and Zn (**B**) concentrations (mean ± SD) in the serum of all examined mares in consecutive months of the year. Lowercase letters (a, b) indicate differences between months at $p < 0.05$. Linear regressions (regression equation and r square) between Cu and Zn concentrations in the serum of all examined mares (**C**) in consecutive months of the year. The difference between the slopes is significant at $p < 0.05$.

The Cu concentration differed throughout the months of the year in the serum of pregnant mares ($p = 0.0002$), but not in non-pregnant mares ($p = 0.09$). In the pregnant group, the Cu concentration was higher in August and September than in May, June, July, and November, with no differences between October and December and the other months. No differences ($p > 0.05$) were found between the pregnant and non-pregnant groups in any individually examined month of the year (Figure 3A). The Zn concentration differed throughout the months of the year in the serum of both pregnant ($p = 0.0007$) and non-pregnant ($p = 0.006$) mares. In the pregnant group, the Zn concentration was higher in September, October, and November than in May and June, with no differences between June, August, and December and the other months. In the non-pregnant group, the Zn concentration was higher in July, August, September, October, November, and December than in May and June. Similarly to the Cu concentration, no differences ($p > 0.05$) were found between the pregnant and non-pregnant groups in each individually examined month of the year (Figure 3B). In the pregnant group, no evidence of a linear relation between the Cu and Zn concentrations was noted (Figure 3C); however, in the non-pregnant group, Cu and Zn showed no different linear behavior ($p = 0.1$) with the common slope of regression value (0.459) (Figure 3D).

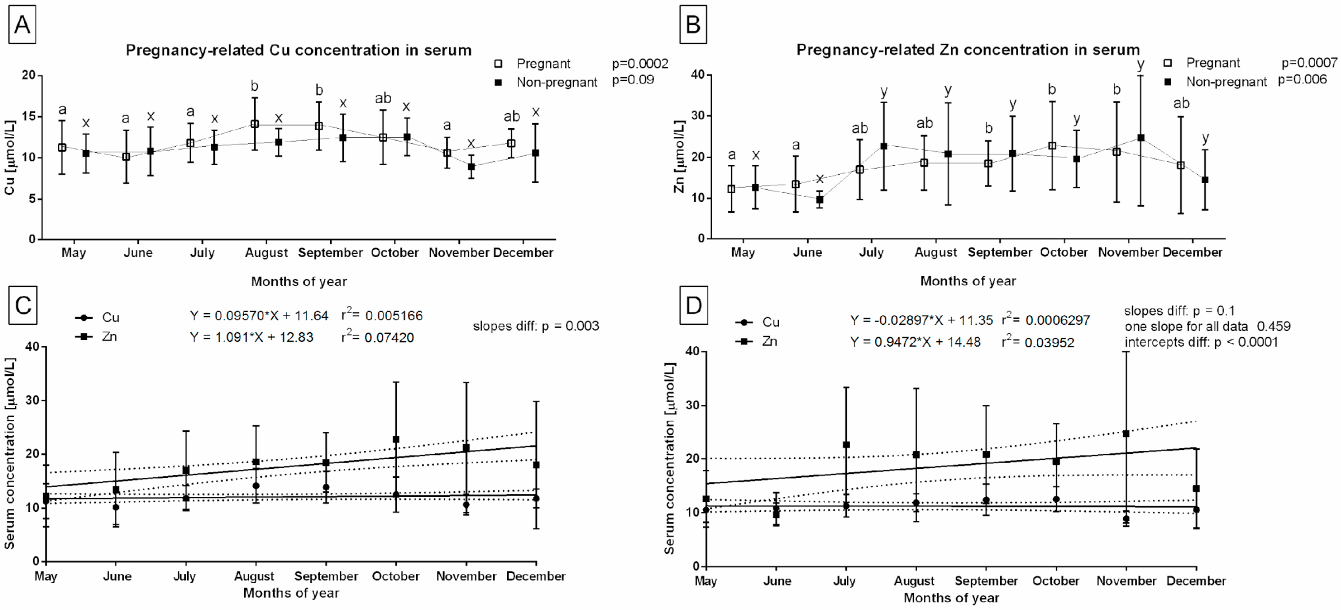

Figure 3. Pregnancy-related Cu (**A**) and Zn (**B**) concentrations (mean ± SD) in the serum of pregnant and non-pregnant mares examined in consecutive months of the year. Lowercase letters indicate differences between months at $p < 0.05$ for pregnant (a, b) and non-pregnant (x, y) groups separately. Linear regressions (regression equation and r square) between Cu and Zn concentrations in the serum of pregnant (**C**) and non-pregnant (**D**) mares examined in consecutive months of the year. Differences between the slopes and intercepts are significant at $p < 0.05$. For slopes that were not significantly different, one slope was calculated.

The Cu concentration did not differ throughout the months of pregnancy in the serum of mares in either examined group ($p = 0.1$) (Figure 4A). The cumulated Cu concentration for the pregnant group was 12.08 ± 3.01 µmol/L, and for the non-pregnant group, it was 11.23 ± 2.51 µmol/L. However, the Zn concentration differed throughout the months of pregnancy in both the pregnant group ($p = 0.02$) and the corresponding non-pregnant group ($p = 0.006$). In the pregnant group, the Zn concentration was higher in the 8th and 11th months of pregnancy than in the 1st, 2nd, 3rd, 4th, and 5th months of pregnancy, with no differences between the 6th, 7th, and 9th months of pregnancy. In the non-pregnant group, the Zn concentration was compared between periods corresponding to months of pregnancy in pregnant mares. The Zn concentration was higher in the 6th, 7th, and 8th months than in 1st and 2nd months, with no differences between 3nd, 4th, 5th, 9th, 10th, and 11th months (Figure 4B). The cumulated Zn concentration for the pregnant group was 18.02 ± 9.03 µmol/L, and for the non-pregnant group, it was 18.59 ± 10.07 µmol/L. For both the Cu and Zn concentrations, no differences ($p > 0.05$) were found between the pregnant and non-pregnant groups in any of the examined months of pregnancy and corresponding periods. Moreover, no evidence of linear relations between Cu and Zn concentrations was observed in either the pregnant (Figure 4C) or non-pregnant (Figure 4D) group.

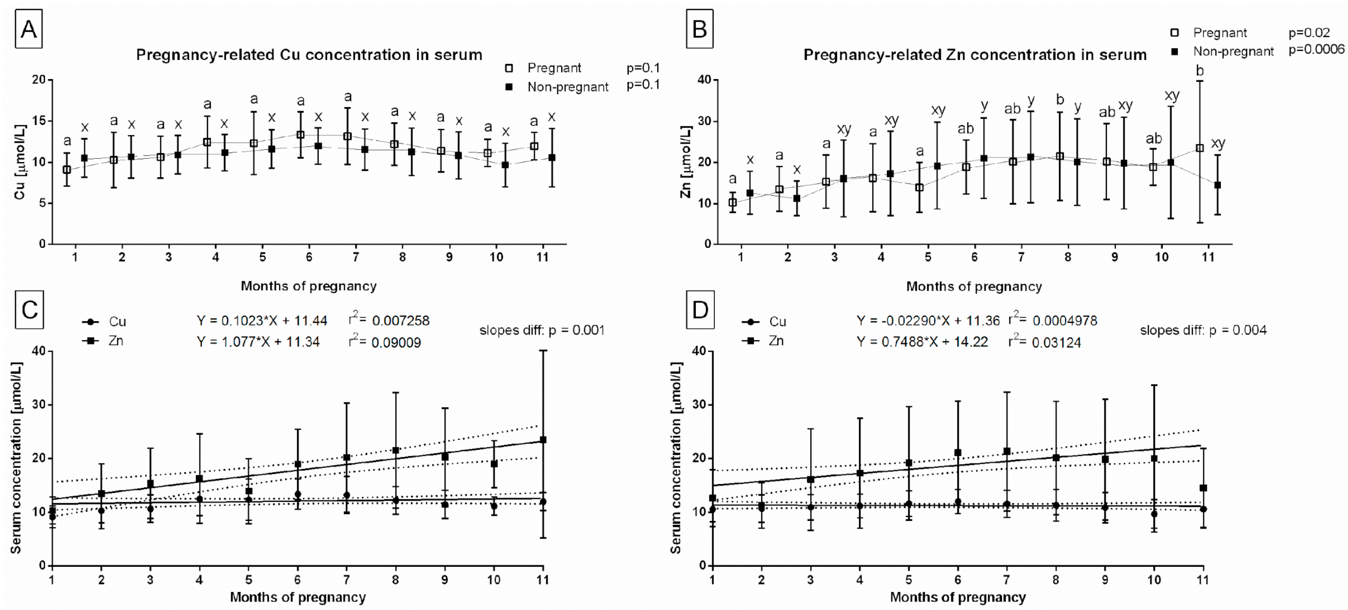

Figure 4. Pregnancy-related Cu (**A**) and Zn (**B**) concentrations (mean ± SD) in serum of pregnant and non-pregnant examined mares in consecutive months of pregnancy. Lowercase letters indicate differences between months at $p < 0.05$ for pregnant (a, b) and non-pregnant (x, y) groups separately. Linear regressions (regression equation and r square) between Cu and Zn concentrations in the serum of pregnant (**C**) and non-pregnant (**D**) examined mares in consecutive months of pregnancy. Differences between the slopes and intercepts are significant at $p < 0.05$.

4. Discussion

4.1. The Most Relevant Results

As the current study aimed to evaluate monthly Cu and Zn serum concentrations throughout the year and during pregnancy, the most relevant results are summarized as follows. The serum Cu and Zn concentrations varied throughout the months of the year; however, this variation did not differ between the pregnant and non-pregnant groups in any individually examined month of the year. Therefore, concerning all studied mares, one may observe that the Cu concentration was higher in late summer and early autumn (August and September) compared to spring (May and June), early summer (July), and late autumn (November), whereas Zn concentration was higher in late summer (August), autumn (September, October, and November), and winter (December) compared to spring (May and June) and early summer (July). Moreover, the Cu concentration remained consistent throughout the months of pregnancy, while the Zn concentration exhibited variations. The Zn concentration was higher in the third trimester of pregnancy compared to the first trimester. Thus, one may carefully conclude that, in an equine breeding herd kept in an extensive breeding system, the Cu and Zn concentrations appear to be affected by seasonality, but not by reproductive status.

4.2. Cu and Zn Concentrations in Mares' Serum

Based on the available search results, specific data on the serum Cu and Zn concentrations in mares during pregnancy and postpartum are very limited. However, recent studies have focused on the serum mineral profiles of mares during various reproductive phases. According to Ali et al., serum Cu concentration is significantly higher in pregnant mares than in subfertile and fertile mares, and serum Zn concentration is higher in cyclic mares than in pregnant and lactating mares [10]. Their study evaluated the serum mineral profile in various reproductive phases of mares. Although the specific findings regarding Cu and Zn concentrations were not mentioned, the study concluded that the last third of the pregnancy, foaling, and lactation period exerts a major influence on the biochemical constituents of serum [10].

Horse serum can include different amounts of Cu and Zn depending on the breed, age, and presence of clinical signs of diseases. The normal ranges of Cu and Zn concentrations in horse serum have been examined in many studies. One study conducted in the United Kingdom found marked differences in the mean serum Cu and Zn concentrations among thoroughbreds housed in different stables [29]. Another study in southeast Queensland assessed the Cu and Zn statuses of farm horses and training thoroughbreds, reporting a plasma Cu concentration of 0.56 ± 0.20 µg/mL (8.81 ± 3.15 µmol/L) and a plasma Zn concentration of 0.47 ± 0.11 µg/mL (7.19 ± 1.68 µmol/L) in brood mares at pasture [26]. Another study conducted in Bavaria evaluated the intake and status of Cu, Zn, and Se in horses, reporting a mean Cu concentration within the normal range expected for horses [25]. Furthermore, a study conducted in Urmia assessed the serum trace mineral concentration in horses and mules, reporting a mean serum Cu concentration of 14.5 µg/dL (22.8 µmol/L) and a mean serum Zn concentration of 7.48 µg/dL (11.4 µmol/L) [30]. One may observe that all currently reported Cu concentration values have been within the normal range from 0.56 µg/mL (8.81 µmol/L) to 1.77 µg/mL (27.85 µmol/L) [22,25,26]. Moreover, all currently reported Zu concentration values have been within the normal range from 7.48 (11.4 µmol/L) to 40.20 µg/dL (61.5 µmol/L) [22,25,26], which suggests a lack of Cu and Zn deficiencies in the examined mares. It is important to note that these studies provide a range of values for Cu and Zn concentrations in the serum of horses; the specific normal ranges may vary, and continuous individual monitoring could be helpful. Therefore, it is recommended to consult with a veterinarian for specific information on the normal ranges of Cu and Zn concentrations in the serum of horses and to evaluate individual physiological ranges. Regarding the impact of mares' nutrition during pregnancy on foal health, it is important to consider the role of minerals such as Cu and Zn. While there may not be specific studies directly linking the concentrations of Cu and Zn in the serum of mares to foal health, it is known that these minerals play crucial roles in various physiological processes, including growth, development, and immune function.

Based on the information provided, the normal ranges of Cu and Zn concentrations in the serum of horses can vary throughout different months of the year and during pregnancy. The Cu concentrations in the serum of all examined mares differed throughout all months of the year, with higher levels in August and September compared to May, June, July, and November. The Cu concentrations in the serum of pregnant mares also differed throughout all months of the year, with higher levels in August and September compared to other months. The Zn concentrations in the serum of all examined mares differed throughout all months of the year, with higher levels in August, September, October, November, and December compared to May, June, and July. These variations could be caused by changing mineral contents in plants constituting fodder. In the summer months, plant absorption of Cu and Zn is higher, possibly resulting in a delayed increase in serum concentrations; the lowest values are reached in spring [31]. Considering the geographical location of the studied herd in the Kuyavian-Pomeranian Voivodeship in the northwest of Poland and the climatic data summarized in Table 1, one may observe higher Cu and Zn blood concentrations in the months following a period of high average air temperatures, moderate humidity, and long sunny days. As climate could significantly impact trace mineral concentrations [32], potential environmental factors, especially seasonal variations in forage, that might affect Cu and Zn concentrations in mares should be considered.

No differences in Cu concentration were found between pregnant and non-pregnant mares in any examined month of the year. No evidence of a linear relation between Cu and Zn concentrations was observed. The Zn concentrations in the serum of pregnant mares differed throughout all months of the year, with higher levels in September, October, and November compared to May and June. The Zn concentrations in the serum of non-pregnant mares differed throughout all months of the year, with higher levels in July, August, September, October, November, and December compared to May and June. No differences in Zn concentration were found between pregnant and non-pregnant mares in any examined month of the year. While supplementation may be favorable for foal development, serum concentration does not vary between pregnant and non-pregnant mares, suggesting that mares' demand is rather stable. However, to draw more precise conclusions, an evaluation of liver supply throughout pregnancy would be useful [33–35]. These findings may carefully imply that pregnancy has a degree of impact on Cu and Zn [2,3] metabolism which is within the reference variability range. It can be suspected that the supply of Cu and Zn in the feed is sufficient to cover the needs of both pregnant and non-pregnant Konik Polski mares.

Furthermore, no evidence of a linear relation between the Cu and Zn concentrations was observed. The obtained result indicates that neither the Cu nor the Zn concentration changed linearly or changed linearly with a different slope of the curve. Upon careful examination of the regression curves, it can be concluded that the first postulate is valid. Further research should be conducted to fit another regression model, such as a regression curve, to depict changes in the concentration of the studied trace minerals over an extended period. Consequently, the biological significance of this observation cannot be unambiguously assessed at this point.

4.3. Limitations

The study preliminarily covered the area of the needs of pregnant mares in terms of selected microelements; however, the proposed experimental design suffers from many limitations. First of all, it cannot be clearly stated that the Konik Polski herd represents all breeding mares or that it is universal for assessing the dynamics of monthly and pregnancy-dependent changes in the concentrations of selected microelements in the serum. The Konik Polski breed represents a hardy breed of horses [36], which was selected due to its extensive breeding system [37]. The mares in the studied stud farm did not receive concentrated or large doses of concentrated feed, and their diet was based primarily on own-produced grass and hay. In warmblood breeding studs, the sport and racing mares are fed in a more diversified way that is individually adapted to the needs of the mare [38–40], which makes grouping much more difficult and complicates the selection criteria. Nevertheless, examining another herd of horses of the same breed that is kept in a similar system, but in a different location, would be advisable to assess the repeatability of the results.

The second major limitation pertains to the lack of a comprehensive analysis of the composition and quantity of the feed consumed. The amount of feed consumed by each mare was estimated by the knowledgeable herd staff, but a more precise evaluation of the feed intake is necessary. Additionally, it is crucial to precisely determine the content of Cu and Zn in grass, grassy hay, and oats, as low or high dietary intake of these minerals can significantly impact the serum results, as indicated in previous studies [1,21–23,25,26]. Furthermore, the use of mineral licks should be quantified or restricted for the duration of the study and a period of several months before the study. However, considering that the nutrition of the mare during pregnancy has a significant influence on the health of the foal [8], the manager of the equine breeding herd could not impose such a restriction due to its potential adverse effects. This limitation was imposed by organizational challenges and COVID-19 restrictions. As these factors were not detailed in the presentation of the study, the results should be considered preliminary and illustrative. Therefore, further research is required that takes into account the chemical composition of each consumed feed type, including parameters such as crude protein, ether extract, crude fiber, exact trace mineral content, and ash [41], as well as the palatability, and, thus, feed intake, of each consumed feed type [42].

Moreover, due to COVID-19 restrictions, blood sampling was conducted from May to December, excluding January, February, March, and April. Consequently, the findings presented herein do not fully capture the annual variations in the examined trace minerals. Further research is needed to augment our understanding of the winter and early spring periods, during which high-quality hay plays a pivotal role in horse nutrition [22,31,43]. This will enable the reported patterns to be applied to research on the nutrition of ponies [44] and horses, including primitive [36], warm-blooded [21,26,45], and cold-blooded breeds [41,46], as well as other equids [5,30,47]. Since these factors were not extensively detailed in the study's presentation, the results should be considered preliminary and illustrative.

Furthermore, the age of the mare and her parity number should be considered when comparing the results, as previous studies have indicated variations in the concentrations of both Cu and Zn depending on the mare's age [48,49]. This variability may help to explain the high degree of variance observed within the groups in this study. However, it is worth noting that age-dependent grouping was not conducted in this study due to the limited sample size (another limitation), which prevented meaningful statistical analysis. In defense of the small sample size, it is important to highlight that the research was conducted on the largest equine breeding herd of Konik Polski horses in Poland. Therefore, conducting a study on a larger group would require considering multiple horse breeds and more than one stud farm, introducing additional variables and making it more challenging to evaluate these preliminary data.

4.4. Further Directions

Subsequent studies should address all the limitations described in this study. A further study involving a larger group of mares, considering both primiparous and multiparous mares to account for the effects of age and the number of foals, should be planned. Additionally, the research should cover an extended period of time, with more frequent blood and feed sampling. This is particularly crucial because a significant portion of Cu and Zn is stored in visceral organs such as the liver [50,51]. In terms of Cu and Zn serum homeostasis and their relationship to liver stores, it is important to note that a deficiency in these microelements may not become apparent until after a prolonged period of nutritional restrictions. Therefore, future research efforts should expand in terms of time, sample size, and horse breeds, and improve the study design to enable a comprehensive investigation across various horse breeds. This would allow for comparisons of results obtained within the same experimental model.

Furthermore, for the practical implementation of the findings from this study, it is essential to consider the economic implications of potential supplementation strategies. In extensive farming systems, exemplified by the Konik Polski herd in this study, mares are expected to meet 80% of their overall nutritional needs during the 7-month grazing period [4]. In slightly more intensive farming systems, winter feeding involves hay and low concentrate supplementation [4]. Despite extensive feeding [4,36], the examined mares did not exhibit deficiencies in Cu or Zn, suggesting that this robust breed [37] of horses may not require additional supplementation of these trace minerals during pregnancy. Maintaining a breeding herd of Konik Polski mares appears to be cost-effective; however, the cost-effectiveness of Cu and Zn supplementation in other breeds, geographic regions, and herds necessitates further research.

5. Conclusions

One may conclude that the month of the year, rather than pregnancy, affects the serum Cu and Zn concentrations in the studied group of mares. Throughout the entire long-term study, fluctuations in Cu and Zn concentrations remained within normal ranges. This suggests that these specific Konik Polski mares, representing a robust breed, did not require additional supplementation of the examined trace minerals. Maintaining these mares in an extensive system seems sufficient to cover the demand for Cu and Zn, even in pregnant mares. However, further research that considers other robust breeds, geographic regions, and herds, as well as the detailed composition and amount of feed, is necessary in order to confirm and practically implement the summarized generalizations.

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