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Effect of maca supplementation on bovine sperm quantity and quality followed over two spermatogenic cycles

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Abstract

Maca (*Lepidium meyenii* Walpers), is an Andean crop that grows between 3,800 and 4,500 m a.s.l. The persistent interest in this plant is based on its assumed effects on fertility of male mammals due to the prevalence of certain, partially specific, secondary compounds. The present study aimed at evaluating the effect of maca supplementation on quality and quantity of semen, mating behavior, and clinical status of peripubertal breeding bulls. The experiment followed a cross-over design lasting for 23 wk with 3 wk of adaptation and baseline measurements, and 2 × 10 wk of treatment feeding thus covering two times the complete 8-wk spermatogenic cycle. Seventy-eight 55 wk to 84 wk old breeding bulls received either no maca (control) or maca (233 mg dried hypocotyls/kg body weight/day) for 10 wk followed by 10 wk without maca (maca early) or maca only in the last 10 wk (maca late). Measurements were always made in the last 2 wk of each period. Apart from standard analyses, ejaculates were analyzed by flow cytometry. Data was evaluated by analysis of variance considering the repeated measurement structure of the data. Significant treatment by measurement period indicated direct or carry-over effects of maca. Maca supplementation had no direct effect on body weight, testes circumference, rectal temperature, mating behavior, and ejaculate volume. However, supplementing maca in the first 10 wk period increased the number of sperms in the second 10 wk period, i.e., when the animals no longer received maca. The DNA fragmentation index and the visually assessed motility of the sperms of bulls, that initially showed a borderline sperm quality, were significantly improved with early maca supplementation, while no such effect was observed in the two other groups. No effects occurred in the proportion of intact sperm plasma membranes or acrosomes or both. In conclusion, maca supplementation seems to improve sperm quantity and quality of bulls to a certain degree, while mating behavior appears unaffected.

Keywords: Breeding bulls; DNA fragmentation index; Sperm count; Sperm quality; Maca

1. Introduction

When screening for natural feed supplements enhancing male fertility, maca (*Lepidium meyenii* Walpers), turned out to be one potential candidate. It is a traditional Andean crop of the Brassicaceae family that grows best at altitudes between 3,500 and 4,500 m a.s.l and is cultivated especially in the Peruvian highlands. The below-ground storage organ, the hypocotyl, is the part of the plant assumed to improve fertility. These properties of maca were first described in 2000 [1], a publication which triggered extensive further research [2–7]. Findings included that maca increases male sexual drive in mice and rats [1,8] and sperm production in

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men [3]. Gonzales et al. [9] noted that maca treatment of rats prevented high altitude-induced spermatogenic disruption. More recently, it has been reported that there are differences among hypocotyl colour types in their effects on spermatogenesis [5,7]. So far only one study, an unpublished thesis from the highlands of Peru [10; cited in 11], investigated effects of maca on fertility of bulls. That study indicated increased sperm count and motility after maca supplementation. Maca is characterized by various secondary metabolites. Macaene and macamides are specific for the maca plant but maca also contains substantial amounts of campesterol and β-sitosterol, the most common phytosterols, and a range of glucosinolates. All these secondary metabolites or a distinct combination of them may explain the potential effects of maca on male fertility.

Milk, beef and semen producers would profit likewise from a higher sperm yield and, especially, from an improved quality of the semen used in artificial insemination. A higher productivity in the collection center would reduce the housing costs of the bulls and contribute indirectly to reduced costs per insemination. Furthermore, the proportion of 5–10% of the young sires which are typically culled before starting intensively with semen production as a consequence of their low semen quality (observations made by Swissgenetics), might be reduced if supplementary feeding would improve semen quality, and the genetic potential of these bulls would not get lost. The improved quality could additionally reduce the costs for keeping unproductive cows even when there is only a slightly higher non-return rate. Conventional criteria for semen assessment are limited in their accuracy in predicting the actual sperm quality. Since recently, flow cytometer techniques offer new possibilities for describing sperm quality more objectively. Traits obtained include the sperm plasma membrane and acrosome integrity assay (SMAIA; [12]), which determines the integrity of these structures in the sperms, and the sperm chromatin structure assay (SCSA; [13]), which evaluates the chromatin integrity.

The hypothesis tested in the present study was that maca supplemented over a sufficiently long period will improve economically viable fertility traits of breeding bulls with adequate sperm quality and especially of those with borderline sperm quality. For this purpose, various fertility traits were assessed, among them variables of sperm quality determined by in-depth flow-cytometric analyses.

2. Materials and methods

2.1. Experimental design

The experiment was based on a cross-over design lasting for 23 wk including three periods: 3 wk of adaptation and baseline measurements in wk 2 and 3, and 2 $\times$ 10 wk of treatment feeding. The length of these periods was determined by the attempt to have the full spermatogenic cycles of 54 days [14] completed. This was the case with the measurements being scheduled in wk 9 and 10. There were three treatment groups, (i) a control group where bulls never received maca, (ii) a group offered maca during the first 10 wk period and no maca in the second 10 wk period (further on called ‘maca early’), and (iii) a group supplemented with maca not before the second 10 wk period (‘maca late’).

2.2. Source and supplementation of maca

The batch of maca hypocotyl meal used for the experiment was purchased directly from Agronaturales, Lima, Peru. This company, specialized in export of maca, collected the hypocotyl harvests in the surroundings of the village Oca, district of Junin, in the Andean highlands of Peru (4,100 m a.s.l.). The 1000 kg batch purchased consisted of a mix of hypocotyls of different colors with approximately half being of yellow and one quarter each being of reddish and of black color type. This ensured that known color-specific differences in composition [15] and biological effects [5,7] were widely excluded which allowed more general conclusions to be drawn from the study. The maca hypocotyls forming the basis of the batch had been grown and dried traditionally, and had been subsequently transported to Lima where they were milled to a powder with a particle size of 0.8 mm. The company furthermore thoroughly mixed the entire batch prior to shipment. For compositional analysis, five samples were taken from individual 10 kg bags. The powder was mixed into a basal concentrate formulated for breeding bulls at a proportion of 91 g/kg dry matter. The same, but non-supplemented, basal concentrate was used for the control treatments. Maca-treated bulls in the experiment were intended to receive maca in an amount of 233 mg/kg body weight. This level was chosen as an equivalent dose had shown beneficial effects on spermatogenesis in rats [3,9]. Supplementation of the maca containing concentrate in the amounts required was performed after allocating bulls to body weight classes formed in steps of 100 kg. The ingredient composition of the basal concentrate was (g/kg): oats, 165; mill byproducts, 150; maize, 100; dried distillers grain solubles,

Table 1
Analyzed composition of the experimental feeds.

	Hay	Concentrates	
		− maca	+ maca
Proximate contents (g/kg dry matter)			
Dry matter[1]	897	921	922
Total ash	77	98	98
Crude protein	98	169	166
Ether extract	17	43	47
Neutral detergent fibre	572	275	259
Acid detergent fibre	355	122	118
Secondary metabolites (μmol/kg dry matter)			
Total glucosinolates	—[2]	0.50	1.08
Macaene	—	—	0.54
Macamide compound 1	—	—	0.06
Macamide compound 2	—	—	0.09

[1] In original substance.
[2] Not analyzed.

100; oat mill feed, 80; molasses, 60; sorghum, 50; wheat, 50, wheat bran, 50; soybean meal, 30; beet pulp, 30; canola expeller, 30; mono-calcium phosphate, 22; palm oil, 22; maize gluten, 20; sodium chloride, 20, calcium carbonate, 14, vitamin-mineral premix, 6; propionic acid, 1. The amounts of minerals and vitamins provided per kg of concentrate were: Ca, 10.6 g; P, 10.0 g; Mg, 3.3 g; Na, 8.5 g; Zn, 410 mg; Cu, 40 mg; Se, 1 mg; vitamin A, 40,000 IU; vitamin B_1, 10 mg; biotin, 0.1 mg; niacin, 40 mg; vitamin D_3, 5,000 IU; and vitamin E, 100 mg. The concentrates were pelleted at a temperature of <60 °C to avoid heat destruction of maca's secondary constituents. The tolerance by breeding bulls of maca supplementation had been confirmed in a preliminary experiment in four breeding bulls in comparison to four control bulls. Complementary to the concentrate, which was offered in the morning, the bulls had *ad libitum* access to grass hay. Samples of the experimental concentrates and of the hay were collected at two times and analyzed for gross nutrient composition [16; methods 923.01; 934.01; 992.15; 2002.03], macaene and macamides [17] and total glucosinolates [18; with small modifications] (Table 1).

2.3. Experimental site and breeding bulls

The study was conducted from September 2007 to January 2009 at the Swissgenetics semen collection centre Mülligen, Switzerland. Six batches of six to 17 peripubertal breeding bulls were included in the experiment. Batches arrived at the collection center in intervals of 2–3 mo. They were of comparable weight (476 ± 43 kg), differed to some extent in age (68 ± 6 wk, ranging from 55 to 84 wk) and originated from different dairy breeds (Holstein, 24; Red Holstein 7; Brown Swiss, 12; Swiss Fleckvieh, 21; Simmental, 14). Bulls of each new batch arriving at the station were allocated to the treatment groups as balanced as possible with respect to breed. As the bulls within the batches were rather homogenous in age and body weight, larger group differences could be prevented in these criteria. Only bulls with sufficient or borderline sperm quality (>300 × 10^3 sperms/μl, >40% motility after thawing; standard operation procedure (SOP) of Swissgenetics) were included in the experiment. Target numbers of bulls had been 30 per treatment, and the realized numbers were 23 for control, 29 for maca early and 26 for maca late.

2.4. Sample and data collection

During the entire experiment, the bulls were subjected to a bi-weekly semen collection schedule using an artificial vagina. In the three 2 wk measurement periods, bulls were only collected once per collection day. This resulted in a total of four ejaculates per period per bull later used for various analyses. The health condition was controlled weekly. Bulls were weighed once per measurement period. During the measurement periods, libido, mounting activity, erection, and ejaculatory thrust as indicators for the mating behavior were assessed always by the same bull handler and by applying the grading scheme given in Table 2.

2.5. Ejaculate analysis before freezing

Immediately after collection, the undiluted ejaculates were transferred to a water bath at 32 °C and evaluated within 20 min after ejaculation for volume (1.05 × weight; [19]), sperm count and motility. The sperm count in the ejaculate was estimated by density measured after dilution with Na-citrate (1:200) with the help of a spectrophotometer (PCB 6121, Eppendorf-Neheler-Hinz GmbH, Germany). Sperm motility was visually assessed per sample in approximately 4–6 fields of 200 spermatozoids each by experienced laboratory technicians under a phase contrast microscope (Axiolab, Carl Zeiss AG, Feldbach, Switzerland) with 200 times magnification. Data is expressed as proportion of forward motile of total spermatozoids.

2.6. Freezing of the straws

Diluted ejaculates were prepared within 60 min after ejaculation with a standard TRIS-egg extender, consisting (2:1) of egg yolk (max. 2 d old) and tryladil (Minitüb, Tiefenbach, Germany). Subsequently, the di-

Table 2
Valuation of the mating behavior.

	Valuation grade			
Behavior	1	2	3	4
Libido	No interest	Mounts too vigorous	Mounts after 5 minutes	Mounts within 5 minutes
Mounting activity	Does not mount	Launches himself at the teaser	Moves cautious on the teaser (clinch in the ischium region)	Targeted and controlled (good clinch of the teaser)
Erection	Penis not visible	Only peak of penis visible	Penis dug, but incomplete erection	Penis normal dug and good erection
Ejaculatory thrust	Refusal of the artificial vagina	Frictions in the artificial vagina	Ejaculatory thrust without dismount	Ejaculatory thrust with dismount

luted ejaculate was cooled to 22–24 °C, and filled and sealed in French mini straws (IMV-technologies, L'Aigle, France) in portions of approx. 15×10^6 sperms. The straws were first stored at 4 °C for 4 h, then frozen and finally stored in cryotanks in liquid nitrogen at −196 °C. This protocol followed SOP of Swissgenetics.

2.7. Flow cytometer analyses

Further sperm analyses were performed on a flow cytometer (model FC500 MPL, Beckman Coulter, Nyon, Switzerland) equipped with an air-cooled argon ion 488 nm laser and red diode laser (635 nm) as excitation source. Analysis of the flow cytometric data was done using the FlowJo software (version 8.5.3, FlowJo, Ashland, USA).

2.7.1. Sperm plasma membrane and acrosome integrity assay (SMAIA)

The SMAIA method applied was based on Wu [12] with small modifications. Briefly, the contents of three straws of frozen semen per measurement day and bull were thawed during 30 s at 38 °C in a water bath and subsequently pooled. The three fluorescent stains used for that analysis were propidium iodide (PI) (Invitrogen, Basel, Switzerland), a combination of peanut agglutinin and fluorencence isothiocyanat (PNA-FITC) (Biozol, Eching, Germany), as well as SYTO 60 (Invitrogen, Basel, Switzerland). Before application, PI, SYTO-60 and PNA-FITC were diluted in ratios of 1:1,000, 1:10,000 and 1:4,000, respectively, with TALP-Hepes (pH 7.4), containing (mmol/l): NaCl, 100; Hepes (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), 40; lactate, 21.6; $NaHCO_3$, 10; KCl, 3.1; $Ca.Cl_2$, 2; sodium pyruvate, 1; polyvinyl alcohol, 1 mg/ml; $MgCl_2$, 0.4; $MgCl_2$, 0.4; EDTA, 0.4; NaH_2PO_4, 0.3. Sperms and staining solution were mixed a ratio of 1:20. Samples were then incubated at room temperature for 15 min. The 633 nm laser excited the SYTO-60 (emission of 755 nm) while the 488 nm laser excited PI (620 nm) and PNA-FITC (525 nm), respectively. From each sample, 5000 individual sperms were evaluated at a flow rate of 300 to 500 sperms/s.

The principle of this assay is as follows. The PI binds to the DNA and thus marks sperms with damaged plasma membrane. The PNA-FITC binds selectively to the outer acrosome membrane by this way labeling sperms with ruptured acrosome. The SYTO 60, which can permeate the membrane, accumulates around the DNA of the sperms which allows the separation of sperms and egg yolk particles originating from the TRIS-egg extender. The combination of these three fluorescent stains thus allows excluding artifacts from the egg yolk and the classification of sperms into those with intact plasma membrane and acrosome, those with defect plasma membrane but intact acrosome and those where both plasma membrane and acrosome are defect. The frequency of sperms with intact plasma membrane and defect acrosome was very low and therefore neglected here.

2.7.2. Sperm chromatine structure assay (SCSA)

The latest SCSA methodology, as described in detail by Evenson [13], was applied in the present study with small modifications. Biefly, 10 μl of thawed semen was diluted in 100 μl of TNE buffer (pH 7.4), containing (mmol/l) Tris-HCl, 0.01; NaCl, 0.15; EDTA, 0.001; and then mixed with 200 μl acid detergens (pH 1.2) for 30 s. Afterwards, 0.6 ml of Acridine Orange (AO) (Sigma-Aldrich, Buchs, Switzerland) solution (6 μg/ml AO in phosphate citrate buffer) was added and the sample was analyzed on the flow cytometer after 3 min of staining time. The AO (excited by the 488 nm laser) binds at the DNA and emits a green fluorencence (525 nm) in sperms with intact DNA and a red fluorescence (675 nm) in sperms with fragmented DNA. From each sample, 5000 individual sperms were evaluated at a flow rate of about 250 sperms/s. In doing that, sperms were gated by the software program in a way that the

influence of egg yolk particles was excluded. The DNA fragmentation index (% DFI; i.e., the percentage of sperms with defect DNA) of the gated sperms was analyzed in previously compensated data in a histogram of the red fluorescence.

2.8. Statistical analysis

The procedure MIXED of SAS (version 9.1.3; SAS Institute, Inc., Cary, NC) was used for ANOVA applying the following model:

$$y_{ijk} = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + \varepsilon_{ijk}$$

where α = maca treatment effect, β = period effect, and ε = residual error. The period effect was considered as repeated measurement, and reflected general time trends. Indications for maca treatment effects were evaluated via the treatment by period interaction, as this interaction excluded time trends. In case this interaction was significant, multiple comparisons among individual treatment by period means were performed and adjusted with Tukey's method. The tables and the figure give Least Square means, the standard errors (SE) of the individual means and *P*-values for time, treatment, and the interaction.

Sperm quality traits determined with flow cytometry and motility were analyzed separately for bulls with borderline and sufficient sperm quality where for motility only the classification made by SMAIA was used. This subdivision was based on the results of the first measurement period before the start of treatment feeding. In case of SMAIA, criteria set to classify ejaculates as to be of borderline quality were that bulls presented either at least three of the four ejaculates with <45% sperms having an intact plasma membrane and an intact acrosome or, in case only two of the four ejaculates fulfilled this criterion, when the average of all four ejaculates was <45% in these traits. Concerning SCSA, bulls with >6% of sperms with a defect chromatin structure were considered to present borderline sperm quality based on the same ejaculate number excluding scheme as applied with SMAIA. The threshold levels were deliberately set rather high (for the plasma membrane integrity) and low (for the % DFI) as compared to those suggested by Rybar et al. [20] in order to increase the number of bulls with borderline semen quality to be statistically evaluated for their response to maca treatment. This strict setting ensured that all bulls with potentially questionable semen quality were included.

3. Results

3.1. Intake of experimental concentrate and body weight

The experimental concentrates were generally consumed with only minimal amounts of refusals if any. Bulls of all treatment groups were still growing during the experiment (period effect, $P < 0.001$).

3.2. Clinical status

As expected from the increasing body weights, also testes circumference increased with time across all treatment groups ($P < 0.001$; Table 3). There were no ($P > 0.05$) treatment differences. Rectal temperature was constant across all treatments and measurement periods with an overall average of 38.3 °C.

3.3. Mating behavior

In the control bulls, there was a weak trend that libido increased with time (Table 3). Increases with time were higher in maca treated bulls than in control concerning mounting activity and erection, and this resulted in a time effect in all three variables (period effect, $P < 0.05$). The ejaculatory thrust was not changing ($P > 0.05$) with time in any of the groups. There was an interaction of treatment by period ($P < 0.05$) for mounting activity, erection, and ejaculatory thrust. These interactions were based on different responses, though. Mounting activity increased exclusively in the 'maca late' bulls. Erection and ejaculatory thrust of control and 'maca early' bulls decreased after the baseline period while this was opposite in the 'maca late' bulls.

3.4. Sperm quantity

Ejaculate volume and sperm density increased ($P < 0.001$) with time in control and maca treated bulls (Table 3). The same was true for sperm density and total sperm amount per ejaculate. There was an interaction of treatment by period ($P < 0.05$) for sperm density. However, this was mostly due to the particularly low value found in the control group in period 1. As for the density, the interaction of treatment by period ($P < 0.05$) in total sperm count per ejaculate ($P < 0.05$) was partially the result of the low initial low value of the control group in the period 1 as well. However, also the comparably small increase in total sperm count in the 'maca early' bulls occurring directly when maca was fed and the large increase in the subsequent period contributed to this interaction (difference in the mean values of this group between periods 2 and 3, $P < 0.05$).

Table 3
Effect of maca supplementation (+: supplementation of maca; −: no supplementation) in different periods (P0 = before start of maca treatment; P1 = weeks 1-10; P2 = weeks 11–20) as measured at the end of the periods on body weight, clinical status, mating behavior and productivity of breeding bulls.

Treatment (T)	Control (n = 23)			Maca early (n = 29)			Maca late (n = 26)					
Period (P)	0	1	2	0	1	2	0	1	2	P-level[1]		
Maca treatment sequence	−	−	−	−	+	−	−	−	+	T	P	T × P
Body weight (kg)												
Mean	466	499	549	467	505	557	475	512	564		‡	
SE	9.2	10.5	9.8	8.6	9.1	10.6	7.6	8.4	8.0			
Clinical status												
Testes circumference (cm)												
Mean	35.7	36.8	37.9	35.7	37.2	38.3	35.9	37.1	38.4		‡	
SE	0.31	0.05	0.24	0.23	0.04	0.18	0.21	0.05	0.23			
Rectal temperature (°C)												
Mean	38.3	38.4	38.4	37.9	38.3	38.3	38.4	38.3	38.4			
SE	0.08	0.31	0.05	0.52	0.23	0.04	0.06	0.21	0.05			
Mating behavior[2]												
Libido												
Mean	3.52	3.64	3.61	3.62	3.72	3.72	3.80	3.88	3.88		‡	
SE	0.075	0.068	0.074	0.051	0.042	0.042	0.044	0.031	0.031			
Mounting activity												
Mean	3.83^{ab}	3.83^{ab}	3.83^{ab}	3.79^{ab}	3.79^{ab}	3.79^{ab}	3.93^{b}	3.96^{a}	3.96^{a}		*	†
SE	0.067	0.067	0.067	0.038	0.038	0.038	0.025	0.019	0.019			
Erection												
Mean	3.85^{ab}	3.82^{ab}	3.83^{ab}	3.95^{a}	3.90^{b}	3.90^{b}	3.98	4.00	4.00		*	†
SE	0.067	0.069	0.067	0.021	0.028	0.028	0.014	0	0			
Ejaculatory thrust												
Mean	3.86	3.83	3.83	3.98	3.97	3.97	3.98	4.00	4.00			*
SE	0.065	0.067	0.067	0.012	0.017	0.017	0.014	0	0			
Semen quantity[3]												
Volume (ml)												
Mean	3.14	3.59	3.69	3.67	3.66	4.26	3.57	3.76	4.12		‡	
SE	0.142	0.156	0.145	0.142	0.138	0.171	0.129	0.108	0.137			
Density (sperms/μl)												
Mean	1292^{b}	1611^{a}	1642^{a}	1353^{ab}	1426^{ab}	1537^{ab}	1594^{ab}	1681^{a}	1628^{ab}		‡	*
SE	65.3	58.5	60.6	59.0	45.3	50.6	63.5	67.9	55.6			
Sperms (10^9/ml ejaculate)												
Mean	4.19^{c}	5.79^{ab}	6.02^{ab}	4.91^{bc}	5.20^{bc}	6.54^{a}	5.89^{abc}	6.23^{ab}	6.60^{ab}		‡	*
SE	0.326	0.343	0.310	0.266	0.265	0.324	0.362	0.277	0.289			

Values are presented as LSmeans and standard error (SE).

[abc] Within a row, values marked with unequal superscript differed ($P < 0.05$).

[1] * = $P < 0.05$; † = $P < 0.01$; ‡ = $P < 0.001$.

[2] Scale of 1–4; see Table 2 for the valuation.

[3] Control, n = 17; maca early n =24; maca late n = 23.

Table 4
Effect of maca supplementation (+: supplementation of maca; −: no supplementation) in different periods (P0 = before start of maca treatment; P1 = weeks 1 -10; P2 = weeks 11–20) as measured at the end of the periods on sperm quality of breeding bulls.

Treatment (T)			Control (n = 21)			Maca early (n = 29)			Maca late (n = 26)					
Period (P)			0	1	2	0	1	2	0	1	2			
Maca treatment sequence	Group[1]		−	−	−	−	+	−	−	−	+	P-level[2] T	P	T × P
Motility (%)[3]	A	Mean	85.2	88.1	87.7	85.2	87.2	87.2	85.3	87.1	88.5		‡	
		SE	0.005	0.010	0.004	0.003	0.003	0.010	0.006	0.005	0.003			
	B	Mean	80.9^{ab}	85.1^{a}	84.6^{a}	75.9^{b}	86.2^{a}	86.0^{a}	85.9^{a}	86.8^{a}	87.7^{a}		‡	*
		SE	0.007	0.010	0.011	0.024	0.005	0.004	0.007	0.015	0.012			
Status of plasma membrane/ acrosome (% of sperms)[4]														
Intact/intact	A	Mean	52.1	58.3	57.9	52.3	58.1	57.0	52.8	58.5	58.2		‡	
		SE	0.012	0.012	0.011	0.010	0.008	0.011	0.010	0.011	0.010			
	B	Mean	36.1	50.5	52.8	33.2	44.6	48.0	31.7	44.9	45.5		‡	
		SE	0.020	0.017	0.019	0.018	0.015	0.015	0.024	0.022	0.025			
Defect/intact	A	Mean	22.6	17.3	16.6	21.8	16.6	16.5	22.7	16.9	15.7		‡	
		SE	0.008	0.009	0.008	0.007	0.006	0.009	0.009	0.008	0.007			
	B	Mean	34.5	22.8	21.7	37.5	24.7	24.5	41.6	27.5	25.5		‡	
		SE	0.020	0.014	0.011	0.021	0.013	0.014	0.025	0.019	0.020			
Defect/defect	A	Mean	25.0	24.2	25.3	25.7	25.2	26.4	24.3	24.5	26.0		†	
		SE	0.007	0.007	0.008	0.006	0.006	0.008	0.006	0.006	0.007			
	B	Mean	29.2	26.5	25.4	39.1	29.7	27.5	26.7	27.5	28.8			
		SE	0.013	0.010	0.015	0.011	0.007	0.009	0.009	0.009	0.011			

Values are presented as LSmeans and standard error (SE).
[ab] Within a row, values marked with unequal superscript differed ($P < 0.05$).
[1] A: bulls with adequate quality; B: bulls with borderline quality; for motility the classification made by status of plasma membrane and acrosome was used.
[2] * = $P < 0.05$; † = $P < 0.01$; ‡ = $P < 0.001$.
[3] Visually determined; A/B: control, n:14/3; maca early, n:17/7; maca late, n: 20/3.
[4] A/B bulls- control, n:19/4; maca early, n: 22/7; maca late, n: 22/4.

3.5. Sperm quality

On the basis of the baseline results, 15 (19%) and 20 (26%) out of 78 bulls were classified as having borderline sperm quality with the two assays, SMAIA and SCSA, respectively.

The percentage of sperms expressing motility increased with time ($P < 0.001$) in control and maca treated bulls, eventually reaching 87.8% on average across all three groups in the bulls with an initially adequate quality. The corresponding average value for all bulls with borderline quality was only 86.1%. Starting from lower levels in the bulls with initially borderline sperm quality, sperm motility was increased ($P < 0.05$) by the early maca treatment, whereas this was not the case in bulls with adequate quality (Table 4).

In bulls with both adequate and borderline sperm quality, the percentage of sperms which had intact plasma membrane and acrosome increased ($P < 0.001$) with time in control and maca treated bulls attaining, after completing the two spermatogenic cycles, average values across all groups of 57.7% and 48.7%, respectively. There were no significant effects of any of the maca treatments.

The frequency of sperms with damaged chromatin structure (% DFI) was declining with time ($P < 0.01$) in control and maca treated bulls, but this only in those with initially borderline quality (Fig. 1). In these bulls there was an interaction of treatment by period ($P < 0.05$). This interaction reflects that there was a reduction ($P < 0.05$) of % DFI from the start of maca feeding by 40% in the maca early group, which continued to be low in the final 10 wk period. This time trend was not obvious in the two other treatment groups. In the bulls with initially adequate sperm quality, % DFI tended to be lowest in the second period and there was a weak trend for an increase in the last period ($P < 0.05$).

4. Discussion

In the present study, a natural feed supplement, maca, was tested for its effects on male cattle fertility in

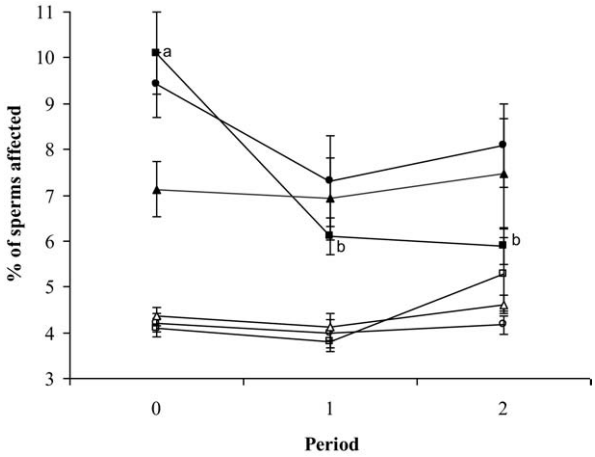

Fig. 1. Effect of maca supplementation on the DNA fragmentation index of sperms from bulls with initially either adequate (open symbol) or borderline quality (closed symbol). Control bulls (○/●) did not receive maca at all, 'maca early' bulls (□/■) received maca in Period 1 and 'maca late' bulls (△/▲) received maca in Period 2, with Period 0 = before start of maca treatment; Period 1 = weeks 1–10; Period 2 = weeks 11–20). Control, n = 18/5; maca early, n = 18/11; maca late, n = 22/4 bulls with adequate/borderline quality. Values are presented as LS means and standard errors. The 'maca early' treatment means marked with unequal superscripts are different at $P < 0.05$.

order to expand knowledge that currently mainly concentrates on reports from human fertility. Recently introduced methods to objectively measure sperm quality in commercial semen production have been applied to determine feeding-induced effects in breeding bulls for the first time known to the authors. The sperm chromatine structure assay (SCSA), for instance, has already been demonstrated to be positively correlated with sperm quality traits such as heterospermic performance, motility, acrosome integrity, morphology of spermatozoa [21], and non-return rate after 56 d [22]. The sperm plasma membrane and acrosome integrity assay (SMAIA) as well as SCSA have been used in the present study to classify bulls for adequate and borderline sperm quality. With the rather strict threshold values applied, approximately 20% of the bulls were at risk of being removed from production at the beginning, presenting quite an economic loss. The thresholds actually applied by artificial insemination companies may be co-determined by their individual production strategies.

4.1. Changes in fertility traits with time

The design of the study offered the opportunity to detect changes in semen quality and behavioral traits over two complete spermatogenic cycles in peripubertal breeding bulls independent of the different modes of supplementary feeding. Factors involved in that time effect were experience and age (maturity). The 2 wk baseline measurement was started 1 wk after the first semen collection had taken place for each bull. At that time, the bulls were almost completely inexperienced, and their sexual function was not yet fully developed. Their daily sperm output corresponded with the indicator values specified by Amman [23] for 11–12 month old Holstein bulls. As expected, the time effect (period) was significant for almost all variables measured, indicating constant improvements. Exceptions, as expected, were rectal temperature, which is not a fertility trait anyway, and ejaculatory thrust. Although significant for almost all mating behavior traits, changes were limited in extent and there was individual variation, especially in libido and mounting activity. This is consistent with the statement of Silver and Price [24] that yearling bulls still have to learn the correct mount orientation through mounting experiences. Over the two spermatogenic cycles, ejaculate volume, sperm density, and total sperm count per ejaculate were increased by, on average, 16, 13, and 28%, respectively. When distinguishing between bulls by initial sperm quality, the proportion of viable sperms (here defined as those with intact plasma membrane and intact acrosome) was increased with time in the bulls with initially borderline quality more than in those with adequate quality (33 vs. 10%). The bulls with initially adequate quality expressed a decrease and a subsequent increase in % DFI (cf. Fig. 1) during the two spermatogenic cycles (−6 and +10%, respectively). Still, on average, the value stayed below the defined critical threshold of 6%. These small variations with time, which may be due to changes in outside temperature [25], are common and, though statistically significant, not relevant. For the bulls with a borderline quality, however, % DFI significantly decreased by 24 and 20%, respectively, in the two spermatogenic cycles.

4.2. Immediate effects of maca supplementation

Clear immediate maca supplementation effects on the variables investigated were rare in the present study. There were several trends but these often differed between the group receiving maca early and that getting maca late. This difference may at least partly be explained by a different susceptibility to maca of bulls with different age and experience. The maca dose (233 mg/kg body weight and day of uncooked and otherwise unprocessed maca meal) applied is assumed to have been sufficiently high to provoke effects. Gonzales et al. [3,26,27] reported fertility effects either with much

lower doses both in men (about 20–40 mg/kg body weight and day of unprocessed maca) and in rats or when applying the same per kg body weight dose (as aqueous extract) as used in the present study [4,9]. In other experiments, dosages of maca extracts (aqueaous, chloroformic, hexanic, alcoholic) applied to rats were higher, ranging up to 1 g/kg body weight and day [2,6,7,28,29]. Processing of maca such as boiling or subjecting maca to different extraction processes might influence the effect of maca on male fertility. However, this has not yet been studied and, as long as the active principles are unknown, it cannot be predicted which treatment might have which effect by (selectively) reducing or concentrating certain secondary compounds. Another factor which might contribute to the decision about the presence or absence of a maca effect on male fertility is hypocotyl color [5,7]. We therefore used a mix of different colors. Apart from colors with a presumed effect, one quarter of the mixture also consisted of reddish maca where no beneficial effect, e.g., on sperm count, could be expected [5,7]. The active metabolites responsible for the fertility effects of maca demonstrated *in vivo* are still unknown. Nevertheless, macaene, macamides, or glucosinolates are presumed to play a decisive role [1]. As intended, the compositional analysis confirmed that concentrations of these three groups of secondary compounds in the batch used (5.39 ± 0.24, 1.63 ± 0.04, and 11.0 ± 0.6 μmol/g dry matter of macaene, macamides, and glucosinolates, respectively) were rather in the middle of values reported elsewhere [15,17,30]. As the concentrations of these metabolites largely vary between batches [30], it would be important to have this information also from other studies. Unfortunately, these ingredients were rarely analyzed when maca was investigated in *in vivo* studies.

In the peer-reviewed literature, maca effects involved various traits. The research about this plant can be grouped into four approaches: (i) studies about its primary and secondary metabolites [15,17,31]; (ii), studies about effects on sexual behavior [2,8], (iii) studies about effects on fertility (see above), and (iv) studies about other effects of maca [27,32]. Maca supplementation was shown to increase male sexual drive in mice and rats [1,2,8] and to enhance sexual desire in men [26], without changing the hormone profile [27]. In breeding bulls, Matos [10] reported a positive influence of maca supplementation on motility and sperm count. Others demonstrated that maca supplementation may increase sperm production in men [4] as well as testes and epididymis weight in rats [3]. Gonzales et al. [9] noted that maca treatment of rats prevented high altitude-induced spermatogenic disruption. Another study [33] demonstrated that maca could enhance spermatogenesis following spermatogenic damage caused by intoxication with malathion (an organophosphorous pesticide). It also seems that differently colored hypocotyls differ in their effects on spermatogenesis, with black maca being superior to reddish and yellow maca [5,7].

For the present study, it has to be stated that the variation between and within individuals between single measurements of individual traits was quite large, and that the groups were not completely balanced in some fertility variables in the baseline period. This may have contributed to mask potentially clearer effects of maca. It was also not possible to balance groups completely for breed representatives, which may have additionally contributed to variation especially in libido [34]. Furthermore, it is important to note that achieving large improvements in bulls with good baseline fertility is difficult anyway. This is why the differentiation made between effects in bulls with adequate sperm quality and those with borderline quality appears helpful. The differentiation showed that two important sperm quality traits, the visually assessed motility and the % DFI, were selectively and massively improved in borderline bulls by early maca supplementation. The failure of this effect to occur with late maca supplementation indicates that the best period to achieve improvements might be early in peripubertal bulls. From the levels found in various traits it actually seems that bulls initially characterized with borderline quality for this trait were not as well developed in terms of semen quality. As the biological variation is high in this development phase, maca supplementation could have slightly helped these bulls to develop faster. Another explanation for the lack of a similar response in borderline bulls in the ‘maca late’ group might have been that these bulls (unintentionally) had a lower initial average % DFI already. However, this trait had declined in the ‘maca early’ bulls to even lower values than those found in the ‘maca late’ bulls. No improvement by early maca supplementation was found in the integrity of sperm plasma membrane and acrosome. It has to be stated that these effects nevertheless have to be treated with care considering the limited number of bulls available with borderline sperm quality.

4.3. Carry-over effects of maca supplementation

Considering the long time required for spermatogenesis of about 8 wk, it can be assumed that any maca effects may be carried on to a period where maca was no longer

fed (type I of carry-over effects) or even get fully expressed only in that period due to a previous lag time in the effect to get manifested (type II of carry-over effects). The experimental designed allowed such effects to be followed in the 'maca early' group in comparison with control and the 'maca late' group. One clear type I carry-over effect was found in % DFI of bulls with borderline sperm quality which stayed low after maca supplementation had been terminated. The most prominent type II carry-over effect was found in sperm count across all bulls of the 'maca early' group, an economically highly important criterion as it affects the number of straws to be produced per bull. Bulls of that group were the only ones presenting a significant increase in total sperm count per ejaculate (by 26%) in the second 10 wk period and they reached the level of the 'maca late' bulls in that period which had started with a 20% higher baseline value in that trait. The increase is arithmetically mostly due to the increase of the ejaculate volume. This finding suggests that maca supplementation is beneficial only when applied sufficiently long and might be even more efficient when supplemented longer than for 10 wk. This is consistent with maca supplementation studies [6] conducted in rats, where the daily sperm production was not yet increased after a supplementation of one spermatogenic cycle. Additionally, Gasco et al. [7] described that the epididymal sperm count was increased after 84 d of treatment, but this was still not obvious in daily sperm count.

5. Conclusions

Apart from improvements in mating behavior, sperm quantity, and quality resulting from age and experience, the present study demonstrated some positive effects of supplementing ground dried hypocotyls of the Andean plant maca in peripubertal bulls. It is also important to note that this strategy, where poor Andean farmers could profit from a new market, apparently has no negative effects on either feed intake or health or distinct sperm quality traits. Still further research is needed before a definitive recommendation of this approach is justified. This could include the use of higher maca doses, the application of the probably most effective form of maca, black hypocotyls, and the extension of the experiment by employing more bulls of borderline sperm quality.

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