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Effects of boric acid and rock salt supplementation on endocrine, mineral, and reproductive responses in ewes during the breeding period

Abstract

The aim of this study is to assess the effects of boric acid and rock salt supplementation on reproductive hormones and serum minerals at different physiological stages, and on mating behavior and reproductive performance during the breeding period. The study groups were formed as follows: Control (40 ml drinking water), Salt (S) (10 g/day of rock salt), B20 and B40 (20 and 40 mg/day boric acid), BS20 and BS40 (10 g/day rock salt + 20 and 40 mg/day boric acid). All supplements were dissolved into 40 ml of water and given by oral route. Supplementation was initiated 17 days before mating and continued until the ewes exhibited estrus and were mated. Blood samples were collected from 10 ewes at four time points: before supplementation, at estrus, and on days 7 and 40 after mating. Estrous behaviors were monitored in 25 ewes, and reproductive performance was evaluated in 60 ewes per group. Serum estrogen (E2) concentrations showed a significant group × time interaction ($p < 0.05$), with higher E2 levels particularly observed in the high-boron groups during estrus. Progesterone (P4) concentrations were elevated in the B40, BS20, and BS40 groups, respectively ($p = 0.01$). Anti-Müllerian hormone (AMH) concentrations did not differ among the experimental groups ($p > 0.05$). The E2 and AMH concentrations were higher in estrus than in anestrous ewes ($p < 0.001$). As pregnancy progressed, serum P4 concentrations increased significantly ($p < 0.001$). Supplementation increased serum boron (B), calcium (Ca), magnesium (Mg), zinc (Zn) ($p < 0.001$) and selenium (Se) ($p < 0.05$) concentrations, with the most pronounced increases observed in the BS40 group. Significant alterations in mineral metabolism were also detected during the estrous period ($p < 0.001$). The dietary supplementation did not exert a significant effect on mating behavior ($p > 0.05$). Although overall reproductive performance did not differ among groups ($p > 0.05$), a relatively higher proportion of female lambs was observed in the boron-supplemented groups, particularly in the B20 group ($p = 0.009$). In conclusion, boron and salt supplementation affected serum mineral and reproductive hormone profiles in ewes, but had limited effects on reproductive performance.

Keywords: Boric acid, Endocrine responses, Mating behavior, Mineral homeostasis, Reproductive performance, Rock salt

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INTRODUCTION

Small ruminant production, particularly sheep farming, occupies a strategic position among sustainable livestock production systems due to its low investment requirements, capacity to utilize marginal lands, adaptability to diverse climatic conditions, and high biological efficiency. Sheep production contributes not only to red meat supply but also plays a significant role in sustaining rural economies through milk, wool, and various by-products [1]. Enhancing productivity in sheep farming requires a combined evaluation of genetic, physiological, and behavioral factors. Within sustainable sheep production systems, nutritional strategies—particularly mineral supplementation—play a key role in optimizing animal health and production performance [2]. Minerals are involved in numerous fundamental biological processes, including enzymatic reactions, endocrine regulation, immune system function, and reproductive physiology [3]. Among these minerals, boron and sodium contribute to different aspects of physiological function. Boron is considered a potentially beneficial trace element that may contribute to mineral metabolism, antioxidant defense, endocrine activity, and reproductive physiology, partly through its interactions with calcium, magnesium, and other minerals [4]. Sodium, in contrast, is an essential macroelement required for maintaining osmotic pressure, fluid and acid–base balance, membrane potential, nerve impulse transmission, and muscle function [5]. Insufficient or imbalanced the mineral intake may result in not only growth retardation, failure to maintain metabolic homeostasis and productivity losses in sheep farming but also disruptions in the synthesis and secretion of hormones, behavioral alterations, and heterogeneous performance outcomes across the flock [6,7].

In recent years, although boron has not been classified as an essential mineral, it has drawn increasing scientific attention for its regulatory role in the endocrine system [4]. This is primarily due to its capacity to interact with specific molecular structures known as diol groups, enabling it to take part in many different biochemical processes [8]. Through this binding capacity, boron can affect many interconnected physiological processes [4]. At the endocrine level, boron has been reported to indirectly affect the activity of enzymes involved in steroidogenesis, the sensitivity of estrogen and progesterone receptors, and the tissue bioavailability of reproductive hormones [4,9,10]. Consistent with the biological benefits of boron, studies in small ruminants have shown that dietary supplementation with various boron derivatives can improve male reproductive parameters, particularly spermatogenesis [11,12]. Although the effects of boron derivatives on steroid hormone regulation in females have been reported [9,12], their impact on reproductive performance traits in female animals has not yet been fully elucidated and requires further investigation.

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Rock salt (sodium chloride), a fundamental mineral source in ruminant nutrition, plays a critical role in maintaining physiological and metabolic functions [13]. In addition to sodium, natural rock salts contain a range of trace minerals. Because of this, they are seen as more than simple salt sources and can help support overall mineral balance in the body [14]. When there is not enough sodium, problems like low appetite, changes in drinking behavior, and higher stress hormone levels can occur. On the other hand, providing enough sodium can improve metabolism, and support overall well-being [15].

The physiological response to boron may vary with sodium availability, as both minerals contribute to maintaining mineral homeostasis and normal physiological functions. Boron is mainly associated with mineral metabolism and reproductive functions, whereas sodium plays an important role in fluid and electrolyte balance [4,13]. Changes in sodium intake may therefore alter the physiological conditions under which boron is absorbed, distributed, and utilized [16, 17]. At the same time, the effects of boron on mineral metabolism may influence how the animal responds to changes in electrolyte balance [4,18]. Therefore, evaluating boric acid and rock salt administered separately or together may help characterize the physiological responses associated with these supplements in ewes.

In this study, the potential effects of boron and salt supplementation on mineral metabolism, reproductive hormone levels, and reproductive performance in ewes were investigated. The experimental groups included boric acid and rock salt administered separately or together. Accordingly, the present study was designed to evaluate the effects of these supplements on reproductive hormones, including estradiol, anti-Müllerian hormone, and progesterone, and mineral levels at different physiological stages, as well as on mating behavior and reproductive performance in ewes.

MATERIALS AND METHODS

Establishment of the experimental design

The study was conducted at a farm located in the Baskil district of Elazig, where 510 Şavak Akkaraman ewes were reared. Prior to the experiment, all ewes were evaluated for health status, and their ages and body condition scores (BCS) were determined. 360 ewes were selected from these animals based on age and BCS. Sixty ewes of similar age and BCS were randomly appointed for each group (Table 1). The groups were identified using ear tags in different colors (yellow, red, blue, green, orange, and black). To avoid problems if the tags were lost and to make the animals easier to recognize from a distance, the ewes were also marked with oil-based paint in the same colors as their ear tags. As the study was conducted under on-farm conditions and the farmer did not have an enclosed shelter,

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the two existing shelters were improved and covered with tarpaulins to protect the animals from direct sunlight and maintain animal welfare throughout the experimental period. Each shelter was divided into three similarly sized compartments, resulting in six compartments for the six experimental groups. Each treatment group was housed in a single compartment, and independent replication at the compartment level was not available. The allocated space per ewe was approximately 1.60 m^2 of floor area, 50 cm of feeder space, and 20 cm of water trough space. Thus, all animals were maintained under comparable housing conditions throughout the experimental period. The rams used in the experiment were 3 to 3.5 years old, in good body condition, and were checked for fertility and overall health. Before the supplements were administered, semen was collected from the rams for three consecutive days, and the rams were distributed into groups with similar semen characteristics (Sperm volume (ml) and sperm density ($x10^6$ spt), respectively; Control:1.02 and 1719.4; Salt: 1.01 and 1672.9; B20:1.04 and 1619.6; B40: 1.01 and 1603.6; BS20: 0.99 and 1636.3; BS40: 1.00 and 1721.5). During the study, similar management conditions were applied to the rams used across all experimental groups. Mating was conducted at a ratio of 20 ewes per ram [19]. The rams assigned to each experimental group were allowed to mate freely with the ewes within their respective groups under a natural mating system. Mating occurred randomly (free mating) within each group throughout the breeding period. The mating season commenced on 15 August and continued for 35 days.

Feeding and management of the experimental groups

During the breeding period, the ewes were fed a daily ration consisting of barley (500–550 g/ewe) and straw (600–700 g/ewe). Feed was distributed into the feeders based on the number of animals per group and feeding was carried out twice daily (morning and evening) by the project personnel. Water was provided *ad libitum* throughout the experimental period. Dietary supplements were administered to the study groups as follows; C: 40 ml of drinking water; S: 10 g/day of rock salt; B20: 20 mg/day of boric acid; B40: 40 mg/day of boric acid; BS20: 20 mg/day of boric acid + 10 g/day of rock salt; and BS40: 40 mg/day of boric acid + 10 g/day of rock salt.

Boric acid supplementation for the experimental groups was prepared by taking into account the recommendations of Sani et al. [20], Cortés et al., [21] and Krishnan et al., [22]. Salt supplementation was determined based on the recommendations of sheep producers and Yorgancılar [23]. Supplements were prepared daily as stock solutions by considering the number of animals in each group (60 ewes × supplement × 40 ml drinking water) and were administered every day at 10:00 a.m. to the ewes at a dose of 40 ml per animal using 50-ml syringes. The supplements were administered orally to each animal individually throughout the experimental period. In

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determining the dose of the boric acid, the elemental boron content was taken into account. The elemental boron levels were calculated based on the molecular weight of boric acid (H_3BO_3 = 61.83 g/mol). According to the calculations, the elemental boron content corresponded to 3.5 mg in the B20 groups and approximately 7 mg in the B40 groups. The boric acid used in the study had the following specifications: Boron trioxide (B_2O_3) ≥56.0%; sulfate (SO_4) ≤0.02%; chloride (Cl) ≤0.001%; and iron (Fe) ≤0.0007%; purity ≥99.5%. Boron intake from feed and water was not considered, as it was similar among the groups. The rock salt used in this study was obtained from the Çankırı Rock Salt® deposit (Çankırı, Türkiye). The product was natural, unrefined rock salt and contained sodium chloride (NaCl) as its major component. Approximately, in addition to sodium (37%) and chloride (58%), the salt naturally contains trace amounts of minerals and elements, including calcium (1626 mg/kg), iron (504 mg/kg), magnesium (23.4 mg/kg), potassium (19.9 mg/kg), manganese (12.9 mg/kg), chromium (2.3 mg/kg), zinc (2.0 mg/kg), copper (1.9 mg/kg), selenium (0.23 mg/kg), molybdenum (0.1 mg/kg), and among others. Supplementation in ewes was initiated 17 days before mating season. It was designed to encompass the entire estrous cycle, which lasts approximately 16-17 days in sheep, thereby covering the developmental window of the follicle destined for ovulation, including critical stages of follicular waves and oocyte maturation. This approach allowed to assess the potential effects of the supplements on the follicle-oocyte unit [24]. Supplementation was continued in ewes until estrus was detected. Ewes that were successfully mated were removed from the experiment, released to pasture, and no longer received supplementation. Supplementation was continued for the remaining unmated ewes until they exhibited estrus and were successfully mated. The supplementation processes was maintained until 17 days before mating season + 35 days during the breeding period. During this time, all ewes were mated. Supplementation was also administered to the rams by considering spermatogenesis period [25]. Accordingly, supplements were provided 45 days before mating season based on the supplementation dose of the respective group, and supplementation of rams was continued throughout the entire breeding season (45 days before mating season + 35 days during the breeding period).

Behavioral monitoring

Behavioral observations were conducted following ram introduction at the onset of the breeding season. Mating behaviors were monitored daily starting at sunrise (from 05:30 a.m.) to 06:00 p.m., and the mating behaviors of mating animals were recorded using the video camera function of mobile devices. For each mating event, the corresponding recording forms were completed simultaneously (Table 2) [26]. Throughout the experimental period,

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25 mating behaviors were recorded from each group. To ensure consistency, all video recordings and behavioral assessment forms were evaluated by the same trained observer.

Blood sampling

Blood samples were collected from the jugular vein of 10 ewes with similar age and body condition scores (BCS) from each group into gel serum tubes (yellow-capped) at the following time points: Before the initiation of supplementation (day 0) during anestrus (AE) , immediately after mating during estrus (E), 7 days after mating (GD7), and 40 days after mating (GD40). For hormone and mineral analyses, blood samples were collected from the same ewes at each sampling time point. The collected blood samples were transported to the laboratory and centrifuged at 3,000 rpm (730 x *g*) for 15 minutes at room temperature. After serum separation, samples were stored at −60 °C until analysis. Serum estrogen (E2) and anti-Müllerian hormone (AMH) concentrations were analyzed in samples obtained before supplementation and immediately after mating. In contrast, progesterone levels (P4) were determined in serum samples collected on days 7 and 40 post-mating.

Mineral analyses

Serum samples were transported to the Bingöl University Central Laboratory under cold-chain conditions, and mineral analyses were performed through outsourced laboratory services. Analyses were conducted for the macro minerals calcium (Ca) and magnesium (Mg), as well as the micro minerals cobalt (Co-could not be detected), iron (Fe), copper (Cu), manganese (Mn), selenium (Se), zinc (Zn), and boron (B). Elemental analyses were carried out using an ICP-MS NexION® 2000 C instrument (PerkinElmer®, USA) equipped with a quartz nebulizer, a cyclonic spray chamber, and an integrated autosampler. Wash solutions were prepared as a mixture of 1% (v/v) suprapure nitric acid and ultrapure water using 18.2 MΩ ultrapure water obtained from a Sartorius™ Arium Pro Ultrapure Water System. To ensure analytical accuracy, ^89Y was used as an internal standard at a concentration of 25 ppb. Instrument tuning, data acquisition, and data analysis were performed using Syngistix for ICP-MS software (version 2.2). Method validation was carried out by evaluating the calibration curves, R^2 values, LODs, and calibration ranges for each element (Table 3).

Hormone analyses

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For hormone analyses, serum samples and commercial kits were transported to the laboratories of the Department of Biochemistry at Malatya Turgut Özal University under appropriate storage conditions, and analyses were performed through outsourced laboratory services. Serum samples stored at -60 °C were gradually thawed on the day of analysis, after which E2, P4, and AMH levels were determined. E2 and AMH levels were analyzed at two different physiological stages, AE and E, whereas P4 levels were analyzed at two different physiological stages, GD7 and GD40. Serum E2, P4, and AMH concentrations were measured using commercial Enzyme-Linked Immunosorbent Assay (ELISA) kits: sheep estrogen (Bioassay Technology Laboratory, Cat. No: EA0016Sh, China), sheep progesterone (Bioassay Technology Laboratory, Cat. No: E0015Sh, China), and sheep anti-Müllerian (Bioassay Technology Laboratory, Cat. No: E0088Sh, China), in accordance with the manufacturer's instructions. The reported detection ranges of the ELISA kits were 0.2-60 ng/L for estrogen, 0.05-15 ng/mL for progesterone, and 0.05-15 ng/mL for AMH, respectively. Serum samples were analyzed without dilution and as single determinations. The measurement ranges of the sheep E2, P4, and AMH ELISA kits were 0.2–60 ng/L, 0.05–15 ng/mL, and 0.05–15 ng/mL, respectively. The analytical sensitivities were 0.11 ng/L, 0.027 ng/mL, and 0.02 ng/mL, respectively. The manufacturer-reported intra-assay and inter-assay coefficients of variation (CVs) were <10% and <12% for E2, <8% and <10% for P4, and <8% and <10% for AMH, respectively. All ELISA kits used were sheep-specific and intended for the quantitative determination of the respective analytes in serum samples.

Monitoring of lambing and evaluation of reproductive performance parameters in ewes

With the onset of lambing, births were monitored by the flock owner and the project team, and from this point onward, data on lambing ewes were recorded, including the type of birth, sex of lambs, survival of lambs during the first 72 hours, animals experiencing abortion, and barren ewes. Records of births occurring at night or on pasture were kept by the shepherd and documented on the same day by the project personnel. Based on the collected data, reproductive performance indices in ewes were calculated as proportions using the following formulas [24,27]: Pregnancy rate = Number of pregnant ewes / number of ewes exposed to rams; Lambing rate = Number of lambing ewes / number of ewes exposed to rams; Reproductive failure = Number of ewes with infertility or abortion / number of ewes exposed to rams; Twin birth rate = Number of ewes with twin births / number of lambing ewes; Female lamb ratio = Number of female lambs / total number of lambs; Lamb yield per ewe exposed to rams = Number of live-born lambs / number of ewes exposed to rams; Lamb yield per birth = Number of live-born lambs / number of lambing ewes; Survival rate = Number of lambs surviving for 72 hours / total number of lambs.

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Statistical analyses

A power analysis was conducted using G*Power (version 3.1.9.7) to evaluate the adequacy of the sample sizes. For blood parameters, the repeated-measures ANOVA (within–between interaction) revealed a statistical power of 0.80 (effect size f = 0.244, $\alpha = 0.05$, six groups, two measurements, n = 60). For behavioral traits, ANOVA (fixed effects, omnibus) performed a power of 0.80 (effect size f = 0.30, $\alpha = 0.05$, six groups, n = 150). For reproductive performance, the chi-square test (contingency tables) also achieved a power of 0.80 (effect size w = 0.19, $\alpha = 0.05$, six groups, n = 360). These results indicate that the sample sizes were sufficient to detect the observed effects with adequate statistical power. A repeated-measures analysis of variance (repeated-measures ANOVA) was used to assess the effects of treatment group (G: C, S, B20, B40, BS20, BS40), measurement time (T: AE and E or GD7 and GD40), and their interaction (G x T) on reproductive hormone concentrations and mineral parameters. The analyses were performed using IBM SPSS Statistics for Windows (IBM Corp., Armonk, NY, USA). Differences among means were evaluated using Bonferroni-adjusted multiple comparisons, whereas behavioral traits were analyzed using the Kruskal–Wallis test when parametric assumptions were not met. Reproductive performance was analyzed using the chi-square (χ^2) test or Fisher's exact test when expected cell frequencies were insufficient. For sex ratio, pairwise *p*-values were adjusted using Holm's sequential Bonferroni procedure, with ordered *p*-values compared against $\alpha/(m - i + 1)$, where "m" is the number of comparisons and "i" is their rank; significance was set at $p < 0.05$.

RESULTS

Serum reproductive hormone concentrations

Data related to reproductive hormone levels are presented in Table 4. While the main effect of supplementation (G) on E2 concentration was not significant ($p > 0.05$), E2 levels increased significantly during the estrous period compared with the beginning of the experiment (T) ($p < 0.001$). However, the G × T interaction effect was found to be significant ($p = 0.048$). Consistent with the significant G × T interaction, prior to supplementation (at the beginning of the experiment), the BS20 group exhibited lower E2 concentrations. On the other hand, during estrus, serum E2 concentrations were significantly elevated in the B40 and BS40 groups compared with the control and BS20 groups ($p < 0.05$). Dietary supplementation had no significant effect on AMH hormone levels. However,

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serum AMH concentrations were found to be significantly higher in ewes exhibiting estrus compared with the anestrus period ($p < 0.001$). Although no significant differences (G x T) were detected among the treatment groups in serum P4 concentrations on days 7 and 40 of pregnancy ($p > 0.05$), the overall mean P4 concentration differed significantly among groups ($p = 0.01$), with the highest values observed in the BS40, BS20, and B40 groups and the lowest value in the control group. Furthermore, serum P4 concentrations increased significantly as pregnancy progressed ($p < 0.001$).

Serum mineral concentrations

The effects of the experimental groups on serum mineral concentrations in ewes are presented in Table 5. Serum B levels were similar among the groups before supplementation ($p > 0.05$). Following supplementation, serum B concentrations increased significantly in the BS40, B40, and BS20 groups ($p < 0.001$). Similar results were also observed in overall boron concentrations ($p < 0.001$). After supplementation, serum Ca levels increased significantly in the BS40, BS20, and B40 groups ($p < 0.001$). Similar results were also found for overall Ca concentrations ($p < 0.001$). Serum Mg concentrations after supplementation and overall Mg levels were highest in the BS40 > B40 ≥ BS20 groups ($p < 0.001$), while the other groups exhibited similar values. Serum Se concentrations was highest in the BS40 group in both the second measurement and the overall assessment ($p = 0.034$). In the second and overall evaluations, serum Zn concentrations were highest in the BS40 group, followed by the B40 and BS20 groups, and lowest in the other groups ($p < 0.001$). In the mean time-dependent levels, serum B, Ca, Mg, and Zn levels increased significantly after supplementation compared with before supplementation values, whereas Cu, Fe, Mn, and Se concentrations decreased ($p < 0.001$).

Mating behavior

Mating behaviors of the experimental groups are presented in Figure 1. As shown in Figure 1, no statistically significant differences were observed among the groups in terms of the ewe's circling behavior around the ram, approach to the ram, turning the head toward the ram, and the ewe's response to the ram's courtship behavior ($p > 0.05$).

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Reproductive performance

Table 6 present the reproductive performance parameters of the experimental groups. No significant differences were observed among the experimental groups in terms of pregnancy rate (p = 0.995). Pregnancy rates ranged from 91.66% to 95.00%, with the highest values recorded in the Salt and B40 groups (95.00%) and the lowest in the Control group (91.66%). Similarly, lambing rate was not significantly affected by the treatments (p = 0.996). Similar results were also obtained for reproductive failure (p = 0.996). Twin lambing rates were also similar among the groups (p = 0.466). When female lamb ratios were evaluated, a generally higher proportion of female lamb was observed in the boron-supplemented groups, with the highest female lamb ratio recorded in the B20 group (p = 0.009). Lambing yield per ewe exposed to rams ranged between 1.01 and 1.11, with no significant differences among the experimental groups (p = 0.999). The number of lambs per birth ranged from 1.08 to 1.21, and no differences were observed among the groups for this parameter (p = 0.998). No lamb mortality occurred within the first 72 hours in any of the experimental groups.

DISCUSSION

In sheep farming, the accurate and timely expression of mating behaviors is among the fundamental determinants of mating success at the flock level, pregnancy rates, and consequently the continuity of production. Adequate manifestation of estrous behaviors in ewes facilitates ram-ewe interactions, thereby enhancing the efficiency of natural mating and reducing labor requirements and time losses in flock management. In this sense, mating behaviors are regarded not only as a reflection of individual reproductive physiology but also as a factor that directly influences the economic and biological efficiency of sustainable sheep production systems [28]. Determining the potential effects of nutrition-based interventions on mating behaviors and reproductive performance in ewes is of importance for the development of practical production strategies.

Accordingly, reproductive hormone profiles (Table 4) were evaluated to facilitate the interpretation of the observed reproductive responses. The findings suggest that endocrine activity changes throughout the breeding season, resulting in variations in the concentrations of reproductive hormones as ovarian activity resumes [29]. This transitional phase involves critical physiological events such as accelerated follicular development, triggering of ovulation, and establishment of luteal function; therefore, the endocrine system becomes highly sensitive to

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environmental and nutritional stimuli. During this period, nutrition-based applications have been reported to influence energy–protein balance as well as hormone synthesis and metabolism through modulation of mineral homeostasis [30]. In this study, the administration of boric acid and rock salt was associated with changes in reproductive hormonal responses in ewes, with the observed responses varying across different time points. E_2 and AMH were measured before supplementation and immediately after mating to assess the effects of supplementation on ovarian function and reproductive endocrine status, whereas progesterone was measured on days 7 and 40 after mating, as these time points are physiologically relevant for evaluating early luteal function and pregnancy maintenance [9, 31]. With respect to E2 concentration, a statistically significant difference was detected among the groups at estrus, with the highest E2 concentration observed in the BS40 group. In this study, boric acid and rock salt supplementation was associated with variations in reproductive hormonal responses in ewes across different time points [32]. The elevation of E2 during estrus is consistent with dominant follicle development and the preovulatory physiological peak and represents an expected endocrine response in ewes [33]. Within this framework, the elevated E2 level observed in the BS40 group may reflect a physiological alteration in ovarian hormonal response associated with the dietary administration of boric acid and rock salt. In addition, boric acid has been reported to support processes involved in steroid hormone synthesis through its potential effects on cellular and metabolic functions [12]. Furthermore, rock salt may contribute to normal cellular and metabolic functions involved in reproductive processes [34]. The significant increase observed in the B40 group indicates that the administration of boric acid alone also affected E2 synthesis through similar pathways. The absence of statistically significant differences in AMH levels among the groups suggests that the applied interventions did not exert a marked short-term effect on AMH secretion, which reflects the follicular pool [35]. Nevertheless, the increase in AMH levels at estrus may be considered a physiological response parallel to enhanced follicular activity [36]. With respect to P4, no significant differences were observed among the groups on either day 7 or day 40 of mating. However, P4 concentrations increased significantly from day 7 to day 40. In contrast, the significantly higher overall mean P4 levels observed in the BS40 and BS20 groups suggest that the administration of boric acid and rock salt was associated with differences in P4 concentrations. Başeğmez et al. [37] reported that boron compounds can modulate P4 responses and that this effect may exhibit a dose-dependent character; in their study, low, medium, and high-dose boric acid was administered orally to female rats for 21 days. They reported that P4 levels increased at high doses and that this increase was accompanied by dose-dependent tissue alterations. In the present study, the observed

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changes in progesterone-related responses were associated with the concurrent administration of boric acid and rock salt.

As shown in Table 3, the calibration data demonstrated satisfactory linearity for all analyzed elements across the selected calibration ranges. The obtained limits of detection (LOD) indicate that the method was sensitive to detect the target elements at the concentrations investigated. Although Zn showed slightly lower linearity than the other elements, its calibration curve remained acceptable for quantitative determination within the selected concentration range. The estrous period in ewes is characterized by increased endocrine activity and elevated metabolic demands associated with follicular development, ovulation, and uterine tissue remodeling, in which mineral metabolism plays a dynamic role. During this period, the balanced supply of minerals such as Ca, Mg, Zn, and Se, as well as trace elements like B, is essential for maintaining cellular function, metabolic processes, and overall physiological activity in reproductive tissues [38,39]. The similarity of serum B and Ca levels among the groups at the beginning of the experiment reflected more clearly the effects of the supplementation interventions (Table 5). Such increase in serum boron levels in the boron-supplemented groups after treatment indicated that boric acid administration expanded the systemic borate pool and this response may have varied depending on the dose administered. This finding is biologically consistent with the literature describing the regulatory role of boron in estrogen metabolism, steroid hormone synthesis, and calcium homeostasis [9,18]. Moreover, the higher boron levels observed in the boron and salt supplemented groups may indicate improved boron availability [4,18]. In ruminants, increased salt intake can stimulate water consumption and salivary secretion, thereby promoting a more stable ruminal environment that may enhance mineral solubility, microbial activity, and overall ruminal function. These improvements may increase nutrient intake and optimize gastrointestinal conditions, thereby facilitating the absorption and utilization of dietary minerals in the small intestine [17]. As shown in Table 5, serum Ca levels elevated in the B40 and BS groups following supplementation. Similarly, Kan et al. [40] reported that the effects of boron on mineral metabolism become particularly pronounced along the Ca-Mg axis, indicating that boron can elevate serum Ca and Mg levels, which may be related to PTH activity. The emergence of significant differences among Mg, Se, and Zn levels of the groups after supplementation, and the marked increases observed especially in the BS40 group, suggest that the high-dose boric acid-rock salt supplementation may be associated with a response in mineral status. Selenium plays a critical role in antioxidant defense through selenoenzymes, while Zn is essential for DNA synthesis, cell division, and the functional maintenance in reproductive tissues [41,42]. Kan et al. [40], reported that boron supplementation can enhance the absorption/retention of Ca, Mg, and Zn in different species; however, the strength of this effect may

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vary depending on animal species, the form of boron, and feeding conditions. On the other hand, the lack of significant post-supplementation differences in Cu, Fe, and Mn concentrations among the groups suggested that the applied interventions had only a limited influence on these minerals. This finding is consistent with the reports of Kan et al. [40], indicating that boron supplementation can increase Ca/P retention while reducing Mn and Fe retention. Moreover, when compared to the beginning of the experiment, serum B, Ca, Mg, and Zn levels generally elevated at the onset of the breeding season; whereas, Cu, Fe, Mn, and Se levels lowered (Table 5). This pattern may be associated with physiological processes of mineral utilization during the breeding season. These findings are compatible with the observations reported by Kan et al. [40]. Furthermore, evaluation of the serum mineral profiles demonstrated that the concentrations of all analyzed minerals remained within the reference ranges reported for sheep and did not reach levels considered indicative of mineral toxicity [43].

Although boron has been reported to play direct or indirect roles in endocrine-related physiological processes [4,44], supplementation with boric acid or rock salt did not result in significant differences in estrous behavior compared with the control group (Figure 1). Maldonado et al. [28], reported that the behavioral expression of estrus in ewes was modulated not only by endocrine stimulation but also by multiple factors such as the presence of the ram, flock dynamics, environmental conditions, and individual temperament. Therefore, changes in hormone levels may not always lead to equally noticeable changes in behavior [45]. However, the absence of behavioral differences among the groups may be attributed to the limited impact of the supplements on behavioral outcomes [18]. Furthermore, the passive nature of estrous behavior in ewes suggests that the applied supplements did not affect the observability of the evaluated behavioral parameters. Indeed, estrous signs in ewes have been reported to exhibit a relatively passive pattern, and behavioral scoring has been shown to display high variability, particularly under field conditions [46], which is thought to have contributed to the absence of detectable differences in estrous behavior among the experimental groups.

Reproductive performance parameters, including pregnancy, lambing, barren, and abortion rates, as well as the distribution of single and twin births, are important indicators of flock productivity and the economic sustainability of sheep production. Although these parameters are associated with endocrine and metabolic status, they may also be influenced by various management, environmental, and mating-related factors under field conditions [47]. In the present study, no statistically significant differences were observed among the treatment groups in pregnancy, lambing, barren, abortion, or twin birth rates (Table 6). Although numerically higher values were observed in some

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of the boron- and salt-supplemented groups, the relatively high baseline reproductive performance may have limited the potential to detect statistically significant differences. Pizzorno [48] and Agarwal et al. [49] suggested that boron may support the bioavailability of reproductive hormones and ovarian function. However, such physiological effects may not necessarily translate into measurable improvements in overall reproductive performance under field conditions. Accordingly, the absence of significant differences in pregnancy and lambing rates in the present study may be consistent with this possibility. Likewise, the absence of differences in the proportions of twin births does not provide evidence that boron supplementation increased twinning rates [48,50]. The relatively high reproductive performance observed across the experimental groups may also have been influenced by the management conditions of the study. Close monitoring of mating activities by the research team may have facilitated the detection and management of mating events, while active involvement during the lambing period and early lamb care may have contributed to the early survival observed in the present study, as no lamb mortality was recorded within the first 72 h after birth in any of the experimental groups. However, these observations should be considered as possible management-related influences rather than established causal effects.

With regard to lamb sex ratio, a higher numerical proportion of female lambs was observed in the boron-supplemented groups, with a statistically significant difference particularly in the B20 group. Previous human studies have reported associations between paternal boron exposure and offspring sex-related outcomes [51,52]. However, comparable evidence in sheep and other livestock species remains limited, and these findings cannot be directly extrapolated to the present experimental conditions. Therefore, the observed difference in female lamb proportion should be interpreted cautiously and regarded as an exploratory finding rather than definitive evidence of a treatment-related effect. In addition, lamb sex ratio may be influenced by biological variation as well as by factors such as the number and distribution of mating events, ram characteristics, timing of mating, and environmental conditions [53]. Because mating was conducted under group-housing and free-mating conditions, potential clustering within treatment compartments and the possible influence of ram assignment cannot be completely excluded. These factors may have contributed to variation in reproductive outcomes, particularly lamb sex ratio, and should therefore be considered when interpreting the present findings.

A limitation of the present study was the absence of independent pen-level replication for each treatment group. Each treatment was housed in a single compartment; therefore, treatment effects could not be completely separated from potential compartment effects. Although boric acid and/or rock salt supplementation was administered

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individually to each ewe, the shared housing environment and free-mating conditions may have contributed to the observed reproductive responses. In addition, the allocation of rams and mating opportunities could not be fully separated from treatment groups. Accordingly, the reproductive findings, particularly those related to lamb sex ratio, should be interpreted cautiously at the treatment level. Further studies incorporating independent pen-level replication and a controlled mating design are warranted to confirm these observations.

CONCLUSION

In conclusion, supplementation affected serum estrogen and progesterone concentrations, with the highest hormone levels generally observed in the BS40 group. Despite these hormonal changes, no significant differences were detected in estrous behavior or in most reproductive performance traits. A higher proportion of female lambs was observed in the boron-supplemented groups; however, further studies with larger sample sizes are required to confirm this observation. Serum mineral concentrations were also significantly influenced by both measurement time and supplementation treatment. These results suggest that boron and salt supplementation can alter hormonal and mineral profiles in ewes during the breeding season. Although the biological significance of these changes remains unclear, the findings provide a basis for future studies exploring the potential role of boron in sheep nutrition and reproduction.

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REFERENCES

1. Paolino R, Di Trana A, Coppola A, Sabia E, Riviezzi AM, Vignozzi L, et al. May the Extensive Farming System of Small Ruminants Be Smart? Agriculture. 2025;15(9):929. https://doi.org/10.3390/agriculture15090929
2. Ponnampalam EN, Jairath G, Alves SP, Gadzama IU, Santhiravel S, Mapiye C, et al. Sustainable livestock production by utilising forages, supplements, and agricultural by-products: Enhancing productivity, muscle gain, and meat quality-A review. Meat Sci. 2025;109921. https://doi.org/10.1016/j.meatsci.2025.109921

3. Stefanache A, Lungu II, Butnariu IA, Calin G, Gutu C, Marcu C, et al. Understanding how minerals contribute to optimal immune function. J Immunol Res. 2023;1:3355733. https://doi.org/10.1155/2023/3355733
4. Khaliq H, Juming Z, Ke-Mei P. The physiological role of boron on health. Biol Trace Elem Res. 2018;186(1):31-51. https://doi.org/10.1007/s12011-018-1284-3
5. Farag MA, Abib B, Qin Z, Ze X, Ali SE. Dietary macrominerals: Updated review of their role and orchestration in human nutrition throughout the life cycle with sex differences. Curr. Res. Food Sci. 2023;6:100450. https://doi.org/10.1016/j.crfs.2023.100450
6. Vieira-Neto A, Lean IJ, Santos JEP. Periparturient mineral metabolism: implications to health and productivity. Animals. 2024;14(8):1232. https://doi.org/10.3390/ani14081232
7. Digby SN, Chadwick MA, Blache D. Salt intake and reproductive function in sheep. Animal. 2011;5(8): 1207-1216. https://doi.org/10.1017/S1751731111000152
8. Kremer D, Post A, Seidel U, Huebbe P, van der Veen Y, Groothof D, et al. Boron Intake and decreased risk of mortality in kidney transplant recipients. Eur. J. Nutr. 2022;61(2):973-984. https://doi.org/10.1007/s00394-021-02702-0
9. Estevez-Fregoso E, Kilic A, Rodríguez-Vera D, Nicanor-Juárez LE, Romero-Rizo CEM, Farfán-García ED, et al. Effects of boron-containing compounds on liposoluble hormone functions. Inorganics. 2023;11(2):84. https://doi.org/10.3390/inorganics11020084
10. Elkomy AE, Abd El-hady AM, Elghalid OA. Dietary boron supplementation and its impact on semen characteristics and physiological status of adult male rabbits. Asian J Poultry Sci. 2015;9:85-96. https://doi.org/10.3923/ajpsaj.2015.85.96
11. Ibrahim TB, Abdel-Wahab A, Aziz RLA, El-Anwar AH, Ibrahim SS. Dietary boron supplementation and its impact on testicular function, thyroid activity and serum calcium in rams. Small Ruminant Research. 2019;174:156-162. https://doi.org/10.1016/j.smallrumres.2019.03.008
12. Abdel-Wahab A, Ibrahim SS, El-Anwar AH, Mabrook EA, Ibrahim TB, Abdel-Razik AH. Effects of dietary boron supplementation on the testicular function and thyroid activity in male goats: involvement of CYP17A1 gene. Reprod Domest Anim. 2022;57:1353-1362. https://doi.org/10.1111/rda.14212

ACCEPTED

13. Thomas DT, Rintoul AJ, Masters DG. Sheep select combinations of high and low sodium chloride, energy and crude protein feed that improve their diet. Appl Anim Behav Sci. 2007;105(1-3):140-153. https://doi.org/10.1016/j.applanim.2006.05.015
14. Lee BH, Yang AR., Kim MY, McCurdy S, Boisvert WA. Natural sea salt consumption confers protection against hypertension and kidney damage in Dahl salt-sensitive rats. Food Nutr Res. 2017;61(1):1300375. https://doi.org/10.1080/16546628.2017.1264713
15. Crintea IN, Cindrea AC, Mederle OA, Trebuian CI, Timar R. Electrolyte Imbalances and Metabolic Emergencies in Obesity: Mechanisms and Clinical Implications. Diseases. 2025;13(3):69. https://doi.org/10.3390/diseases13030069
16. Park M, Li Q, Shcheynikov N, Zeng W, Muallem S. NaBC1 is a ubiquitous electrogenic Na+ -coupled borate transporter essential for cellular boron homeostasis and cell growth and proliferation. Mol Cell. 2004;16(3):331-41. https://doi.org/10.1016/j.molcel.2004.09.030
17. White HC, Davis NG, Emon MLV, DelCurto-Wyffels HM, Wyffels SA, DelCurto T. Impacts of increasing levels of salt on intake, digestion, and rumen fermentation with beef cattle consuming low-quality forages. J Anim Sci. 2024;102:skae284. https://doi.org/10.1093/jas/skae284
18. Nielsen FH. Update on human health effects of boron. J Trace Elem Med Biol. 2014;28(4):383-387. https://doi.org/10.1016/j.jtemb.2014.06.023
19. Cunningham K, Van Burgel A, Kelman KR, Macleay CM, Paganoni BL, Thompson AN. Interactions between ewes and rams during mating can be used to predict lambing dates accurately, but not sire. Animals. 2022;12(13):1707. https://doi.org/10.3390/ani12131707
20. Sani A, Sani A, Sharifimoghadam S, Bahari M, Emamvirdi M, Emamvirdi A, et al. The Effects of Boron on Sperm Qualities and Testicular Histopathology in Animal Studies: A Systematic Review. J Urol Surg. 2025; 12(4):202-212. https://doi.org/10.4274/jus.galenos.2025.2024-9-7
21. Cortés S, Espinoza-Navarro O, Ferreccio C. High exposure to boron in drinking water and sperm parameters in Chilean young people. Int J Morphol. 2017;35:99-104. https://doi.org/10.4067/S0717-95022017000100017
22. Krishnan BB, Selvaraju S, Gowda NKS, Subramanya KB, Pal D, Archana SS, Bhatta R. Dietary boron supplementation enhances sperm quality and immunity through influencing the associated biochemical

ACCEPTED

parameters and modulating the genes expression at testicular tissue. J Trace Elem Med Biol. 2019;55:6-14. https://doi.org/10.1016/j.jtemb.2019.05.004

23. Yorgancılar MÜ. Small ruminant breeding systems: Environmental conditions, shelters In: Erdem H, Çiftci E, Işık MK, editors. Academician Bookstore: İstanbul, Türkiye, 2021; p. 35.
24. Özyurtlu N, Bademkıran S. Estrus synchronization and induction of estrus methods in sheep. Dicle Univ Vet Fac J. 2010;1(1):17- 22.
25. Lekola KP, Ngcobo MS, Lehloenya KC. Selenium efficiency in protecting sperm quality and testicular parameters of South African indigenous Zulu rams exposed to heat stress. Trop Anim Health Prod. 2024;56(8):349. https://doi.org/10.1007/s11250-024-04207-4
26. Maurya VP, Naqvi SMK, Gulyani R, Joshi A, Mittal JP. Effect of thermal stress on sexual behaviour of superovulated Bharat Merino Ewes. Asian-Australas J Anim Sci. 2005;18(10):1403-1406. https://doi.org/10.5713/ajas.2005.1403
27. Şen M, Uğurlu M. Reproductive Traits, Survivability, Growth Performance and Several Body Size Characteristics in Romanov Sheep. Ataturk Univ J Vet Sci. 2021;16(2):155-163. https://doi.org/10.17094/ataunivbd.813491
28. Maldonado JG, Valverde GR, Lara RRD, Sánchez JG, Ruiz JA. Ram sexual preferences and estrous behavior expression in ewes with different reproductive status. Turk J Vet Anim Sci. 2021;45(6):1074-1079. https://doi.org/10.3906/vet-2012-80
29. Grosbois J, Srsen V, Grande AM, Picton HM, Telfer EE. Reproductive seasonality influences follicle dynamics and the ovarian extracellular matrix structural properties in ewes. Reproduction. 2025;169(6). https://doi.org/10.1530/REP-25-0010
30. Corsetti G, Pasini E, Scarabelli TM, Romano C, Singh A, Scarabelli CC, et al. Importance of energy, dietary protein sources, and amino acid composition in the regulation of metabolism: an indissoluble dynamic combination for life. Nutrients. 2024;16(15):2417. https://doi.org/10.3390/nu16152417
31. Xu Q, Wang C, Wang L, Feng R, Guo Y, Feng S, et al. Correlation analysis of serum reproductive hormones and metabolites during multiple ovulation in sheep. BMC Vet Res. 2022;18(1):290. https://doi.org/10.1186/s12917-022-03387-1

ACCEPTED

32. Başer ÖF, Karapehlivan M, Kükürt A, Uysal AY, Kafkas E, Dağ S. Protective effect of boric acid against 3-NPA-induced ovarian damage. Biol Trace Elem Res. 2026;204:1008-1021. https://doi.org/10.1007/s12011-025-04725-8
33. Ramesh V, Devi LS, Joshi V, Mech M, Khate K, Khan MH. Ovarian follicular dynamics, hormonal profiles and ovulation time in Mithun cows (Bos frontalis). Reprod Domest Anim. 2022;57(10):1218-1229. https://doi.org/10.1111/rda.14196
34. Minegishi S, Luft FC, Titze J, Kitada K. Sodium handling and interaction in numerous organs. Am J Hypertens. 2020;33(8):687-694. https://doi.org/10.1093/ajh/hpaa049
35. Iwase A, Hasegawa Y, Tsukui Y, Kobayashi M, Hiraishi H, Nakazato T, Kitahara Y. Antimüllerian hormone beyond an ovarian reserve marker: The relationship with the physiology and pathology in the life-long follicle development. Front Endocrinol. 2023;14:1273966. https://doi.org/10.3389/fendo.2023.1273966
36. Shahrokhi SZ, Kazerouni F, Ghaffari F. Antimüllerian hormone: Genetic and environmental effects. Clin Chim Acta. 2018;476:123-129. https://doi.org/10.1016/j.cca.2017.11.027
37. Başeğmez M, Doğan N, Demirel HH, Doğan MF. Effects of boric acid on reproductive hormones and utero-ovarian tissues in female rats: A dose-response study. J Boron. 2025;10(4):150-155. https://doi.org/10.30728/boron.1761512
38. Jyani V, Mudgal V, Gupta M, Bharadwaj A. Supplementation of critical micro-nutrients in peri-parturient dairy buffaloes improves lactation performance. J Trace Elem Miner. 2024;8:100136. https://doi.org/10.1016/j.jtemin.2024.100136
39. Kapper C, Stelzl P, Oppelt P, Ganhör C, Gyunesh AA, Arbeithuber B, Rezk-Füreder M. The impact of minerals on female fertility: A systematic review. Nutrients. 2024;16(23):4068. https://doi.org/10.3390/nu16234068
40. Kan F, Kucukkurt I. The effects of boron on some biochemical parameters: A review. J Trace Elem Med Biol. 2023;79:127249. https://doi.org/10.1016/j.jtemb.2023.127249
41. McDowell LR. Minerals in animal and human nutrition. 2nd ed. Amsterdam. The Netherlands: Elsevier Science BV. 2003

ACCEPTED

42. Abdelnour SA, Abd El-Hack ME, Noreldin AE, Batiha GE, Beshbishy AM, Ohran H, et al. High salt diet affects the reproductive health in animals: an overview. Animals. 2020;10(4):590. https://doi.org/10.3390/ani10040590

43. Poppenga RH, Ramsey J, Gonzales BJ, Johnson CK. Reference intervals for mineral concentrations in whole blood and serum of bighorn sheep (Ovis canadensis) in California. J Vet Diagn Investig 2012;24(3):531-538. https://doi.org/10.1177/1040638712441936

44. Akarsu S, Şimşek ÜG, Erişir M, Sönmez M, Güngör IH, Acisu TC, et al. Rock salt and boron supplementation: Effects on oxidative stress in blood and semen of rams. J Hellenic Vet Med Soc. 2025;76(4):9929-9936. https://doi.org/10.12681/jhvms.40490

45. Cameron JL. Hormonal Mediation of Physiological and Behavioral Processes That Influence Fertility. In: National Research Council (US) Panel for the Workshop on the Biodemography of Fertility and Family Behavior; Wachter KW, Bulatao RA, editors. Offspring: Human Fertility Behavior in Biodemographic Perspective. Washington (DC): National Academies Press (US); 2003. 5. Available from: https://www.ncbi.nlm.nih.gov/books/NBK97279/

46. Dodd CL, Pitchford WS, Edwards JEH, Hazel SJ. Measures of behavioural reactivity and their relationships with production traits in sheep: A review. Appl Anim Behav Sci. 2012;140(1-2):1-15. https://doi.org/10.1016/j.applanim.2012.03.018

47. Shorten PR, Edwards SJ, Juengel JL. The role of reproductive loss on flock performance: a comparison of nine industry flocks. Transl. Anim. Sci. 2021;5(1):txab013. https://doi.org/10.1093/tas/txab013

48. Pizzorno L. Nothing boring about boron. Integr Med (Encinitas). 2015;14(4):35.

49. Agarwal A, Aponte-Mellado A, Premkumar BJ, Shaman A, Gupta S. The effects of oxidative stress on female reproduction: A review. Reprod Biol Endocrinol. 2012;10:49. https://doi.org/10.1186/1477-7827-10-49

50. Bolt HM, Başaran N, Duydu Y. Effects of boron compounds on human reproduction. Arch Toxicol. 2020;94(3):717-724. https://doi.org/10.1007/s00204-020-02700-x

51. Robbins WA, Wei F, Elashoff DA, Wu G, Xun L, Jia J. Y:X sperm ratio in boron-exposed men. J Androl. 2008;29(1):115-121. https://doi.org/10.2164/jandrol.107.003541

ACCEPTED

52. Scialli AR, Bonde JP, Brüske-Hohlfeld I, Culver BD, Li Y, Sullivan FM. An overview of male reproductive studies of boron with an emphasis on studies of highly exposed Chinese workers. Reprod Toxicol. 2010;29(1):10-24. https://doi.org/10.1016/j.reprotox.2009.10.006
53. Ungerfeld R, Alexander BM. Determinants of ram sexual behavior and its impact on sheep breeding. Anim Reprod Sci. 2024;270:107599. https://doi.org/10.1016/j.anireprosci.2024.107599

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Table 1. Age and body condition scores (BCS) of ewes in the experimental groups

	Control	Salt	B20	B40	BS20	BS40
Age of ewes, years	3.61±0.136	3.35±0.138	3.57±0.170	3.19±0.162	2.90±0.153	3.79±0.179
BCS of ewes	3.00±0.017	3.17±0.100	3.33±0.087	3.03±0.075	3.06±0.092	3.25±0.075

Values are presented as mean ± SEM.

Table 2. Forms used to determine mating behaviour in rams and ewes [26]

Date…….
Sheep ID……
Treatment Group ……
ROTATIONAL MOTION Status
0 – No rotation
1 – Very slow rotation
2 – Slow rotation
3 – Medium speed rotation
4 – Rapid rotation
5 – Spinning very fast
HEAD ROTATION MOVEMENT
0 – No turning of the head or showing interest
1 – The female looks at the ram without turning her head.
2 – The female turns her head and looks briefly at the ram.
3 – The female turns her head and gazes at the ram for a long time.
4 – The female turns her head and gazes at the ram for a long time, touching her head to a single spot on his body.
5 – The female turns her head and gazes at the ram for a long time, pressing her head against one specific area for an extended period.
APPROACH WITH CAUTION
0 – No approaching the ram
1 – The sheep only makes eye contact with the ram.
2 – The sheep moves slowly towards the ram.
3 – The sheep moves quickly towards the ram
4 – The sheep runs towards the ram.
5 – The sheep runs around the ram.
THE SHEEP'S REACTION TO THE RAM'S INTERVENTION
0 – No interest in the ram
1 – When the ram touches the sheep, the sheep runs away from the ram.
2 – When the ram touches it, it slowly runs away.
3 – The ram retreats when touched
4 – It stops when the ram touches it
5 – When the ram approaches, stand with a normal posture.

Table 3. Calibration parameters for the identified elements

Identified elements	R^2 (Coefficient of Determination)	LOD (Limit of Detection, ppb)	Calibration Range (ppb)
B	0.999914	0.231689	0.581–50.258
Ca	0.999113	27.682189	32.499–1265.992
Cu	0.999958	0.033890	0.691–50.159
Fe	0.999853	0.114332	16.596–1260.173
Mg	0.999810	0.211305	10.879–1261.041
Mn	0.999920	0.026537	0.604–50.273

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Se	0.999964	0.569613	0.675–49.913
Zn	0.991754	0.929144	0–49.726

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Table 4. The effect of research groups on reproductive hormone levels in ewes

Features		Control	Salt	B20	B40	BS20	BS40	Average time dependent levels	SEM	*p*-values		
										G	*T*	*GxT*
Estrogen (E2), ng/L	AE	9.67^{a}	8.67^{a}	9.69^{a}	9.37^{a}	7.44^{b}	8.82^{a}	8.94^{Y}	0.31			
Estrogen (E2), ng/L	E	12.49^{b}	14.37^{ab}	13.85^{ab}	14.82^{a}	13.65^{b}	15.98^{a}	14.13^{X}	0.12	0.109	< 0.001	0.048
Average E2 level, ng/L		11.08	11.52	11.78	11.90	10.55	12.40	11.53	0.19			
Anti-müllerian (AMH), ng/mL	AE	3.11	3.02	2.85	3.31	3.05	2.62	2.99^{Y}	0.08			
Anti-müllerian (AMH), ng/mL	E	3.29	3.74	3.43	4.45	3.36	3.79	3.68^{X}	0.13	0.075	< 0.001	0.288
Average AMH level, ng/mL		3.20	3.38	3.14	3.88	3.20	3.20	3.33	0.08			
Progesterone (P4), ng/mL	GD7	1.20	1.36	1.27	1.54	1.51	1.66	1.43^{Y}	0.06			
Progesterone (P4), ng/mL	GD40	1.38	1.55	1.68	2.25	2.35	2.45	1.94^{X}	0.09	0.01	< 0.001	0.121
Average P4 level, ng/mL		1.29^{B}	1.45^{AB}	1.47^{AB}	1.89^{A}	1.93^{A}	2.06^{A}	1.68	0.06			

Control: No additives; Salt: 10 g/day rock salt; B20: 20 mg boric acid/day; B40: 40 mg boric acid/day; BS20: 20 mg boric acid + 10 g rock salt/day; BS40: 40 mg boric acid + 10 g rock salt/day; G: Group; T: Measurement time; GxT: Interaction of Group x Measurement time; AE: Anestrus, day 0 of the study before supplementation; E: Day of estrus; GD7: Gestation day 7; GD40: Gestation day 40; SEM: Standard error of the mean; *p*: Statistical significance

$^{A,B:}$ Different superscript letters indicate significant differences among groups (main effect of group (G)).

^{X,Y}: Different superscript letters indicate significant differences over time (main effect of time (T))

^{a,b}: Different superscript letters within the same row indicate significant differences between the levels of the G × T interaction

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Table 5. The effect of research groups on serum mineral levels in ewes

Features (levels, ppm)		Control	Salt	B20	B40	BS20	BS40	Average time dependent levels	SEM	*p*-values		
										G	*T*	*GxT*
B	(AE)	0.404	0.409	0.410	0.413	0.407	0.422	0.411^{Y}	0.003			
B	(E)	0.452^{b}	0.468^{b}	0.493^{b}	0.634^{a}	0.631^{a}	0.690^{a}	0.562^{X}	0.014	< 0.001	< 0.001	< 0.001
Average B		0.428^{B}	0.438^{B}	0.452^{B}	0.524^{A}	0.519^{A}	0.556^{A}	0.487	0.007			
Ca	(AE)	30.663	31.101	31.859	33.086	33.006	32.180	31.999^{Y}	0.335			
Ca	(E)	32.770^{b}	30.544^{b}	31.787^{b}	53.856^{a}	55.679^{a}	56.326^{a}	43.693^{X}	1.712	< 0.001	< 0.001	< 0.001
Average Ca		31.716^{B}	30.882^{B}	31.823^{B}	43.471^{A}	44.343^{A}	44.253^{A}	37.846	0.901			
Cu	(AE)	1.751	1.686	1.705	1.775	1.937	1.786	1.776^{X}	0.041			
Cu	(E)	0.414	0.394	0.445	0.451	0.419	0.498	0.426^{Y}	0.012	0.474	< 0.001	0.556
Average Cu		1.082	1.040	1.075	1.113	1.178	1.142	1.106	0.021			
Fe	(AE)	1.889	1.849	1.943	1.933	2.029	1.934	1.931^{X}	0.022			
Fe	(E)	0.808	0.786	0.728	0.803	0.681	0.790	0.765^{Y}	0.019	0.941	< 0.001	0.059
Average Fe		1.349	1.318	1.335	1.368	1.355	1.362	1.348	0.014			
Mg	(AE)	8.035	7.909	8.121	8.396	8.533	8.424	8.241^{Y}	0.086			
Mg	(E)	8.250^{c}	7.335^{c}	8.613^{c}	12.939^{b}	9.552^{bc}	17.722^{a}	10.716^{X}	0.566	< 0.001	< 0.001	< 0.001
Average Mg		8.143^{C}	7.622^{C}	8.367^{C}	10.668^{B}	9.043^{BC}	13.073^{A}	9.478	0.294			
Mn	(AE)	0.064	0.059	0.068	0.065	0.057	0.059	0.062^{X}	0.001			
Mn	(E)	0.009	0.011	0.008	0.012	0.011	0.011	0.010^{Y}	0.001	0.517	< 0.001	0.067
Average Mn		0.036	0.035	0.038	0.038	0.034	0.035	0.036	0.001			
Se	(AE)	0.091	0.096	0.096	0.093	0.098	0.091	0.094^{X}	0.002			
Se	(E)	0.064^{c}	0.061^{c}	0.071^{bc}	0.083^{ab}	0.062^{c}	0.097^{a}	0.073^{Y}	0.003	0.034	< 0.001	0.002
Average Se		0.077^{B}	0.078^{B}	0.083^{AB}	0.088^{AB}	0.080^{AB}	0.094^{A}	0.083	0.001			
Zn	(AE)	0.244	0.252	0.261	0.243	0.245	0.235	0.247^{Y}	0.003			
Zn	(E)	0.234^{c}	0.223^{c}	0.261^{c}	0.915^{ab}	0.750^{b}	1.338^{a}	0.623^{X}	0.067	< 0.001	< 0.001	< 0.001
Average Zn		0.239^{C}	0.238^{C}	0.261^{C}	0.579^{B}	0.497^{B}	0.787^{A}	0.435	0.033			

Control: No additives; Salt: 10 g/day rock salt; B20: 20 mg boric acid/day; B40: 40 mg boric acid/day; BS20: 20 mg boric acid + 10 g rock salt/day; BS40: 40 mg boric acid + 10 g rock salt/day; boron (B); calcium (Ca); copper (Cu); iron (Fe); magnesium (Mg); manganese (Mn); selenium (Se); zinc (Zn); G: Group; T: Measurement time; GxT: Interaction of Group x Measurement time; AE: Anestrus, day 0 of the study before supplementation; E: day of estrus; SEM: Standard error of the mean; *p*: Statistical significance

$^{A,B,C:}$ Different superscript letters indicate significant differences among groups (main effect of group (G)).

^{X,Y}: Different superscript letters indicate significant differences over time (main effect of time (T))

^{a,b,c}: Different superscript letters within the same row indicate significant differences between the levels of the G × T interaction

ACCEPTED

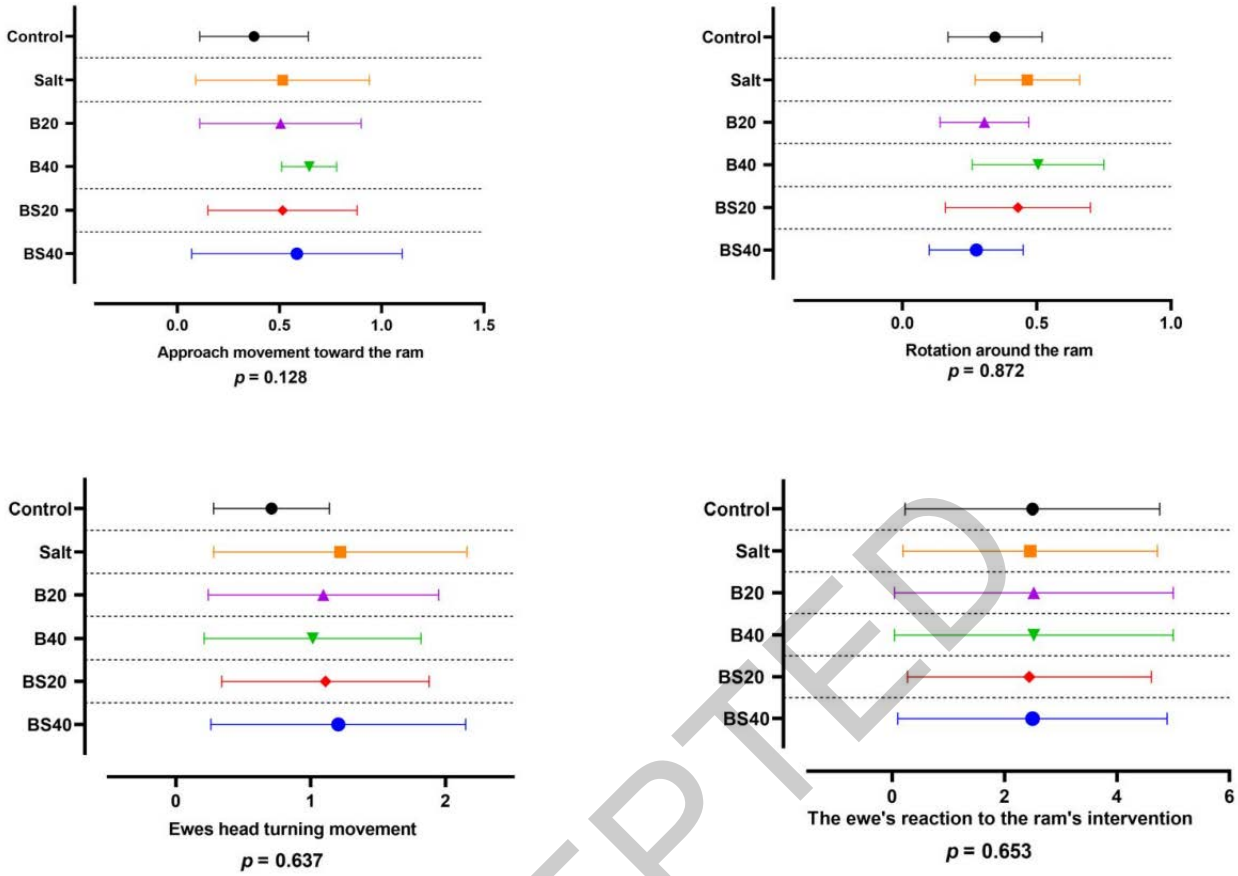


Figure 1. Behavioral characteristics of sheep in research groups

Control: No additives; Salt: 10 g/day rock salt; B20: 20 mg boric acid/day; B40: 40 mg boric acid/day; BS20: 20 mg boric acid + 10 g rock salt/day; BS40: 40 mg boric acid + 10 g rock salt/day; *p*: Statistical significance; Values are presented as mean ± SEM

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Table 6. The effect of research groups on reproductive performance in ewes

Features	Control	Salt	B20	B40	BS20	BS40	*p*-values
Pregnancy rate	55/60 (91.66 %)	57/60 (95.00 %)	56/60 (93.33 %)	57/60 (95.00 %)	56/60 (93.33 %)	56/60 (93.33 %)	0.995
Lambing rate	55/60 (91.66 %)	57/60 (95.00 %)	56/60 (93.33 %)	56/60 (93.33 %)	55/60 (91.66 %)	56/60 (93.33 %)	0.996
Reproductive failure	5/60 (8.34 %)	3/60 (5.00 %)	4/60 (6.67 %)	4/60 (6.67 %)	5/60 (8.34 %)	4/60 (6.67 %)	0.996
Twin lambing rate	12/55 (21.81 %)	7/57 (12.28 %)	5/56 (8.92 %)	9/56 (16.07 %)	9/55 (16.36 %)	11/56 (19.64 %)	0.466
Proportion of female lambs	26/67 (38.80^{b} %)	30/64 (46.87^{ab} %)	41/61 (67.21^{a} %)	40/65 (61.53^{ab} %)	36/64 (56.25^{ab} %)	30/67 (44.77^{ab} %)	0.009
Lamb yield per ewe	67/60 (1.11)	64/60 (1.06)	61/60 (1.01)	65/60 (1.08)	64/60 (1.06)	67/60 (1.11)	0.999
Lamb yield per birth	67/55 (1.21)	64/57 (1.12)	61/56 (1.08)	65/56 (1.16)	64/55 (1.16)	67/56 (1.19)	0.998

Control: No additives; Salt: 10 g/day rock salt; B20: 20 mg boric acid/day; B40: 40 mg boric acid/day; BS20: 20 mg boric acid + 10 g rock salt/day; BS40: 40 mg boric acid + 10 g rock salt/day; Data for each group are presented as n/N (%); *p:* Statistical significance; ^{a,b}: Differences between proportions denoted by different letters in the same row are significant ($p < 0.05$).

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