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Effect of Semen Collection in the Metabolite and Hormonal Content of Rabbit Seminal Plasma

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ABSTRACT

Blood serum (BS) and seminal plasma (SP) share a plethora of compounds that might present an individual and/or temporal concentration variation. We aimed to determine whether BS and SP concentrations of albumin, calcium, citrate, creatinine, fructose, glucose, lactate, total protein, urea, zinc, cortisol, anti-Müllerian hormone (AMH) and testosterone are related to weekly collections in New Zealand White (NZW) adult rabbit bucks. During a 12-week study, blood samples were obtained at the beginning and the end of the study period, and semen samples were taken twice a week from four NZW adult rabbit bucks, starting at 6–7 months of age. After semen collection, the sperm motility was subjectively assessed, and SP was obtained by centrifugation. BS and SP were evaluated for the above-mentioned metabolites using a Biosystems BA400 automated analyser with commercial-specific kits or enzyme immunoassay (EIA) kits, assessing the effects of the male and time of collection. In addition, a correlation analysis aimed at disclosing associations between parameters in BS and SP was performed. Male effect was not significant for BS, but it was significant for SP albumin, citrate, fructose, glucose, lactate and total protein. In addition, all the correlations in BS were positive, whereas they were more balanced in SP, being close to half of the correlations. In conclusion, variations of some metabolites (albumin, citrate, fructose, glucose, lactate and total protein) appear to be potential biomarkers for rabbit SP, although further studies should test their usefulness for sperm fertility assessment.

Manuel Álvarez-Rodríguez and Laura García-Calvo contributed equally to this study.

1 | Introduction

Rabbit semen is composed of spermatozoa, seminal plasma (SP) and the gelatinous mass (Holtz and Foote 1978; Mukherjee et al. 1951). The SP is not only the fluid in which spermatozoa bathe, but it also has key functions in sperm function and nutrition and immunomodulatory roles in the female genital tract (Maxwell et al. 1996; Rodriguez-Martinez et al. 2021). It is composed of a mixture of secretions from the testis, epididymis and the accessory sexual glands (Mann and Lutwak-Mann 1981; Rodriguez-Martinez et al. 2021), and it is produced at the time of ejaculation when the different glands mix their secretions, which combine with the spermatozoa being expelled from the cauda epididymis (Mann and Lutwak-Mann 1981). The main components of SP are (besides water) proteins, nutrients for spermatozoa, decapacitating factors, key modulatory proteins, ions, hormones and extracellular vesicles (Davis 1973, 1974; Davis and Hungund 1976; Parra et al. 2023; Rodriguez-Martinez et al. 2021), among many others. Being the rabbit doe an ovulation-induced animal (Bakker and Baum 2000), the presence of a specific ovulation induction factor in rabbit SP is a current matter of research (Adams and Ratto 2013; Silva et al. 2011). In addition, it has been reported that SP plays an important role in rabbit semen preservation (Aksoy, Cankat-Lehimcioglu, and Akman 2010; Domingo et al. 2018).

The accessory glands produce their secretions partly by transporting components directly from the bloodstream, whereas the secretory cells also show a prominent biosynthetic and regulatory ability (Mann and Lutwak-Mann 1982). Thus, there is an intimate relationship between both blood plasma and SP constituents (Mann and Lutwak-Mann 1982, 1976). In a healthy organism, body homeostasis maintains bloodstream component concentrations within a fixed range, which is particular for each species and preserved through time (Benson and Paul-Murphy 1999). However, SP component concentrations may vary with the season or ejaculation frequency/collection rhythm and other factors (Bencheikh 1993, 1995; Lankri et al. 2019; Mocé et al. 2000). Therefore, the hormones, substrates and metabolites present in SP could be useful as fertility biomarkers (Rodriguez-Martinez et al. 2021).

On one hand, albumin, one of the major proteins of SP (Bezerra et al. 2019), presents individual variations in semen in rabbit bucks (Bencheikh 1995). Another interesting metabolite, fructose, provides energy to spermatozoa (Kasimanickam et al. 2019). Metabolites such as zinc (Zn), supplemented on a diet, have been described as related to directly modulated creatinine (El-Gindy et al. 2023), being Zn supplementation related and increased blood testosterone and reduced blood cortisol levels (El-Gindy et al. 2023). Also regarding creatinine, this metabolite, and cortisol and testosterone, is controlled in rabbits by diverse dietary supplements such as plant extract Maca (*Lepidium meyenii*) (Ragab et al. 2023) or proline (Abdelnour et al. 2021).

On the other hand, hormonal levels are physiological indicators of the behavioural, metabolic and sexual status of the animal, with some of them being potential biomarkers for male's fertility. Thus, cortisol is the primary stress biomarker, which is secreted by the adrenal cortex and regulated via the sympathetic-medullary-adrenal axis (Hennessy 1997). Cortisol is functionally involved in controlling animals' behaviour and metabolic, endocrine and immune function to ensure adequate coping strategies and well-being (Pani, et al. 2000). Considering reproduction, sexual hormones, such as testosterone, are essential to maintain the function of the male's accessory sexual glands, including protein and fructose synthesis. In that respect, the seminal vesicles secrete fructose, a critical monosaccharide in SP, and these accessory glands are very sensitive to stimulation of androgens (mainly testosterone [Walker 2009]). Moreover, heat stress in rabbit bucks caused a significant decrease in testosterone concentrations (Huang et al. 2023). In vitro incubating small ruminants' spermatozoa with testosterone before cryopreservation impairs their survival (Martínez-Fresneda et al. 2020). Finally, the anti-Müllerian hormone (AMH), which is produced by Sertoli cells, plays an essential role in the maturation and differentiation of spermatogenic cells and, when assessed as a biomarker in SP, can provide direct information about spermatogenesis (La Marca et al. 2010). Moreover, a higher sperm motility recovery after cryopreservation in men with asthenozoospermia might be associated with lower AMH levels (Nery et al. 2014).

Industrial rabbit meat production relies mainly on artificial insemination (AI) with fresh-extended semen (Castellini 1996). Semen collection depends on the production rhythm, which can be extensive, semi-intensive or intensive (Castellini 1996) with the following collection rhythms: (1) extensive: two successive ejaculates (within 15 min.) once a week; (2) semi-intensive: two successive ejaculates two times/week; (3) intensive: two successive ejaculates three times/week (Bencheikh 1993, 1995), being the most common production rhythm the semi-intensive one (Boiti et al. 2005). However, an excessive collection frequency reduces semen quality by decreasing both sperm quality and sperm concentration (Bencheikh 1993, 1995; Lankri et al. 2019; Mocé et al. 2000). In addition, there is little information in the literature about rabbit buck SP composition and its variation over time with regular collection.

Therefore, this study aimed to determine BS and SP concentrations of albumin, calcium, citrate, creatinine, fructose, glucose, lactate, total protein, urea, zinc, cortisol, AMH and testosterone in rabbit bucks in a regular semen collection regime of two times/week, assessing male and time effects on the concentration variation of those metabolites. We hypothesized that the BS and SP concentrations of these parameters would remain stable over time during a regular semen collection regime; however, differences might be evident, such as discrimination among males.

2 | Materials and Methods

2.1 | Animals and Housing Conditions

Animal husbandry and experimental handling were performed according to the European Directive 2010/63/EU, 22/09/2010, for animal experiments, and Spanish Regulations RD 53/2013, modified by RD 118/2021. Four New Zealand White (NZW) adult rabbit bucks (from 6 to 7 months old) were included in this study. Rabbit bucks come from the nucleus colony at the farm of the *Institut de Recerca i Tecnologia Agroalimentaries* (IRTA, Torre Marimon, Caldes de Montbui, Barcelona, Spain), and they were not related to each other. Animals were transported to

the experimental farm at the Faculty of Veterinary Medicine, Autonomous University of Barcelona (Barcelona, Spain), where this study was performed. Animals were kept under a controlled photoperiod of 16 h of light and 8 h of darkness, and a range of temperature between 20 and 26°C, with a relative humidity of 60%–75% maintained by a forced ventilation system. Each animal was individually housed in a cage ($85 \times 40 \times 30$ cm^3) equipped with plastic footrests, a feeder (restricted to 180 g/day of an all-mash pellet) and free access to water.

2.2 | Experimental Design

Two independent experiments were performed in this study using the same adult rabbit bucks ($n = 4$). The total length of the study was 8 consecutive weeks. Blood samples were collected at the beginning and at the end of the study, whereas semen samples were collected from each rabbit buck twice per week during the whole duration of the study.

2.3 | Blood Serum (BS) Collection and Processing

Blood (2 mL) was collected by venipuncture of the lateral marginal ear vein at the beginning and at the end of the study (8-week interval). After clotting during 30 min at room temperature, blood samples were centrifuged at $1500 \times g$ during 5 min, collecting the BS and storing it at −80°C until analyses.

2.4 | Semen Collection, Motility Assessment and SP Processing

Semen samples were obtained twice per week during the length of the experiment (six times, at 2-week intervals for the 12 weeks of the study). Therefore, a total of 12 samples were obtained for each rabbit buck. Males started to be trained at 4.5 months of age with a commercial polyvinyl chloride artificial vagina (HUMECO, Huesca, Spain) containing water at 50°C. Semen samples were macroscopically assessed to discard insufficient quality criteria, such as alterations in colour and thickness or presenting urine or calcium carbonate deposits. Samples were transported to the laboratory at 38.5°C–39°C within 10 min after collection and immediately assessed for sperm motility. A 5 μL drop of each sperm sample, diluted 1:100 with GALAP (HUMECO, Huesca, Spain), was placed in a pre-warmed (38°C) slide, covered with a coverslip and subjectively assessed under a phase-contrast microscopy at ×10 (M60i, Proiser, Spain) for the subjective assessment of total motility (TMOT, %).

SP was centrifuged at $1500 \times g$ during 10 min at room temperature, and the supernatant was carefully removed, checking the absence of cells. Tubes were stored at −80°C pending analyses.

2.5 | BS and SP Analyses

Due to volume limitations of SP samples for analysis, the two consecutive collections of each male in the same week were pooled (12 SP analyses per male). BS and SP samples were sent in dry ice to the University of León for analysis at Laboratory of Instrumental Analyses, León, Spain.

Biochemical analysis for albumin, calcium, citrate, creatinine, fructose, glucose, lactate, total protein, urea and Zinc was validated for rabbit species and was performed with a BA400 automated analyser and specific kits (Biosystems, Barcelona, Spain) according to manufacturer instructions, as follows:

- Albumin: Bromocresol green method (albumin reaction with bromocresol green at pH 4.1 and absorbance measurement at 630 nm), with a repeatability of 2.1% and a limit of detection of 1.4 g/L.
- Calcium: Arsenazo method (Ca^{2+} reaction with the arsenazo III dye and absorbance measurement at 650 nm), with a repeatability of 1.2% and a limit of detection of 0.2 mg/dL.
- Citrate: Citrate lyase/dehydrogenase method (enzymatic conversion of citrate to oxalacetate and then reduction a malate, consuming NADH; the NADH absorbance was measured at 340 nm), with a repeatability of 2.5% and a limit of detection of 19 mg/dL.
- Creatinine: Alkaline picrate method (creatinine reaction with picrate in alkaline medium and absorbance measurement at 500 nm), with a repeatability of 1.3% and a limit of detection of 0.03 mg/dL.
- Fructose: Hexokinase/phosphoglucose isomerase method (enzymatic conversion of fructose to fructose-6-phosphate, isomerization to glucose-6-phosphate and then oxidation to gluconate-6-phosphate, producing NADH; the NADH absorbance was measured at 340 nm), with a repeatability of 1.4% and a limit of detection of 4 mg/dL.
- Glucose: Glucose oxidase/peroxidase method (enzymatic conversion of glucose to gluconic acid with production of H_2O_2, used in the peroxidation of 4 aminoantipyrine in the presence of phenol, producing quinoneimine; the absorbance was measured at 500 nm), with a repeatability of 1.2% and a limit of detection of 0.23 mg/dL.
- Lactate: Lactate oxidase/peroxidase method (enzymatic conversion of L-lactate to pyruvate with production of H_2O_2, used in the peroxidation of 4 aminoantipyrine in the presence of TOOS, producing quinoneimine; the absorbance was measured at 500 nm), with a repeatability of 0.6% and a limit of detection of 0.35 mg/dL.
- Protein (total): Total protein was estimated by the Biuret method (protein reaction with copper II in alkaline medium and absorbance measurement at 535 nm), with a repeatability of 0.5% and a limit of detection of 0.8 g/L.
- Urea: Urease/glutamate dehydrogenase method (enzymatic hydrolysis of urea producing NH_3, used for producing glutamate from oxoglutarate while consuming NADH; the NADH absorbance was measured at 340 nm), with a repeatability of 1.9% and a limit of detection of 2.5 mg/dL.
- Zinc: Zinc concentration was estimated by the Bromo-PAPS method (Zn reaction with 5-Br-PAPS in alkaline medium and absorbance measurement at 560 nm), with a repeatability of 1.6% and a limit of detection of 176 μg/dL.

As previously described by our group (Gardela et al. 2023), hormonal determinations were assessed by commercial enzyme immunoassay (EIA) kits for cortisol (Cortisol ELISA Kit; Neogen Corporation, Ayr, UK), testosterone (Testosterone ELISA Kit; Neogen Corporation, Ayr, UK) and AMH (AMH; Cloud-Clone Corporation, Katy, USA). The Cortisol ELISA Kit presented cross-reactivity with prednisolone (47.4%), cortisone (15.7%), 11-deoxycortisol (15.0%), prednisone (7.83%), corticosterone (4.81%), 6β-hydroxycortisol (1.37%), 17-hydroxyprogesterone (1.36%), deoxycorticosterone (0.94%), progesterone (0.06%) and all other steroids (<0.06%). The Testosterone ELISA kit presented cross-reactivity with testosterone glucuronide (16.12%), androstenedione (0.86%), bolandiol (0.86%), testosterone enanthate (0.13%), estriol (0.10%), testosterone benzoate (0.10%), estradiol (0.05%), dehydroepiandrosterone (0.04%) and all other steroids (<0.09%). No significant cross-reactivity or interference between AMH and any analogue was observed in the AMH kit. Each EIA was biochemically validated for *Oryctolagus cuniculus* and SP by verifying precision (intra-assay coefficients of variation (CV) from duplicated samples, sensitivity (smallest amount of detected hormone), specificity (linearity of dilution) and accuracy (spike-and-recovery test).

2.6 | Statistical Analyses

Statistical analyses were performed with the R environment version 4.3.1. For analysis, data were separated into BS and SP analysis. Data were checked for normal distribution using the Shapiro–Wilk test. For each of the quantified parameters (TM, albumin, calcium, citrate, creatinine, fructose, glucose, lactate, total protein, urea, zinc, cortisol, AMH and testosterone) in each sample group (BS and SP), both male and time effects were estimated using a linear-mixed effect model followed by pairwise comparisons. The significance level was set at $p \leq 0.05$.

In addition, correlation among the quantified metabolites (TM, albumin, calcium, citrate, creatinine, fructose, glucose, lactate, total protein, urea, zinc, cortisol, AMH and testosterone) was explored in each sample group (BS and SP) by the Spearman's Rank Correlation Test. Correlations were considered significant at $p \leq 0.05$.

3 | Results

3.1 | Validation of the EIA for Cortisol, Testosterone and AMH

As previously reported by our research group (Gardela et al. 2023), cortisol presented and intra-assay CV of 6.36% ± 1.12, whereas testosterone was 3.36% ± 0.76%, and AMH was 4.96% ± 0.84%, all below the acceptable threshold. The sensitivity of the EIA kits was 40 pg for cortisol, 2 pg for testosterone and 937.5 pg for AMH. The dilution test showed high and significant ($p < 0.05$) similarities between the expected and observed levels with r^2 of 0.99 for cortisol, 0.99 for testosterone and 0.92 for AMH. The results of the spike-and-recovery test to measure accuracy presented a mean recovery percentage of 118.70 for cortisol, 124.75 for testosterone and 118.01 for AMH. Results demonstrate that these EIA kits are strongly precise, specific, accurate and sensitive enough for quantification of both BS and SP levels of cortisol, testosterone and AMH in the rabbit.

3.2 | BS Metabolites and Hormones

3.2.1 | BS Metabolites and Hormones Concentration Remained Stable Both Among Males and Time

No significant differences were observed among males during the whole study period (male-effect analysis, Table 1) or between both blood collections (time-effect analysis, Table 2).

3.2.2 | High Correlations Are Present Among Some Key Metabolites and Hormones in BS

Spearman's rank correlation analysis for SP metabolites is depicted in Figure 1 (full correlations and p values in the Table S1). In summary, positive and significant ($p < 0.05$) correlations were observed for albumin and calcium (rho = 0.79), albumin and total protein (rho = 0.84), calcium and lactate (rho = 0.91), calcium and total protein (rho = 0.97), creatinine and fructose (rho = 0.85), creatinine and glucose (rho = 0.85), creatinine and zinc (rho: 0.83), fructose and glucose (rho = 1), fructose and urea (rho = 0.83), fructose and cortisol (rho = 0.82), fructose and testosterone (rho = 0.80), glucose and urea (rho = 0.81), glucose and cortisol (rho = 0.81), glucose and testosterone (rho: 0.80), lactate and total protein (rho = 0.89) and urea and testosterone (rho = 0.71).

3.3 | SP Metabolites and Hormones

3.3.1 | SP Metabolites and Hormonal Analysis Reveal Potential Biomarkers

The male showed a significant effect for albumin, citrate, fructose, glucose, lactate and total protein (male-effect analysis, Table 3). The pairwise analysis highlighted one of the males (identified as buck #1) as showing a different pattern for albumin, fructose, glucose and total protein. On the other hand, no significant effects of the week of collection were found for the other parameters (time-effect analysis, Table 4).

3.3.2 | High Correlations Are Present Among Some Key Metabolites and Hormones in SP

Spearman's rank correlation analysis for SP metabolites is depicted in Figure 2 (p and rho matrix in Table S2). Briefly, positive and significant ($p < 0.05$) correlations are observed for TM and lactate (rho = 0.48), albumin and citrate (rho = 0.44), albumin and lactate (rho = 0.48), albumin and total protein (rho = 0.89), calcium and lactate (rho = 0.50), calcium and testosterone (rho = 0.43), citrate and lactate (rho = 0.48), citrate and total protein (rho = 0.59), creatinine and urea (rho = 0.92), creatinine and cortisol (rho = 0.90), creatinine and testosterone (rho = 0.76), fructose and glucose (rho = 0.82), fructose and zinc (rho = 0.41), glucose and zinc (rho = 0.63), lactate and total protein (rho = 0.45), urea and cortisol (rho = 0.89), urea

TABLE 1 | Blood serum metabolites depicted for the male-effect analysis: Samples collected at the starting point and end point of the experimental procedure.

	Rb1	Rb2	Rb3	Rb4
Albumin (g/L)	42.89 ± 0.22	46.37 ± 0.94	43.64 ± 0.52	42.90 ± 2.84
Calcium (mg/dL)	13.47 ± 0.10	14.11 ± 0.40	13.62 ± 0.68	13.30 ± 1.68
Citrate (mg/dL)	9.38 ± 0.35	9.98 ± 1.54	9.79 ± 0.39	5.32 ± 1.16
Creatinine (mg/dL)	0.99 ± 0.16	0.92 ± 0.00	0.83 ± 0.13	0.60 ± 0.10
Fructose (mg/dL)	167.37 ± 22.44	131.69 ± 3.38	134.55 ± 5.94	117.05 ± 15.72
Glucose (mg/dL)	174.48 ± 24.61	137.39 ± 3.10	140.51 ± 6.26	122.18 ± 18.25
Lactate (mg/dL)	72.94 ± 3.89	84.97 ± 0.37	84.70 ± 24.34	56.27 ± 35.34
Total protein (g/L)	76.29 ± 0.94	80.68 ± 2.68	77.61 ± 2.78	73.35 ± 13.57
Urea (mg/dL)	40.77 ± 0.27	30.85 ± 0.39	32.38 ± 3.43	29.00 ± 2.43
Zinc (μg/dL)	195.19 ± 31.91	225.30 ± 2.58	188.86 ± 60.34	156.46 ± 18.37
Cortisol (pg/mL)	569.23 ± 226.77	256.44 ± 256.44	160.30 ± 108.30	90.26 ± 90.26
AMH (pg/mL)	2037.50 ± 15.07	2667.16 ± 497.03	2579.81 ± 417.18	2152.53 ± 173.32
Testosterone (pg/mL)	264.12 ± 26.16	116.33 ± 3.42	193.38 ± 48.82	92.86 ± 66.17

Note: Values are depicted as mean ± standard error of the mean (SEM).
Abbreviations: AMH, anti-Müllerian hormone; Rb1, rabbit buck 1; Rb2, rabbit buck 2; Rb3, rabbit buck 3; Rb4, rabbit buck 4.

TABLE 2 | Blood Serum metabolites depicted for the time-effect analysis, samples collected at the starting point and end point of the experimental procedure.

	Week 1–2	Week 11–12
Albumin (g/L)	44.82 ± 1.03	43.08 ± 1.15
Calcium (mg/dL)	14.29 ± 0.34	12.96 ± 0.48
Citrate (mg/dL)	9.47 ± 1.07	7.76 ± 1.21
Creatinine (mg/dL)	0.79 ± 0.06	0.88 ± 0.14
Fructose (mg/dL)	135.34 ± 3.46	139.98 ± 18.51
Glucose (mg/dL)	141.26 ± 3.22	146.02 ± 19.85
Lactate (mg/dL)	88.58 ± 8.29	60.86 ± 14.29
Total protein (g/L)	81.97 ± 2.07	71.99 ± 4.13
Urea (mg/dL)	33.17 ± 2.68	33.34 ± 3.05
Zinc (μg/dL)	173.62 ± 20.59	209.28 ± 24.43
Cortisol (pg/mL)	326.12 ± 70.51	212.00 ± 195.05
AMH (pg/mL)	2426.31 ± 252.29	2292.19 ± 238.46
Testosterone (pg/mL)	188.02 ± 31.50	145.32 ± 54.58

Note: Values are depicted as mean ± standard error of the mean (SEM).
Abbreviation: AMH, anti-Müllerian hormone.

and testosterone (rho = 0.71) and cortisol and testosterone (rho = 0.75). On the other hand, negative and significant ($p < 0.05$) correlations are observed for TM and cortisol (rho = −0.47), albumin and fructose (rho = −0.75), albumin and glucose (rho = −0.83), calcium and fructose (rho = −0.41), calcium and AMH (rho = −0.45), citrate and fructose (rho = −0.59), citrate and glucose (rho = −0.42), creatinine and zinc (rho = −0.41), creatinine and AMH (rho = −0.50), fructose and lactate (rho = −0.61), fructose and total protein (rho = −0.78), glucose and lactate (rho = −0.67), glucose and total protein (rho = −0.86), urea and AMH (rho = −0.47), cortisol and AMH (rho = −0.64) and AMH and testosterone (rho = −0.56).

4 | Discussion

In this study, a regular semen collection rhythm of two times/week in rabbit bucks, with an evaluation of both individual differences as well as temporal variation, was assessed for BS and SP concentrations of albumin, calcium, citrate, creatinine, fructose, glucose, lactate, total protein, urea, zinc, cortisol, AMH and testosterone. Our results show that BS parameters were homogeneous in the studied animals, with no significant effects from the male or week of collection. This might indicate that the obtained values are representative for the specific population where the sampled animals come from. In this line and for future works, it would be desirable to increase the number of studied rabbit bucks with the goal of obtaining reference population values and to, potentially, identify breed differences. Furthermore, interesting correlations were obtained from blood analysis, all the significant differences being positive correlations.

Semen collection rhythm can affect semen quality, as it has been observed that an extensive collection rhythm (once a week) is the most convenient for achieving good semen quality (Lankri et al. 2019), but the most common collection rhythm in rabbit production is semi-intensive (twice a week) (Boiti et al. 2005). The latter agrees with our obtained results, as no significant differences were observed among weeks for any of the parameters in our results.

Contrarily, significant differences among rabbit bucks have been observed in SP determinations of albumin, citrate, fructose, glucose, lactate and total protein, reflecting that, even though BS determinations were non different among males, SP presented individual variations. Our results are in agreement with the

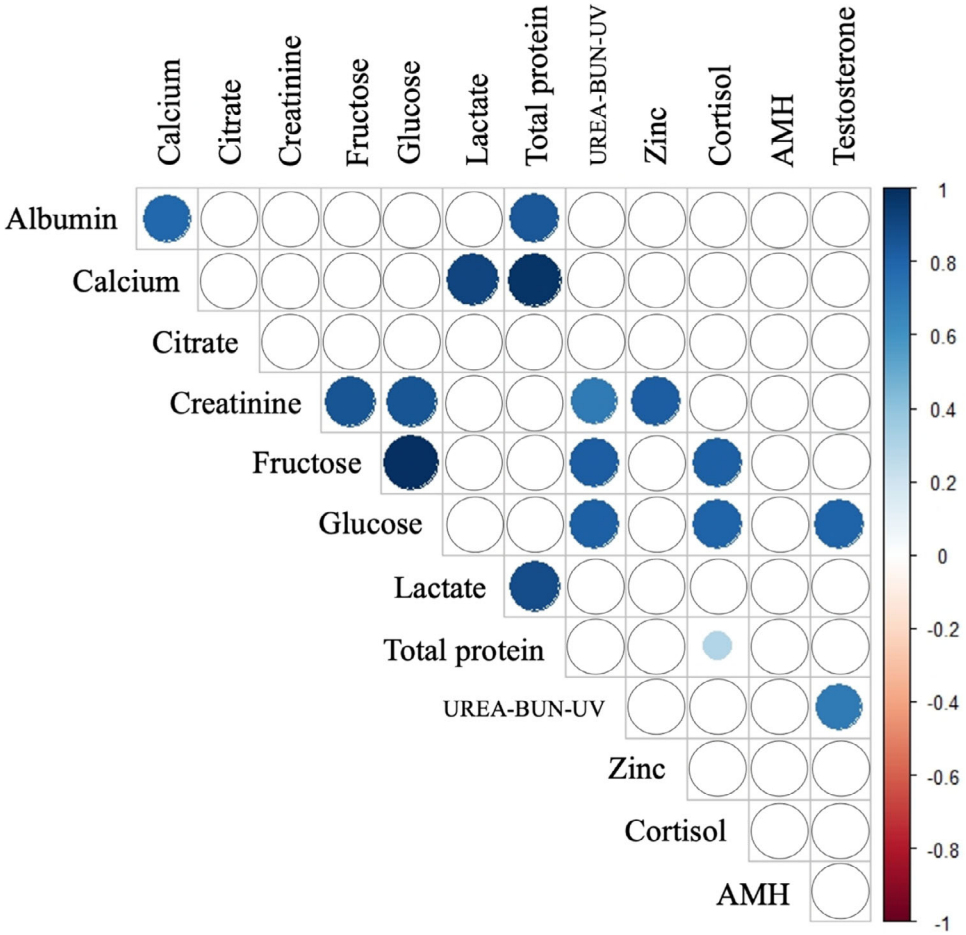

FIGURE 1 | Spearman's rank correlation matrix for blood serum metabolites. Spearman's rho correlation values are directly related to the size of the circle, whereas only significant correlations are represented (p value < 0.05). Blue colours indicate significant ($p < 0.05$) positive correlations, and red colours significant ($p < 0.05$) negative correlations.

literature, as albumin has been reported as one of the major proteins of SP (Bezerra et al. 2019), and individual variations in semen quality have also been observed when assessing semen collection rhythm in rabbit bucks (Bencheikh 1995). Moreover, it has previously been reported that the genotype is related to a specific abundance of SP proteins in rabbits (Casares-Crespo et al. 2018), but the importance of individual variation seems to be higher for the metabolite parameters measured in this study. Specific protein composition of rabbit buck SP, as depicted by SDS–PAGE electrophoresis, is intimately linked with SP and semen quality (Anous et al. 2017), which acquires extreme relevance in terms of rabbit buck selection for semen quality. However, protein determination by SDS–PAGE electrophoresis is a long and expensive method, and, thus, other markers suitable to be detected by alternative laboratory approaches, which are faster and cheaper, shall be elected in order to assess individual rabbit buck semen quality. In addition, feeding and supplementation also affect both BS and SP composition, as it has been shown by soybean lecithin supplementation (Attia and Kamel 2012) and *Acacia saligna* (Yousef 2005) of rabbit bucks diet.

Citrate and lactate showed a clear correlation pattern, and glucose and fructose seemed to follow an inverse pattern, according to our data. Previous studies found a decrease in creatinine with dietary Zn supplementation (El-Gindy et al. 2023), which is related to our results, as we observed a negative correlation in SP between creatinine and Zn. In addition, it has previously been reported that dietary Zn supplementation increased blood testosterone and reduced blood cortisol levels (El-Gindy et al. 2023). However, our results did not find any correlation between Zn and cortisol nor cortisol or testosterone. Moreover, previous studies show a reduction of blood creatinine as well as cortisol and testosterone in rabbits after oral administration of either the plant extract Maca (*L. meyenii*) (Ragab et al. 2023) or proline (Abdelnour et al. 2021). Both studies are in agreement with our results, as our SP analysis resulted in a positive correlation of creatinine with both cortisol and testosterone, whereas AMH levels were negatively correlated with SP creatinine. The seminal vesicles are particularly sensitive to testosterone (Walker 2009), hormone that stimulates those accessory glands to produce fructose. Fructose is an interesting metabolite as it is one of the variety of substances in the SP that provides energy to spermatozoa (Kasimanickam et al. 2019) and whose concentration is reduced in heat stress treatments (Huang et al. 2023). Interestingly, we observed an individual variation in SP fructose concentration among rabbit bucks. This finding makes this metabolite an interesting target for rabbit buck SP quality marker as well as rabbit buck reproductive health indicator.

TABLE 3 | Seminal plasma (SP) metabolites depicted for the male-effect analysis.

	Rb1	Rb2	Rb3	Rb4
Total sperm motility (%)	71.25 ± 5.91	67.08 ± 8.23	56.67 ± 12.19	66.67 ± 9.21
Albumin (g/L)	13.95 ± 1.11[a]	3.49 ± 0.25[b,c,d]	3.02 ± 0.28[b]	4.42 ± 0.33[c,d]
Calcium (mg/dL)	14.16 ± 2.66	13.93 ± 2.33	8.02 ± 0.77	10.85 ± 1.46
Citrate (mg/dL)	102.38 ± 8.70[a]	90.88 ± 10.19[a,b]	56.27 ± 11.92[a,b]	54.90 ± 3.46[b]
Creatinine (mg/dL)	5.52 ± 3.76	5.12 ± 3.36	1.77 ± 1.05	2.94 ± 1.12
Fructose (mg/dL)	35.27 ± 1.80[a]	72.78 ± 8.95[b]	76.94 ± 6.33[b]	89.93 ± 7.74[b]
Glucose (mg/dL)	4.59 ± 1.45[a]	39.80 ± 5.56[b]	35.33 ± 3.12[b]	28.40 ± 4.23[b]
Lactate (mg/dL)	43.36 ± 3.49[a]	37.41 ± 6.42[a,b]	24.41 ± 1.84[b]	37.26 ± 5.00[a,b]
Total protein (g/L)	58.62 ± 3.13[a]	16.57 ± 2.97[b]	16.02 ± 1.51[b]	18.02 ± 3.04[b]
Urea (mg/dL)	88.89 ± 16.90	112.90 ± 4.36	110.82 ± 29.56	138.83 ± 31.76
Zinc (μg/dL)	1322.10 ± 58.35	1740.22 ± 154.29	1444.20 ± 121.55	1378.80 ± 71.42
Cortisol (pg/mL)	149.50 ± 101.80	223.58 ± 181.64	86.08 ± 54.48	174.50 ± 96.63
AMH (pg/mL)	8819.17 ± 628.45	7906.29 ± 1007.05	9276.48 ± 589.17	7507.91 ± 604.47
Testosterone (pg/mL)	127.29 ± 20.59	168.20 ± 30.60	108.14 ± 27.44	123.75 ± 19.47

Note: Values are depicted as mean ± standard error of the mean (SEM). Different letters in the superscript indicate significant ($p < 0.05$) individual differences among rabbit bucks.
Abbreviations: AMH, anti-Müllerian hormone; Rb1, rabbit buck 1; Rb2, rabbit buck 2; Rb3, rabbit buck 3; Rb4: rabbit buck 4.

TABLE 4 | Seminal plasma (SP) metabolites depicted for the time-effect analysis.

	Week 1–2	Week 3–4	Week 5–6	Week 7–8	Week 9–10	Week 11–12
Total sperm motility (%)	58.13 ± 13.03	55.00 ± 12.56	58.13 ± 10.01	68.75 ± 6.04	80.00 ± 4.71	72.50 ± 1.86
Albumin (g/L)	6.99 ± 2.65	6.73 ± 2.83	6.83 ± 3.20	6.42 ± 2.88	4.57 ± 1.41	5.78 ± 2.67
Calcium (mg/dL)	13.72 ± 3.21	10.53 ± 2.33	12.71 ± 3.48	14.66 ± 3.14	9.58 ± 2.02	9.26 ± 0.55
Citrate (mg/dL)	74.55 ± 13.99	56.31 ± 5.64	81.62 ± 16.60	81.13 ± 16.20	86.34 ± 22.20	76.71 ± 16.31
Creatinine (mg/dL)	2.85 ± 1.46	7.08 ± 4.99	3.21 ± 1.46	2.68 ± 1.19	6.52 ± 5.80	0.69 ± 0.29
Fructose (mg/dL)	54.22 ± 12.28	69.16 ± 13.48	69.11 ± 11.83	73.55 ± 16.68	78.99 ± 16.63	67.35 ± 11.99
Glucose (mg/dL)	19.36 ± 7.92	25.21 ± 8.99	31.70 ± 9.53	28.56 ± 9.00	31.96 ± 9.01	25.40 ± 9.29
Lactate (mg/dL)	46.69 ± 9.07	32.35 ± 5.89	29.70 ± 4.91	35.42 ± 6.73	29.23 ± 2.82	40.28 ± 5.35
Total protein (g/L)	31.95 ± 8.53	25.62 ± 9.49	28.98 ± 14.48	25.08 ± 11.09	30.64 ± 14.09	24.67 ± 8.68
Urea (mg/dL)	116.53 ± 33.16	117.75 ± 39.98	156.86 ± 42.74	123.32 ± 18.78	85.39 ± 13.03	77.64 ± 11.88
Zinc (μg/dL)	1445.18 ± 155.87	1325.08 ± 25.19	1547.61 ± 179.07	1561.20 ± 147.92	1522.58 ± 144.84	1426.32 ± 237.17
Cortisol (pg/mL)	92.75 ± 49.34	370.13 ± 259.33	219.00 ± 143.29	99.63 ± 49.28	161.75 ± 158.10	7.25 ± 7.25
AMH (pg/mL)	8597.18 ± 1003.63	6910.56 ± 1126.78	7759.03 ± 636.45	8416.59 ± 926.55	9666.66 ± 911.94	9354.82 ± 281.08
Testosterone (pg/mL)	142.60 ± 33.13	153.89 ± 41.31	152.47 ± 24.98	115.28 ± 21.01	109.28 ± 51.36	102.83 ± 12.93

Note: Values are depicted as mean ± standard error of the mean (SEM).
Abbreviation: AMH, anti-Müllerian hormone.

SP plays an important role in sperm motility in the rabbit (Muller and Kirchner 1978), and interestingly, the negative correlation between TM and cortisol in SP reflects the importance of stress in semen quality. From these results, it is likely that less stressed animals (lower cortisol) present higher total sperm motility. Moreover, it has been previously observed that there is a positive correlation between sperm motility and sperm viability in the rabbit (Bencheikh 1995); thus, according to those previous observations and our study, SP cortisol may be used as a marker for sperm viability in the rabbit, being necessary to increase the number of animals tested.

It would also be interesting to confirm our findings by associating with fertility, as semen quality is related to SP quality, and both are critical for fertility (Bezerra et al. 2019; Castellini et al. 2000, 2013; Chang, Hanada, and Hunt 1971). This study, mapping relevant markers both in BS and SP, seeds new light into the path for rabbit semen quality and fertility markers. Interestingly, all

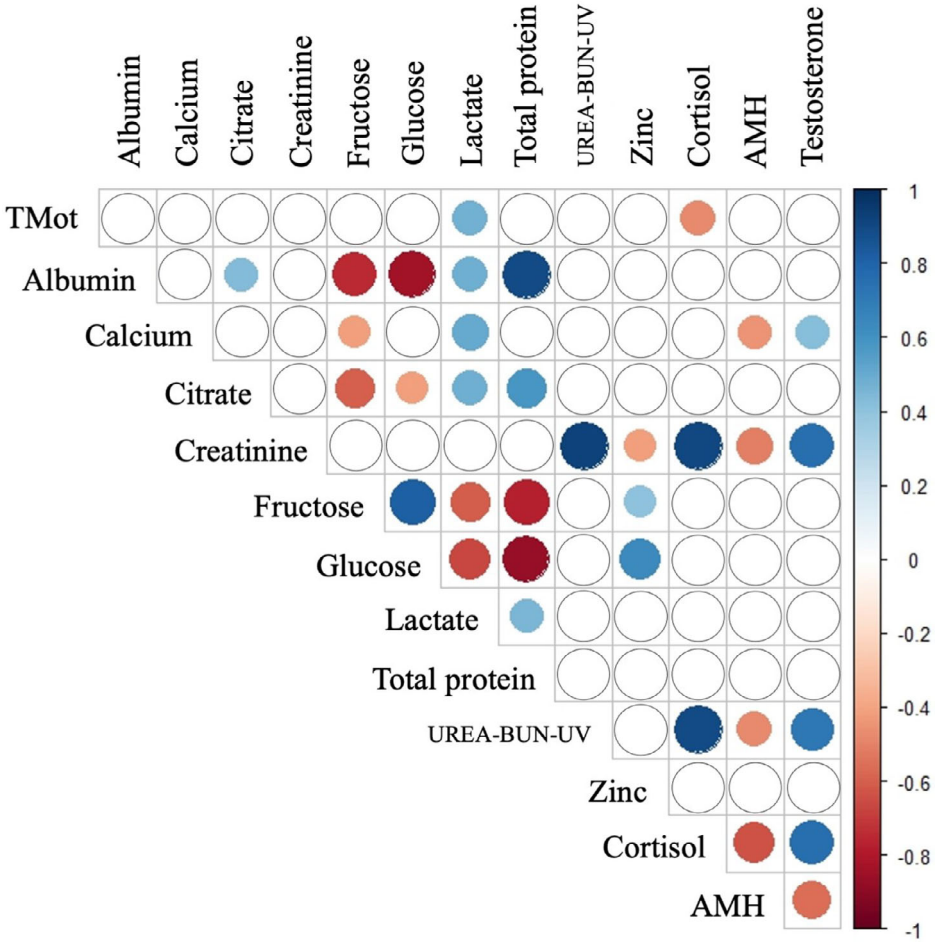

FIGURE 2 | Spearman's rank correlation matrix for seminal plasma (SP) parameters. Spearman's rho correlation values are directly related to the size of the circle, whereas only significant correlations are represented (p value < 0.05) Blue colours indicate significant ($p < 0.05$) positive correlations, and red colours significant ($p < 0.05$) negative correlations. TMOT, total motility.

the significant correlations within BS metabolites are positive, whereas close to half of the significant correlations within SP metabolites are negative. Could this be explained by the excretory nature of SP and the accessory gland functions, with a marked individual effect?

It has previously been reported that rabbit SP does not reach the uterus after AI (Asch, et al. 1977); thus, SP may only take a role in sperm function when natural mating occurs. As such, SP proteins benefit rabbit sperm motility depending of the collection fraction and molecular weight (Muller and Kirchner 1978); however, no significant effect in sperm motility has been reported by incubating rabbit sperm with rabbit serum albumin (Muller and Kirchner 1978). This is in agreement with our results, as no significant correlation between total protein or albumin and total sperm motility has been found in this study. Interestingly, average albumin values in SP, as previously reported (Muller and Kirchner 1978), are slightly lower as the ones presented in this study, even though the same breed has been analysed. These results, albeit separated almost 40 years, may reflect how selection pressure in rabbit bucks can modify SP composition.

In summary, this study confirmed the overall hypothesis, as both BS and SP concentrations of the assessed metabolites remain stable over time in a standard collection regime, although individual differences appeared, and further studies are needed to extrapolate these metabolite variations into fertility trials and exhaustive measurements of sperm quality parameters.

5 | Conclusions

This study showed that a semi-intensive semen collection rhythm of two times/week does not significantly alter SP concentrations of albumin, calcium, citrate, creatinine, fructose, glucose, lactate, total protein, urea, zinc, cortisol, AMH and testosterone in rabbit bucks. BS concentrations of the above-mentioned metabolites did show neither individual nor temporal differences, reflecting a maintained homeostasis in rabbit serum. Finally, SP concentrations of albumin, citrate, fructose, glucose, lactate and total protein appeared to be dependent on each rabbit buck, making of those metabolites promising subjects of study for further studies on rabbit buck fertility and SP quality.

Author Contributions

Manuel Álvarez-Rodríguez: conceptualization, methodology, writing–original draft preparation, project administration, supervision. **Manel**

López-Béjar: conceptualization, methodology, writing–review and editing, supervision. **Felipe Martinez-Pastor**: conceptualization, methodology, writing–review and editing, supervision. **Alejandro Vicente-Carrillo**: conceptualization, methodology, writing–original draft preparation. **Laura García-Calvo**: methodology, writing–original draft preparation. **Jaume Gardela**: methodology, writing–review and editing. **Mateo Ruiz-Conca**: methodology, writing–review and editing. **Sergi Olvera-Maneu**: methodology, writing–review and editing. **Adrián Martín-San Juan**: methodology, writing–review and editing.

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Ethics Statement

Animal husbandry and experimental handling were performed according to the European Directive 2010/63/EU, 22/09/2010, for animal experiments, and Spanish Regulations RD 53/2013, modified by RD 118/2021.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The datasets used and analysed in this study are available from the corresponding author upon reasonable request.

Peer Review

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Supporting Information

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