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Research Article

Structural and metabolic responses of Holstein heifers to rumen undegradable protein supply

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ABSTRACT- This study evaluated the effects of various levels of rumen-undegradable protein (RUP) on performance, blood metabolites, and structural growth of Holstein dairy heifers. Seventy-two Holstein heifers (initial body weight = 128.7 ± 15.5 kg; age = 4 ± 0.31 months) were allocated into 12 pens, with 6 heifers per pen. The pens were randomly assigned to 1 of 3 dietary treatments over a period of 60 days. The dietary treatments consisted of three percentages of RUP (28%, 33%, and 38% of crude protein). Feed intake was recorded from each pen daily. The heifers were weighed every two weeks. Blood samples were taken on day 1, 30, and 60. Body condition score, back fat thickness, and skeletal growth were recorded on day 1 and 60 of the experiment. Increasing dietary RUP levels reduced dry matter, crude protein, and RDP intakes ($P < 0.05$), whereas it enhanced RUP and MP intakes ($P < 0.01$). Feeding different levels of RUP had no effect on final body weight, body condition score (BCS), and back fat thickness ($P > 0.05$). Serum total protein and cholesterol concentrations increased ($P < 0.05$) in response to higher dietary RUP, whereas serum urea concentration decreased ($P < 0.05$). The RUP supplementation positively influenced wither height and body length growth ($P < 0.01$), but it did not affect other growth parameters ($P > 0.10$). Overall, supplementing prepubertal heifer diets with increased RUP levels enhanced the supply of metabolizable protein, improving structural growth and metabolic status without increasing fat deposition.

INTRODUCTION

In dairy farms, heifer raising is one of the most important challenges in today's animal husbandry sector, as these animals guarantee herd sustainability and renewal. However, replacing heifers significantly increases milk production costs. Thus, strategies that facilitate the accelerated growth of heifers from birth to first calving can enhance herd profitability by mitigating the financial impacts associated with heifer management (Geiger et al., 2016). To reach profitability, dairy heifers should be inseminated at ages ranging from 13 to 15 months, and calve between 22 and 24 months (Akins, 2016; NASEM, 2021). This strategy can maximize both milk production and economic longevity. To achieve this goal, feeding and management practices must be effectively implemented to ensure satisfactory growth curves. Both inadequate and excessive daily weight gains can lead to undesirable outcomes (NASEM, 2021). Smaller heifers face increased risks of calving difficulties and lower future milk yields, while those experiencing rapid growth may become overweight, thereby reducing productive lifespan and milk output and increasing the likelihood of calving complications and metabolic diseases. After weaning, heifer growth should proceed with high protein/muscle gain and minimal fat increase, ideally with weight gains of around 0.9 kg/d, which reduces time to insemination and, thus, calving age (Akins, 2016). From the age of 3 to 9 months, the mammary gland grows at a faster rate than other organs (allometric growth) and it becomes sensitive to nutrition during this time. Excessive energy supply decreases epithelial cell proliferation. Thus, fat accumulates in mammary tissues, potentially reducing future milk production (Sejrsen et al., 1982). Additionally, excess energy intake post-weaning can lead to increased mammary parenchymal DNA without improving milk yield (Radcliff et al., 2000). Prepubertal diets for heifers should be formulated with sufficient metabolizable protein (MP) to support muscle growth (Akins, 2016). Protein retention in muscle tissue is the primary goal, making muscle mass accretion a major determinant of protein requirements. However, desirable heifer rearing programs should account for the effect of protein nutrition on structural growth, future

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milk production potential, and feed efficiency (NASEM, 2021).

One approach to increase MP supply in heifer diets is to elevate levels of rumen-undegradable protein (RUP), which might improve daily weight gain and skeletal growth, and avoid excessive body fat accumulation. Although research on nutritional manipulations in Holstein heifers has enhanced our understanding of the effects of MP supply on average daily gain and mammary gland development (Capuco et al., 2004; Albino et al., 2015), less is known about the effect of MP supply during the prepuberty period on structural growth and blood metabolites of Holstein dairy heifers. Moallem et al. (2004) studied the effects of RUP (5.9% and 7.9% of DM) and showed that adding RUP from fish meal improved wither height and hip height. Silva et al. (2018) evaluated different RUP levels (38%, 44%, 51%, and 57% of crude protein) using protected soybean meal in Holstein heifers at various physiological stages (prepuberty and puberty) and reported that 51% RUP supported the highest daily gain and nitrogen retention. To date, no research has investigated the effects of combining plant and animal RUP sources in heifer diets, despite its potential to provide the highest flow of essential amino acids to the small intestine (Ipharraguerre and Clark, 2005). We hypothesized that increasing dietary RUP of per-puberty heifers using a mixture of plant and animal proteins could enhance the supply of MP, likely improving performance, skeletal growth, and metabolic status in dairy heifers. Thus, the objective of this study was to investigate the effects of added RUP in diets of dairy heifers from 4 to 6 months of age on feed intake, average daily gain, blood metabolites, and skeletal growth.

MATERIALS AND METHODS

This experiment was carried out from January to March 2024, in a commercial dairy farm (Namfar Agricultural and Livestock Company, Isfahan, Iran). Animal management was approved by the Animal Care Committee of Isfahan University of Technology. Seventy-two Holstein heifers (initial BW = 128.7 ± 15.5 kg; age = 4 ± 0.31 month) were the subjects in the study. At the time of enrollment, the heifers had no health issues. The study was conducted in a pen-based design, including 12 pens (18 m^2, sand bedding), each housing 6 heifers. The pens (experimental units) were randomly allocated to 1 of 3 experimental diets for 60 days. The dietary treatments were similar in metabolizable energy (ME) and crude protein (CP) but different in RUP contents (28%, 33%, and 38% of CP). The experimental diets were fed as total mixed ration (TMR) and offered ad libitum. The heifers were fed once daily at 08:00 with the goal of maintaining 3% to 5% orts. The diets were formulated using the NASEM (2021) software, and the ingredients and their chemical composition are presented in Table 1. During the experiment, the TMR offered and the orts were recorded daily to measure dry matter intake (DMI). The TMR and orts were sampled weekly and frozen at −20 °C. At the end of the experiment, samples of TMR and orts were dried at 60 °C for 48 h for DM determination. Dried samples were pooled by week, treatment, and pen and analyzed for CP, ether extract (EE), ash (AOAC, 1998), acid detergent fiber (ADF), and neutral detergent fiber (NDF; Van Soest et al., 1991). Nitrogen fractionation (A, B1, B2, B3, and C) was determined in dietary treatments (Licitra, 1991). The RDP and RUP of diets were estimated by the NASEM model (2021). The heifers were weighed weekly, with initial and final weights recorded to calculate the average daily gain. Heifers were scored (Wildman et al., 1982; 1-5-point scale) for body condition by two trained evaluators at day 0, 30, and 60 of the experiment. Ultrasonography was performed monthly on the heifers to measure back fat thickness (BFT) between the 12th and 13th ribs with a frequency of 6.5 MHz (Dramiński iScan, Dramiński SA, Olsztyn, Poland).

Table 1. Ingredients and chemical composition of diets fed dairy Holstein heifers (g/kg DM; unless otherwise stated) before puberty (4 to 6 months of age)

Ingredient	Diets[1]		
	28RUP	33RUP	38RUP
Legume forage hay, mature	238.2	238.2	238.2
Corn silage, normal	56.6	56.6	56.6
Corn grain, ground, dry	380.4	380.4	380.4
Soybean meal, solvent[2]	200.5	141.60	82.70
Poultry by-product meal[3]	0	29.60	59.20
Extruded full-fat soybean[4]	0	29.60	59.20
Wheat bran	72.1	73.00	73.90
Slow released urea	3.90	2.70	1.50
Calcium carbonate	7.90	7.90	7.90
Dicalcium phosphate	2.30	2.30	2.30
Magnesium oxide	2.40	2.40	2.40
Salt	3.20	3.20	3.20
Sodium bicarbonate	14.30	14.30	14.30
Vitamin premix[5]	6.00	6.00	6.00
Mineral premix[6]	6.00	6.00	6.00
Monensin	0.20	0.20	0.20
Bentonite	6.00	6.00	6.00
Chemical composition[7]			
ME (Mcal/kg)	2.64	2.66	2.69
CP (g/kg DM)	175	174	173
RDP (% of CP)	72.29	66.70	61.30
RUP (% of CP)	27.71	33.30	38.70
MP (g/kg DM)	107.2	113.5	118.6
NDF (g/kg DM)	227	223	220
Starch (g/kg DM)	314	317	320
Ca (g/kg DM)	8.20	9.30	10.0
P (g/kg DM)	4.80	5.30	5.80

[1]Diets designated as 28RUP, 33RUP, and 38RUP contained 28%, 33%, and 38% RUP based on % of CP, respectively.
[2]Contained 91 % DM, 40.10 % CP, 20.25 % of CP fraction A, 78.40 % of CP fraction B, 1.35 % of CP fraction C, 7.18 % Ash.
[3]Contained 93.6 % DM, 49.20 % CP, 13.00 % of CP fraction A, 77.00 % of CP fraction B, 10.00 % of CP fraction C, 27.30 % Ash.
[4]Contained 93.6 % DM, 33.65 % CP, 8.00 % of CP fraction A, 82.00 % of CP fraction B, 10.00 % of CP fraction C, 5.61 % Ash.
[5]Premix contained 500,000 IU of vitamin A/kg, 150,000 IU of vitamin D/kg, 4,000 IU of vitamin E/kg, and 50 mg biotin.
[6]Premix contained 105 mg of Co/kg, 3,500 mg of Cu/kg, 190 mg of I/kg, 8,500 mg of Mn/kg, 100 mg of Se/kg, 11,000 mg of Zn/kg, and 5,000 mg Mg.
[7]ME = metabolizable energy; CP = crude protein; RDP = rumen degradable protein; RUP = rumen undegradable protein; MP = metabolizable protein; NDF = neutral detergent fiber.

At days 0, 30, and 60 of the experiment, blood samples were collected from the coccygeal vein 3 to 4 h after the morning feeding using evacuated tubes without anticoagulant (Vacumed, no additive, FL Medical, Torreglia, Italy). Samples were centrifuged at 3000 × g for 15 min at 20 °C, and serum was stored at –20 °C until analysis. Serum concentrations of glucose, total protein, albumin, urea, cholesterol, and triglycerides were determined with commercial kits (Pars Azmoon Laboratory, Tehran, Iran). Beta-hydroxybutyrate (BHB) was analyzed using Randox kits (Randox Laboratories Ltd., Crumlin, UK). A serum spectrophotometer (UNICCO 2100, Zistchemi Co., Tehran, Iran) was used for all metabolite analyses. Structural growth parameters, including body length, abdominal girth, wither height, heart girth, hip width, and hip height, were measured in heifers at days 0 and 60 (Khan et al., 2007). All 72 heifers completed the study, and all collected data were included in the final analysis. No health issues or data collection problems required the exclusion of any animal or measurement. Data were analyzed using the MIXED procedure of SAS. The observational unit was determined by the nature of the response variable: for body weight (BW), average daily gain (ADG), structural growth, BCS, and blood metabolites, the individual heifer was considered the unit of observation and included as a random effect in the model. For nutrient intake variables, the pen was considered the experimental unit and included as a random effect. Time was included as a repeated measure for variables such as nutrient intake and blood metabolites. The model included fixed effects of treatment, sampling time, and their interaction. An autoregressive covariance structure was selected based on the lowest values of Bayesian Information Criterion (BIC), Akaike Information Criterion (AIC), and corrected AIC (AICC). Initial measurements (BW, BCS, and blood metabolites) were used as covariates for the analysis of the corresponding variables. For analyses of changes in BW, BCS, and skeletal growth, the fixed effects of time and treatment × time interaction were removed from the model. Main effects were considered significant at $P < 0.05$, and tendencies were declared at $0.05 < P < 0.10$. Orthogonal contrasts were applied to test linear and quadratic effects of RUP inclusion for the main effect of treatment.

RESULTS AND DISCUSSION

Effects of dietary supplementation with RUP on nutrient intake in Holstein heifers aged 4 to 6 months are presented in Table 2. Increasing dietary RUP decreased intakes of DM (linear, $P = 0.03$), CP (linear, $P = 0.01$), and RDP (linear, P < 0.01), while increasing the intakes of RUP (linear, $P < 0.01$) and MP (linear, $P = 0.02$). ME intake did not differ among dietary treatments ($P = 0.16$). Time had a significant effect ($P < 0.01$) on nutrient intake, with intakes of DM and other nutrients increasing as heifers aged from 4 to 6 months ($P < 0.01$). A treatment × time interaction was detected for RDP ($P = 0.03$) and RUP ($P = 0.01$) intakes: heifers fed 33RUP and 38RUP diets consumed less RDP and more RUP than those fed 28RUP during the study (Fig. 1 and Fig. 2). Compared to postpubertal heifers, prepubertal heifers (< 6 months of age) have lower ruminal microbial activity and are less efficient in utilizing dietary nitrogen for microbial protein synthesis (Silva et al., 2018). Therefore, dietary RUP supplementation appears necessary to ensure an adequate supply of MP to the small intestine of these animals. As the percentage of RUP increased from 28% to 38% of CP, DMI showed a linear decrease, which aligns with Tamlinson et al. (1997), who reported reduced DMI when RUP increased from 31% to 55% of CP using blood meal. Silva et al. (2018), however, observed no effect of increasing dietary RUP from 38% to 57% of CP via processed soybean meal on DMI, organic matter intake, or ME intake in prepubertal and postpubertal heifers. Still, consistent with the present findings, they reported greater RUP intake and lower RDP intake with higher dietary RUP. In contrast, Zanton et al. (2007) found no effect of CP degradability on nutrient intake, and other studies (Gabler and Heinrichs, 2003; Corea et al., 2020) similarly reported no significant effects of dietary RUP proportion on nutrient intake or digestibility when total CP levels were constant. The variability among studies on the effects of RUP on nutrient intake in heifers likely reflects differences in basal diet composition, dietary CP content, RDP-to-RUP ratio, heifer age (prepubertal vs. postpubertal), protein sources, breed, and study duration. Although DMI values in the present study exceeded the 5.5 kg/d estimated by NASEM (2021) for heifers, reduced RDP intake with increasing dietary RUP may have limited maximum microbial fermentation of organic matter in the rumen, thereby contributing to the observed reduction in DMI.

Effects of different levels of RUP on BW, BCS changes, and BFT of Holstein dairy heifers are shown in Table 3. Final BW (P = 0.66), BW change (P = 0.47), average daily gain (ADG; P = 0.47), final BCS (P = 0.10), BCS change (P = 0.66), and BFT (P = 0.54) were not influenced by dietary RUP level. Consistent with the present results, Whitlock et al. (2002) reported that increasing dietary MP from 37 to 44 g per Mcal of ME in prepubertal heifers fed for rapid body growth (1.2 kg/day) did not affect ADG, age at puberty, or BW at puberty or slaughter. In contrast, Silva et al. (2018) found no interaction between physiological stage and RUP level when feeding diets containing 38%, 44%, 51%, and 57% of CP as RUP to Holstein heifers at prepubertal and pubertal stages. They observed that ADG and nitrogen retention were maximized at 51% RUP, although higher RUP levels had no effect on BFT, which is in agreement with the present findings.

Furthermore, Tomlinson et al. (1997) and Corea et al. (2020) reported that increasing RUP levels from 310 to 550 and from 260 to 360 g/kg of CP using blood meal and fish meal over 50 and 70 days, respectively, improved ADG and feed efficiency in heifers. In the present study, the absence of differences in ADG among treatment groups should be considered a favorable outcome. Although DMI decreased with increasing dietary RUP, this reduction did not negatively affect ADG. This outcome is likely explained by the greater flow of MP and potentially improved amino acid availability to the small intestine. The discrepancy between the present findings and those of previous studies may be attributed to the differences in the age and BW of the heifers used. The younger and lighter heifers in this study may have exhibited reduced efficiency in nutrient utilization, a phenomenon previously noted by Silva et al. (2018). The effects of dietary RUP level on serum metabolite concentrations of Holstein heifers are presented in Table 4. Increasing dietary RUP increased serum total protein (linear, P = 0.03) and cholesterol (linear, P = 0.05), while reducing serum urea (linear, P = 0.02). Serum concentrations of glucose (P = 0.18), albumin (P = 0.22), triglycerides (P = 0.52), and BHB (P = 0.20) were not affected by RUP level. Time also influenced serum metabolite concentrations. From 4 to 6 months of age, serum urea tended to decrease (P = 0.09), total protein (P < 0.01), albumin (P < 0.01), and triglycerides (P = 0.02) decreased, whereas cholesterol increased (P < 0.01). A treatment × time interaction was observed for serum albumin (P = 0.05) and cholesterol (P = 0.09): heifers fed 33RUP and 38RUP had higher serum albumin at day 30 (Fig. 3) and higher serum cholesterol at day 60 (Fig. 4) compared with those fed 28RUP.

Table 2. Nutrient intake of dairy Holstein heifers fed different levels of rumen undegradable protein (RUP) before puberty (4 to 6 months of age)

Trait	Diet[1]			SEM	*P*-value[2]			
	28RUP	33RUP	38RUP		L	Q	Time	Diet × Time
Intake[3] (kg/d)								
DM	6.16	5.96	5.87	0.11	0.03	0.62	< 0.01	0.20
CP	1.07	1.03	1.01	0.02	0.01	0.62	< 0.01	0.18
RDP	0.779	0.692	0.622	0.009	< 0.01	0.47	< 0.01	0.03
RUP	0.299	0.346	0.393	0.004	< 0.01	0.98	< 0.01	0.01
MP	0.660	0.677	0.696	0.009	0.02	0.90	< 0.01	0.27
ME (Mcal/d)	16.27	15.87	15.80	0.31	0.16	0.56	< 0.01	0.23

[1]Diets designated as 28RUP, 33RUP, and 38RUP contained 28%, 33%, and 38% RUP based on % of CP, respectively.
[2]L = linear effects of dietary RUP; Q = quadratic effects of dietary RUP; Time = effect of time of collection; Diet × Time = interaction effect between dietary RUP levels and time.
[3]DM = dry matter; CP = crude protein; RDP = rumen degradable protein; RUP = rumen undegradable protein; MP = metabolizable protein; ME= metabolizable energy.

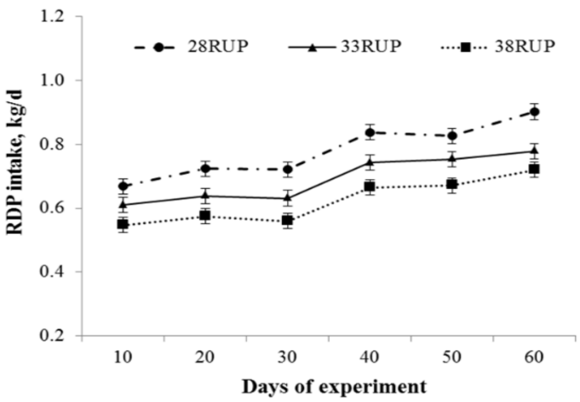

Fig. 1. Interaction effect between dietary rumen undegradable protein (RUP) levels and time on rumen degradable protein (RDP) intake of dairy heifers. Diets designated as 28RUP, 33RUP, and 38RUP contained 28%, 33%, and 38% RUP based on % of crude protein (CP), respectively. RUP, P < 0.01; day, P < 0.01; diet × day, P = 0.03.

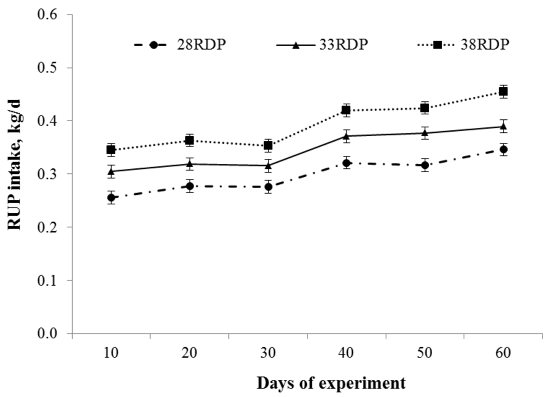

Fig. 2. Interaction effect between dietary rumen undegradable protein (RUP) levels and time on RUP intake of dairy heifers. Diets designated as 28RUP, 33RUP, and 38RUP contained 28%, 33%, and 38% RUP based on % of crude protein (CP), respectively. RUP, P < 0.01; day, P < 0.01; diet × day, P = 0.0.

Table 3. Body weight, body condition score, and back fat thickness (BFT) of dairy Holstein heifers fed different levels of rumen undegradable protein (RUP) before puberty (4 to 6 months of age)

Trait[3]	Diet[1]			SEM	P-value[2]	
	28RUP	33RUP	38RUP		L	Q
BW (kg)						
Initial	125.52	130.46	130.09	4.50	-	-
Final	187.05	191.58	189.70	5.89	0.66	0.54
BW change	61.53	61.11	59.60	2.55	0.47	0.81
ADG	1.09	1.09	1.06	0.04	0.47	0.81
BCS						
Initial	2.94	2.97	3.04	0.05	-	-
Final	3.10	3.18	3.22	0.06	0.10	0.72
BCS change	0.15	0.20	0.18	0.06	0.66	0.50
BFT (mm)	1.93	1.86	1.89	0.06	0.54	0.39

[1]Diets designated as 28RUP, 33RUP, and 38RUP contained 28%, 33%, and 38% RUP based on % of crude protein (CP), respectively.
[2]L = linear effects of dietary RUP; Q = quadratic effects of dietary RUP.
[3]BFT = back fat thickness was measured between the 12th and 13th ribs using ultrasonography with a frequency of 6.5 MHz (Dramiński iScan, Dramiński SA, Olsztyn, Poland).
[3]BW = body weight; ADG = average daily gain; BCS = body condition score; BFT = back fat thickness.

Table 4. Serum metabolites concentrations of dairy Holstein heifers fed different levels of rumen undegradable protein (RUP) before puberty (4 to 6 months of age)

Trait	Diet[1]			SEM	P-value[2]			
	28RUP	33RUP	38RUP		L	Q	Time	Diet × Time
Glucose (mg/dL)	69.54	66.76	63.19	3.18	0.18	0.91	0.62	0.44
Total protein (g/dL)	6.16	6.45	6.88	0.21	0.03	0.67	< 0.01	0.61
Albumin (g/dL)	2.37	2.56	2.61	0.12	0.22	0.63	< 0.01	0.05
Urea (mg/dL)	30.28	26.73	25.83	1.43	0.02	0.48	0.09	0.79
Cholesterol (mg/dL)	139.03	160.68	154.73	4.94	0.05	0.08	< 0.01	0.09
Triglyceride (mg/dL)	172.65	173.91	153.90	20.22	0.52	0.68	0.02	0.96
BHB[3] (mmol/L)	3.11	2.69	2.63	0.24	0.20	0.75	0.61	0.11

[1]Diets designated as 28RUP, 33RUP, and 38RUP contained 28%, 33%, and 38% RUP based on % of crude protein (CP), respectively.
[2]L = linear effects of dietary RUP; Q = quadratic effects of dietary RUP; Time = effect of time of collection; Diet × Time = interaction effect between dietary RUP levels and time.
[3]*Beta-hydroxybutyrate.*

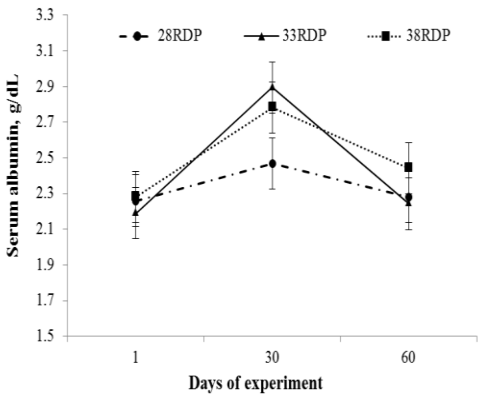

Fig. 3. Interaction effect between dietary rumen undegradable protein (RUP) levels and time on serum albumin concentration of dairy heifers. Diets designated as 28RUP, 33RUP, and 38RUP contained 28%, 33%, and 38% RUP based on % of crude protein (CP), respectively. RUP, $P = 0.22$; day, $P < 0.01$; diet × day, $P = 0.05$.

In the present study, serum total protein concentration increased and serum urea concentration decreased with increasing dietary RUP levels. These changes may be attributed to the greater MP intake and increased amino acid flow to the small intestine. Mohammadzadeh et al. (2021) reported that increasing RUP from 28% to 36% of CP did not affect albumin or total protein concentrations in Holstein dairy calves, although BUN levels decreased post-weaning. Blood urea levels are considered an indicator of nitrogen metabolism (Hammond, 1997). Indeed, serum urea or BUN concentration is influenced by dietary CP content, the degradability of protein in the rumen, and the supply of post-ruminal protein (Jonker et al., 1998). In the current experiment, heifers fed 33RUP and 38RUP consumed less CP and RDP compared with those fed 28RUP. This reduction likely decreased ruminal protein metabolism and ammonia nitrogen production, which in turn contributed to the observed decline in serum urea concentration.

In the present study, serum cholesterol concentration increased with higher dietary RUP levels. Changes in serum cholesterol concentration in response to dietary protein may result from alterations in absorption, biosynthesis, tissue distribution, and excretion, which collectively influence the overall turnover rate of cholesterol in the body (Terpstra et al., 1983). To the best of our knowledge, only one study has investigated the effect of dietary protein degradability on blood cholesterol concentrations in ruminants. Al Ani et al. (2021) reported that replacing soybean protein with whey protein or urea reduced blood cholesterol concentrations.

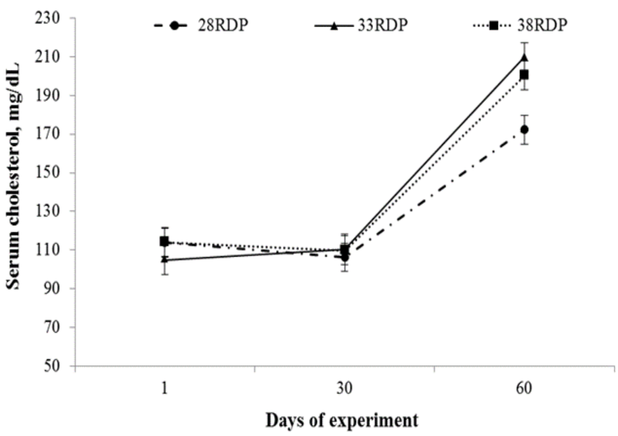

Fig. 4. Interaction effect between dietary rumen undegradable protein (RUP) levels and time on serum cholesterol concentration of dairy heifers. Diets designated as 28RUP, 33RUP, and 38RUP contained 28%, 33%, and 38% RUP based on % of crude protein (CP), respectively. RUP, $P = 0.05$; day, $P < 0.01$; diet × day, $P = 0.09$.

In non-ruminants, dietary protein type has also been shown to influence cholesterol metabolism. Howard and Gresham (1968) reported increased fecal cholesterol excretion in rabbits fed soybean meal compared with those fed a casein-based diet. Similarly, Fumagalli et al. (1978) observed that replacing casein with soybean protein in rabbits increased steroid excretion without affecting bile acid excretion. Reiser et al. (1977) reported that substituting casein with soybean protein in mice doubled hepatic 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) reductase activity, the rate-limiting enzyme in cholesterol synthesis. These findings indicate that the type of dietary protein can markedly influence cholesterol metabolism. Nonetheless, further studies are required to better understand the effects of dietary protein sources on cholesterol metabolism, particularly in ruminants.

The effects of different dietary RUP levels on structural growth of Holstein dairy heifers are presented in Table 5. Increasing RUP did not affect final wither height ($P = 0.17$), but it did increase wither height gain (linear, $P = 0.02$). Similarly, higher dietary RUP increased both final body length (linear, $P < 0.01$) and body length gain (linear, $P < 0.01$). In contrast, abdominal girth, heart girth, hip height, and hip width were not affected by increasing dietary RUP ($P < 0.10$).

In the present study, heifers fed diets with higher RUP levels (33RUP and 38RUP) exhibited greater increases in wither height and body length compared to those receiving 28RUP. Similarly, Zhang et al. (2017) reported a linear increase in wither height as dietary CP content increased from 10.2% to 13.5% of DM via soybean meal supplementation in older Holstein heifers. Moallem et al. (2004) also observed increases in both wither height and hip height when dietary RUP was increased from 5.9% to 7.9% of CP. Growth curves indicated that the effects of supplemental RUP on wither height and hip height were most pronounced between 90 and 150 days of age, declining thereafter. This suggests that protein supplementation during the early post-weaning period can accelerate skeletal growth in dairy heifers. Lammers and Heinrichs (2000) reported a daily increase of 0.019 cm in wither height when dietary protein increased from 11.8% to 15.6% of DM, which is comparable to the increases of 0.033 and 0.069 cm per day observed in the 33RUP and 38RUP treatments, respectively. Notably, they found that skeletal growth rate increased without a corresponding rise in ADG. These findings indicate that prepubertal skeletal growth can be influenced by protein nutrition independently of overall body weight gain. Considering the strong correlation between wither height and milk production during the first lactation (Sieber et al., 1988; Markusfeld and Ezra, 1993), and the fact that skeletal growth rate declines after puberty (Moallem et al., 2004), it can be concluded that the prepubertal period represents the greatest opportunity to enhance skeletal growth through dietary protein supplementation.

The present study observed an increase in serum cholesterol with higher dietary RUP levels. Since cholesterol serves as a precursor for sex hormones such as estrogen and progesterone, which are key regulators of mammary gland development and reproduction (Grummer, 1988; Hawkins et al., 1995), further research is warranted to investigate the effects of prepubertal RUP supplementation on reproductive performance in heifers.

Table 5. Structural growth of dairy Holstein heifers fed different levels of rumen undegradable protein (RUP) before puberty (4 to 6 months of age)

Trait	Diet[1]			SEM	*P*-value[2]	
	28RUP	33RUP	38RUP		L	Q
Wither height, cm						
Initial	94.75	93.29	92.82	1.33	-	-
Final	110.21	110.58	112.09	1.26	0.17	0.61
Change	15.45	17.29	19.27	1.20	0.02	0.24
Abdominal girth, cm						
Initial	132.25	134.50	132.09	2.10	-	-
Final	164.46	165.21	163.13	2.68	0.63	0.56
Change	32.20	30.70	31.04	2.00	0.57	0.61
Heart girth, cm						
Initial	109.75	112.75	111.57	1.60	-	-
Final	137.00	138.87	139.70	1.83	0.17	0.74
Change	27.25	26.12	28.13	1.18	0.48	0.16
Hip height, cm						
Initial	97.62	98.25	99.08	1.21	-	-
Final	115.17	115.42	116.65	1.15	0.23	0.63
Change	17.54	17.16	17.55	0.75	0.98	0.60
Hip width, cm						
Initial	20.20	20.37	20.69	0.49	-	-
Final	28.87	29.07	29.12	0.59	0.68	0.21
Change	8.66	8.70	8.43	0.62	0.71	0.20
Body length, cm						
Initial	93.16	92.12	93.17	1.53	-	-
Final	107.50	112.42	117.39	1.12	<0.01	0.10
Change	14.33	20.30	24.24	1.29	<0.01	0.15

[1]Diets designated as 28RUP, 33RUP, and 38RUP contained 28%, 33%, and 38% RUP based on % of crude protein (CP), respectively.
[2]L = linear effects of dietary RUP; Q = quadratic effects of dietary RUP.

CONCLUSION

The results of this study indicated that increasing dietary RUP linearly decreased intakes of DM, CP, and RDP, while linearly increasing the intakes of RUP and MP. Dietary RUP did not affect final BW, average daily gain, final BCS, BCS changes, or BFT. Increasing RUP linearly increased serum total protein and cholesterol concentrations, while decreasing serum urea. Structural growth was also influenced, with higher RUP levels leading to linear increases in wither height gain, final body length, and body length gain. However, abdominal girth, heart girth, hip height, and hip width were not affected by dietary RUP.

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CRediT AUTHORSHIP CONTRIBUTION STATEMENT

Conceptualization: Tahere Amirabadi Farahani and Najme Eslamian Farsuni; Methodology: Hossein Mohammadkhani; Software: Najme Eslamian Farsuni; Formal analysis: Tahere Amirabadi Farahani and Najme Eslamian Farsuni; Resources: Tahere Amirabadi Farahani; Investigation: Hossein Mohammadkhani and Najme Eslamian Farsuni; Data curation: Najme Eslamian Farsuni and Hossein Mohammadkhani; Writing—original draft preparation: Tahere Amirabadi Farahani; Writing—review and editing: Tahere Amirabadi Farahani; Supervision: Tahere Amirabadi Farahani and Najme Eslamian Farsuni; Project administration: Tahere Amirabadi Farahani; Funding acquisition: Tahere Amirabadi Farahani and Najme Eslamian Farsuni.

DECLARATION OF COMPETING INTEREST

There are no conflicts of interest among authors regarding the publication of this paper.

ETHICAL STATEMENT

Animal procedures were conducted in accordance with the ethical guidelines of the Iranian Council of Animal Care (1995).

DATA AVAILABILITY

Data will be made available upon reasonable request.

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