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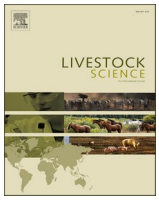

Grazing intensity and associated frequency of human contact, and horn status, influence activity on pasture, physiological pre-slaughter reactions and meat quality in beef heifers

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HIGHLIGHTS

- positive effects of greater grazing intensity on pre-slaughter stress & meat quality.
- presence of horns associated with decreased meat juiciness.
- pre-slaughter heart rates associated with post mortem metabolism & meat quality.

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ABSTRACT

Meat quality is influenced by many factors related to the animal, such as its genetics and health status, farm management, and slaughter and processing conditions. The present study aimed to investigate the effects and interactions of grazing intensity and horn status on behaviour, physiological pre-slaughter stress status and meat characteristics of beef heifers. The study involved 32 horned and 32 disbudded F1 crossbred (Limousin × Swiss Dairy breed) heifers during summer grazing on mountain pastures. Half of the heifers of each horn status were assigned to one of two grazing systems, balanced for live weight, dam and behavioural reactivity: grazing at either high (HI) or low (LI) grazing intensity. HI groups grazed in 3 times smaller paddocks and changed the paddock three times more often than LI groups. The effects of horn status and grazing intensity on physical activity on pasture, pre-slaughter stress and meat quality of the *m. longissimus thoracis* were studied. Compared to HI heifers, LI Heifers walked more when on pasture, showed greater stress levels before stunning, and their meat had greater water losses and greater early troponin levels. The varying pre-slaughter stress levels may be attributed to the differing frequency of human contact resulting from the differing frequency of paddock changes and may explain part of the effects on meat quality. Compared to disbudded heifers, horned heifers had faster heart rates at the abattoir, and their meat had lower cooking loss and was less juicy. Pre-slaughter heart rates showed robust correlations with various meat quality indicators. The study shows that both horn status and grazing management, including human contact, influence meat quality. Part of the effects may be related to different pre-slaughter physiological reactions, which subsequently influence meat quality.

1. Introduction

Grasslands are a sustainable feed resource for ruminant production. The key for optimal grassland use and animal performances is grazing management. Besides botanical and chemical composition, sward height, fertilizer application and others, both stocking density and grazing rotation play a central role (Roca-Fernández and González-Rodríguez, 2013; Andressa Scholz et al., 2021). In comparison to continuous grazing, rotational grazing may increase, decrease or not alter herbage offer and quality and animal performances, such as feed intake, milk production and average daily gain (Thomet and Hadorn, 2000; Abrahamse et al., 2008; Briske et al., 2008; Chen and Shi, 2017).

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Rotational grazing may further increase physical activity, as shown earlier, possibly due to greater avoidance behaviour due to lower space allowance (Walker and Heitschmidt, 1989). Later studies did however not confirm this (Hart et al., 1993; Venter et al., 2019)

Disbudding is a common practice in Swiss cattle farming, legally regulated by the (Federal Assembly of the Swiss Confederation, 2005) Swiss Animal Welfare Act and the Swiss Animal Welfare Ordinance (Federal Assembly of the Swiss Confederation, 2008), and may also alter physical activity. Indoor-housed horned heifers and bulls showed increased general activity levels and different reactivity during controlled reactivity tests compared to their disbudded counterparts (Reiche et al., 2020a) . Horned dairy cows observed in outdoor areas with varying space allowances exhibited slightly higher locomotor activity compared to unhorned cows, irrespective of space allowance (Lutz et al., 2015; Lutz et al., 2019). The increased physical activity might relate to more frequent agonistic social interactions and greater inter-individual distances observed among horned animals (Bouissou, 1972; Lutz et al., 2019; Reiche et al., 2020a).

Meat quality is influenced by many factors related to the animal, including genetics, diet and health, as well as to farm management and slaughter and processing conditions (Devine et al., 2004; Clinquart et al., 2022). As grazing management and disbudding modify animal behaviour during rearing, they may indirectly influence meat quality. Specifically, grass availability and physical activity may influence muscle fibre type composition and meat colour (Dunne et al., 2011; Gangnat et al., 2017a) and horned bulls produced less tender meat than disbudded bulls (Reiche et al., 2019). Part of these influences may be caused by differences in stress reactivity, as stress reactions influence meat quality. Stress during the slaughter period may deplete glycogen reserves, resulting in meat with relatively high ultimate pH, which reduces meat quality and shelf-life (Ponnampalam et al., 2017). Stress immediately preceding slaughter increases metabolic rate causing a faster pH decline and higher muscle temperatures negatively influencing water-holding capacity, shear force, and sensory traits such as juiciness and tenderness (Warner et al., 2007; Bourguet et al., 2010; Reiche et al., 2019; Carrasco-García et al., 2020). The behavioural physiological stress reactions at slaughter and in other situations are influenced amongst others by the rearing experience of the animal (Mounier et al., 2006; Probst et al., 2012; Bourguet et al., 2015; Reiche et al., 2019).

The present study investigated the effects and interactions of grazing intensity and horn status on behaviour, physiological pre-slaughter stress status and meat characteristics of beef heifers. We hypothesized that both grazing intensity and horn status would affect the measured variables and that they interact with each other. Relationships between variables were also studied. While earlier studies investigated correlations between meat quality and stress reactivity measured a few weeks before slaughter, the present study determined stress reactivity several months before slaughter.

2. Animals, material and methods

All experiments respected the Swiss laws of animal protection and were authorized by the cantonal veterinary office of Fribourg, Switzerland (No. 2015_21_FR).

2.1. Animals and housing

The experiment was part of a larger study during which observations in relation to animal behaviour and cortisol responses to ACTH were made (Reiche et al., 2020b; Reiche et al., 2022). Seventy-one F1 crossbred heifers (Limousin (sire breed) × Swiss dairy breed (dam breed, including Swiss Fleckvieh, Red Holstein and Holstein) were purchased in June 2016 at a mean age of 6.5 weeks (mean body weight: 74 kg) in two cohorts, i.e. replicates (first replicate: n=36, second replicate: n=35), which started the experiment at an interval of two weeks. They were housed in groups of 17 to 18 heifers on deep litter in four pens on the experimental farm of Agroscope in Posieux, Switzerland. At the age of 9 weeks, half of the heifers were disbudded under sedation, local anesthesia and systemic analgesia using a hot iron. Animals of each replicate were subsequently allocated to one of two rearing groups including either exclusively horned (H+) or exclusively disbudded (H-) animals, balanced for body weight and dam breed (see (Reiche et al., 2020b)). The four rearing groups of heifers remained in the stable until the age of 13 months. In May 2017, 64 heifers, i.e. 16 heifers of each rearing group, were brought to a pasture area at 1200 m above sea level for summer grazing, where they stayed until slaughter in September. These were the heifers used for the present experiment. The area was located in the Swiss canton of Jura, about 70 km away from the experimental farm in Posieux. Each group of 16 heifers was subdivided into two subgroups of 8 heifers each, balanced for body weight and stress reactivity evaluated at the age of 11 months (Reiche et al., 2020b). One subgroup of each rearing group was assigned to high, or to low grazing intensity (low intensity: LI, high intensity: HI). This resulted in four treatment groups (LI H+, LI H-, HI H+, HI H-) per replicate, i.e. eight groups in total. The eight groups rotated on 64 paddocks on a total surface of 23 ha. Of that, 11.5 ha were dedicated to the LI groups, the other 11.5 ha to the HI groups. The four LI groups (two LI H+ and two LI H- groups) rotated on sixteen paddocks with a surface of 0.72 ha each (Fig. 1). The four HI groups (two HI H+ and two HI H- groups) rotated on 48 paddocks with a surface of 0.24 ha each (Fig. 1). The HI groups changed paddocks 3 times more often than LI groups to respect the target postgrazing sward height of 4-5 cm, measured using plate meters (Farmworks Plate Meter F200, Jenquip, Feilding, NZ). The pastures were permanent pastures composed predominantly of grasses (50-70%).

2.2. Animal-related measurements during the grazing period

Weighing. Animals were weighed every 5 weeks. Animals of one replicate were tested during one morning (between 08:00 and 10:00 AM) of the same day. At each of the two consecutive test days, two of the four treatment groups of the replicate were separately led by two familiar stockpersons into two waiting pens close to the crush (Grüter, Eschenbach, Switzerland) where the regular weighing took place.

Physical activity. During two periods of 14 days each (June and August 2017), heifers were equipped with accelerometers (Rumiwatch®, Itin&Hoch, Liestal, Switzerland) to assess physical activity at 14 and 16 months of age. Thirty-two accelerometers were available. In each 14-d-period, they were used during the first seven days on the four treatment groups of the first replicate, and during the last seven days on the second replicate. Raw data were converted using the Rumiwatch® converter version 0.7.4.5 (Itin+Hoch, Liestal, Switzerland) and the algorithm 00.57 with a 1-hour output resolution. The quantified behaviours included the time spent lying, standing and walking, and the number of stand up events and strides. Interventions to re-fit or replace devices when accelerometers were lost, data logging stopped or battery power was too low, took place between 7:00 AM and 12:00 AM. These hours were excluded from analysis. Data were averaged per animal and measurement day; only complete 19-h measurement days were used for statistical analysis.

Leadership. At two occasions (in July and August 2017), leadership was assessed while the treatment groups were led, separately, to a new paddock (mean duration: 4 minutes). Animals were driven by the same two familiar stockpersons, with one person going in front of and one person following the treatment group. The paths to the new paddocks were fenced-in, and wide enough to allow animals to pass each other within them. Every 30 seconds the two first and two last animals of the group were recorded as “leaders” and “followers”, respectively, using a dictaphone. The four other animals of the group were counted as “intermediate”.

Crush test. The crush test, frequently used in beef cattle, aims at measuring behavioural reactivity (Waiblinger et al., 2006). At the age of 14 mo, crush test scores were assessed using refined scores of Silva et al.

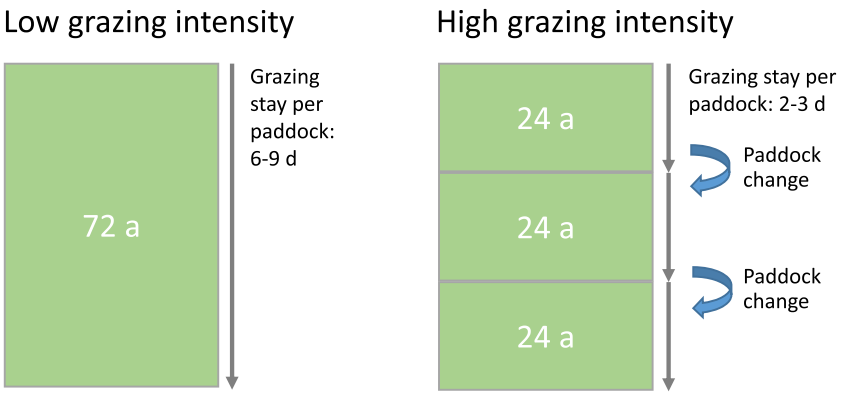

Fig. 1. Schematic representation of the grazing management. Paddocks for LI and HI heifers had a surface of 72 and 24 a, respectively. LI heifers changed paddocks every 6-9 d, HI heifers every 2-3 d.

(2017). The test took place during regular weighing immediately before the reading of the weight. Once in the waiting pen, the animals of the group to be tested were driven into a corridor with a sliding door at its end (Fig. 2). The animals waiting in the corridor were visually separated from the weighing scale. To allow entry of the heifer that was closest (the first in the line in the corridor) into the weighing scale, the corridor's sliding door and the weighing scale door were opened. If the animal to be tested did not enter the scale voluntarily, it was driven from behind into the weighing scale. The test started with closing the weighing scale door and ended 30 seconds after. Three observers, together, evaluated the behavioural reactions from a live video transmission (direct observation). The observed behaviours are described in Table 1. Once all animals of the first two treatment groups were tested, the described procedure was repeated for the other two remaining treatment groups.

2.3. Slaughter, ante and post mortem measurements

The heifers were slaughtered in September 2017 at 17 mo of age over four slaughter days. Each slaughter day, the animals of two treatment groups of one replicate were slaughtered (slaughter day 1: HI H- and LI H+; slaughter day 2: LI H- and HI H+; slaughter day 3: LI H- and HI H+, slaughter day 4: LI H+ and HI H-). The 16 animals to be slaughtered were loaded and transported in a cattle lorry, physically separating the treatment groups, to the slaughterhouse. The lorry left the farm at 09:15 AM and transport time was 55 minutes. After the arrival at the slaughterhouse, animals waited for 20 – 50 min in the lorry and were then unloaded, one treatment group after the other, into the slaughter corridor. The sixteen heifers were directly driven to the stunning box where they were stunned, one after another, within 15 min. Until stunning, heifers were in contact with animals of their treatment group, but not with unfamiliar animals.

Pre- and post-mortem (pm) measurements were similar as published in detail in Reiche et al. (2019). Briefly, heifers to be slaughtered were weighed one hour before loading in the familiar weighing scale. In the weighing scale, the animal's head was restrained in the weighing scale head gate and a saliva sample was taken from the heifer using a cotton swab (Salivette, Sarstedt, Nümbrecht, Germany) for cortisol assays. Immediately thereafter, the heifer was equipped by a Polar® chest belt (Polar Team Pro, Kempele, Finland). At the slaughterhouse, a second saliva sample was taken immediately after stunning. The chest belt was removed after bleeding.

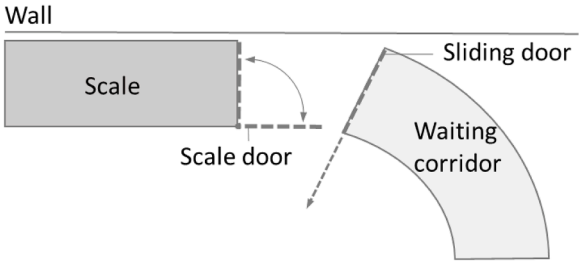

Fig. 2. Schematic representation of the installations used for the crush test. Dotted lines represent the two positions (closed and open) of the corridor and weighing scale doors.

Table 1
Definitions of observed behaviours during the crush test.

Behaviour	Unit	Range	Definition
Entering the crush	ordinal score	0-3	0 - voluntarily 1 - gentle, repeated tapping on the animal's croup 2 - touching, bending or flexing the animal's tail 3 - pushing the animal from behind
Head movement	ordinal score	0-2	0 - no defensive (brusque) movement 1 - one or several defensive movements 2 - continuously defensive movements
Tentatives to turn around	number/ minute	0-6	angle between the median line of head and the body between 90° and 180°
Head on barrier	number/ minute	0-6	head is placed on the barrier of the crush (also during tentatives to turn around and jumping)
Body movement	ordinal score	0-2	0 - no steps 1 - one or several slow steps 2 - one or several sudden steps
Crush movement	number/ minute	0-13	crush is shaking
Jump	number/ minute	0-1	two front legs taken off the ground, then animal falls back to the ground
Tail movement	ordinal score	0-2	0 - no tail movements 1 - one or several tail movements with the tail moving up to the height of the croup 2 - at least one tail movement with the tail moving beyond the height of the croup
Defecation/ miction	binary	0-1	0 - no, 1 - yes
Vocalisations (closed mouth)	number/ minute	0-7	
Vocalisations (open mouth)	number/ minute	0-1	
Crush score	ordinal score	0-26	sum of all variables listed above
Movement score	ordinal score	0-6	sum of scores of head, body and tail movements

Carcass weight and CH-TAX (equivalent to EUROP) classification (conformation and fat cover) were assessed within 30 min after slaughter. Briefly, conformation and fat cover were evaluated on a 7-point (1: lowest muscularity, 7: greatest muscularity) and five-point scale (1: lean, 3: optimal, 5: fat), respectively.

All measurements and samples were taken from the *longissimus thoracis* (LT) muscle at the level of the 10th rib. After chilling of the carcasses for 24 h at 2°C at the slaughterhouse, a 20-cm piece of the LT muscle located between the 10th and the 12th rib was excised and stored in the laboratory at 4°C for the measurements of water losses, T-Bars, shear force and sensory traits. pH and temperature were measured 1h, 2h, 4h, 6h and 48h pm on the LT piece using a probe (WTW 197S, WTW GmbH, Weilheim, Germany). Samples (1-2 g) for determining proteolysis were taken 1, 3, 5, 48 and 168h pm from the LT piece, immediately frozen and stored until analysis at -80°C. Proteolysis analysis was carried out as described by Reiche et al. (2019). The values for metavinculin, 80kDa calpain-1 and Troponin T were expressed as percentages over the intensity of the respective bands in a reference sample, of unautolysed 80 kDa of the total peak area of the calpain-1 bands (80, 78 and 76 kDa), and of 38 and 36 kDa bands over the total intensity of 38, 36, 33 and 30 kDa bands, respectively (see Reiche et al., 2019). Forty-eight hours pm, 2-gram samples for determining glycolytic potential (GP) were collected from the LT piece. At the same time, four 2-cm-thick slices were cut. One slice was used for the measurements 48h pm, whereas the other three were aged and used for ageing and drip loss (first slice), T-Bars, thawing and cooking loss and Warner-Bratzler shear force (WBSF; second slice), and sensory analysis (third slice). Slices were vacuum-packed and frozen at -28°C either immediately for 48-h-analysis or after a 14-d-ageing at 4°C to measure drip loss and ageing loss following Honikel (1998). Warner-Bratzler shear force and cooking loss were determined as described by Reiche et al. (2019), using a grill (Indu-Griddle, SH/GR 3500, Hugentobler, Schönbühl, Switzerland) and a texture analyzer (TA. HDplus, Stable Micro Systems, Godalming, England). Glycolytic potential was calculated according to (Monin and Sellier, 1985): GP = 2 x [(glycogen) + (glucose) + (glucose-6-phosphate)] + lactate.

Sensory analysis was carried out by eight trained panellists according to a standard protocol (International Organization for Standardization (2003) in eight sessions following the descriptions of Reiche et al., 2019. In each session, two plates of four samples were served one after another. The four samples on one plate contained one sample of each of the four treatments and were served to the panellists in a balanced order. The panellists evaluated overall flavour, juiciness and tenderness, graded from zero (lowest grade) to ten (highest grade) and overall liking (graded on a satisfaction scale from one (low satisfaction) to nine (high satisfaction)).

Salivary cortisol concentration was determined by a commercially available ELISA kit (Demeditec, Kiel, Germany).

2.4. Data processing and statistical analysis

Average daily gain was calculated for the whole grazing period by dividing the weight difference by the number of days between the first and last weighing.

The percentages of observations spent in the categories “leader”, “intermediate” and “follower” during the two leadership tests were calculated by dividing the number of times an animal was counted in the respective category by the total number of counts. Percentages were both calculated for each of the two leadership tests and averaged over the two tests.

To evaluate stress reactions during the slaughter periods, the recorded heart rates (HR) were analyzed by animal and pre-slaughter period, i.e. loading, transport, waiting in the lorry, unloading, slaughter corridor and stunning. Where the visual inspection of the data indicated a loss of contact between the electrode and the skin, data were considered as missing values (for the animal and period concerned).

Linear mixed effects model in the R environment (packages lme4 and multcomp) were used to analyze data related to activity on pasture, physiological measures, and physical, chemical and sensory meat quality. Statistical models included the fixed effects horn status and grazing intensity and their interaction, unless the interaction effect was not significant. Replicate and animal (where possible) were introduced as random factors. Where significant interactions were found post-hoc tests (Tukey’s HSD) have been used to evaluate differences between subgroups. Significance was set at $P<0.05$.

Pearson correlations coefficients were computed across all animals and by treatment group using the XLStat Software (2020.5.1). Correlations including more than one treatment group used z-scores calculated over only the concerned groups, by taking into account horn status, grazing intensity and replicate. Correlation coefficients were considered of interest if they had a value of at least 0.40 in at least two coherent treatment groups (same horn status or same grazing intensity) and presented the same direction. Correlations caused by outliers were excluded. When a variable was correlated with several other variables, ANCOVA was used to identify which model described best the former variable (optimal R^2 value). Using the same software, Principal Component Analyses (PCA) were carried out on correlated variables, both across all groups (first PCA) and coherent treatment groups (second to fifth PCA), to visualize clusters of correlations. A sixth PCA was carried out on tenderness, WBSF values, and indicators of proteolysis (levels of calpain-1, troponin, and metavinculin). Only variables with factor loadings of at least 0.50 were maintained in the PCA analyses.

Reactivity tests have been carried out during rearing and were reported in an earlier paper (Reiche et al., 2020). The relationships between reactivity measured in these tests (Novel Object Test: NOT and Food Competition Test: FCT; Crush test), stress reactions during the slaughter period and meat quality traits, are presented graphically using the Graphia software (Freeman et al., 2020) allowing to present clusters of correlations. The 61 variables used in the present paper and 74 of the 153 variables measured during the stress reactivity tests (Reiche et al., 2020) were included in the analysis. The 135 variables included the 56 slaughter-related variables used in the present paper, 2 variables for the Crush test (crush and movement score, see Tables 1), 2 for the FCT test (successful displacement of another animal, or unsuccessful attempt), 3 for leadership (time spent as leader, follower and intermediate) and 72 concerned the NOT (motion, exploration of the test arena and the Novel Object) presented in the earlier paper (Reiche et al., 2020). First, for each treatment group, correlation clusters were produced per group choosing a minimal r-value and a granularity in order to obtain 3 or 4 clusters. Second, an overall analysis using all animals was carried out creating 7 clusters for a graphical presentation. Similarities between groups were evaluated by considering coherence in variables retained in the different clusters. It was considered that there was coherence of variables if variables of a same category (eg. WHC, sensory analyses, or pm metabolism related variables) were retained in a given cluster (see Fig. 4).

3. Results

3.1. Observations during summer grazing

Weight gain. The mean average lifetime daily gain and that until and during summer grazing was not influenced by pasture intensity and horn status (all $P>0.10$; Table S1).

Physical activity. All of the recorded behaviours were influenced by grazing intensity, with mostly interactive effects with period (interaction effects: $P<0.09$, Table 2).

Time spent walking, number of strides and stand-up events were greater in June than in August, with more time spent walking and strides for LI than HI heifers in August and more stand-up events for HI than LI heifers in June ($P < 0.05$ for post-hoc comparisons; Table 2). HI heifers spent more time lying down in August than June, while LI heifers showed intermediate levels ($P < 0.05$ for post-hoc comparisons;

Table 2
Raw means, standard errors and effects of grazing intensity, horn status, observation period and their interactions on physical activity on pasture.

	June							August							P-values				
	HI			LI				HI			LI				Period	GI	Horn status	GI x Horn status*	GI x period
	H+ mean	SE	H- mean	SE	P-hoc	H+ mean	SE	P-hoc	H+ mean	SE	H- mean	SE	P-hoc	H+ mean	SE	H- mean	SE	P-hoc					
Walking (min/d)	57	2.2	70	3.6	ab	50	7.0	74	3.8	ab	47	2.5	51	2.1	ab	49	4.1	60	4.7	ab	**<0.001**	*0.09*	0.12	0.33	*0.074*
Strides (number/d)	1124	60.5	1225	51.9	bc	1226	104	1595	131	bc	1124	60.5	1225	51.9	bc	1226	104	1595	131	bc	**<0.001**	**0.017**	0.11	0.27	**0.022**
Standing immobile (min/d)	503	10.6	516	9.4	a	407	51.3	469	14.0	a	482	7.4	495	8.2	a	504	35.5	456	31.8	a	**0.033**	**0.007**	0.39	**0.006**	**0.001**
Lying down (min/d)	580	12.2	555	12.1	a	470	59.5	597	17.2	a	611	8.6	594	9.1	a	611	8.6	594	9.1	a	**<0.001**	**0.014**	0.28	*0.056*	**0.003**
Stand-up events (number/d)	13	0.6	12	0.5	c	8	1.1	11	0.6	c	7	0.2	8	0.5	c	9	0.8	7	0.6	c	**<0.001**	*0.079*	0.93	0.62	**<0.001**

GI - Grazing intensity; HI and LI - high and low grazing intensity, respectively
a,b,c are based on Tukey's post-hoc tests comparing the involved groups of the interaction grazing intensity x period. Different letters within a row indicate statistically different means.
* For interaction effects, see Table S2

Table 2). Time spent standing immobile was greater and lower for HI heifers in June and August, respectively, than for LI heifers (P = 0.069 and P = 0.059, respectively, for post-hoc comparisons). The influence of grazing intensity on time spent lying and standing depended further on the horn status (Table S2). Across both periods, horned LI heifers tended to spend less time lying and spent more time standing immobile than disbudded LI heifers, while the opposite was observed in the high intensity group. However, most often post-hoc comparisons did not reach significance (P > 0.08) for the latter interactions (Table S2).

Leadership. The percentage of observations spent as leader, intermediate and follower ranged from 0 – 0.93 (mean: 0.25), 0 – 1 (mean: 0.50) and 0 – 0.94 (mean: 0.25), respectively. Percentages of observations spent in each category at the two observation occasions were positively correlated, with moderate to high correlations for time spent as leader and as follower (Table S4), and weak, non-significant correlations for time spent as intermediate.

Crush test. Pasture intensity and horn status had no effect on the observed behaviours. The scores of head and tail movements were positively correlated with the score of body movements (Table S4).

3.2. Pre-slaughter physiology and meat quality

Heifers were slaughtered at a live weight of 444 ± 2 kg. Carcass weights and fat cover were not (P>0.10) influenced by grazing intensity or horn status (Table S1).

Overall, levels of stress indicators were greater for horned than disbudded heifers, while they varied by grazing intensity depending on the stress indicator and pre-slaughter period. Horned heifers had greater salivary cortisol levels before loading, tended to have faster HR during the first transport period and had faster HR at stunning than disbudded heifers (Table 3). Compared to HI heifers, LI heifers had slower HR during loading, faster HR in the period from unloading to stunning and lower salivary cortisol concentrations at stunning. Horned LI heifers tended to have faster HR during the last 10 min before stunning (interaction: P = 0.06; Table 3).

Meat of disbudded heifers was juicier than that of horned heifers. Tenderness, global flavour and overall liking were not affected by grazing intensity or horn status. Indicators of pm energy metabolism, water holding capacity and proteolysis varied by rearing condition and/or horn status depending on the variable (Table 3). Early and ultimate pm temperature and pH were mostly not influenced by grazing intensity and horn status (Table 3, Table S1), except for pH 4h pm, which was lower in horned HI heifers and disbudded LI heifers, compared to the other groups (Table S2). A similar tendency was observed for pH 6h pm (Table S2). Thawing loss 48h pm was lower in meat of HI than of LI heifers and cooking loss 48h pm was lower in meat of horned than disbudded heifers (Table 3). Thawing loss 14d pm was lower in horned HI heifers than disbudded HI heifers, and greater in horned LI than disbudded LI heifers (Table 3 and Table S2). An opposite tendency was observed for cooking loss 14d pm (Table 3 and Table S2). Warner-Bratzler shear force tended to be lower in HI than in LI heifers. Drip loss and water loss during ageing were not affected by grazing intensity or horn status (Table 3).

80 kDa Calpain-1 levels were greater 3h pm in disbudded HI than in disbudded LI heifers, while levels in horned heifers were similar (Table 3 and Table S2); a similar tendency was observed for 80 kDa calpain-1 levels 5h pm (Table 3 and Table S2). For disbudded heifers, Troponin degradation 3h pm was greater for HI than LI animals (Table 3 and Table S2). For both disbudded and horned heifers, troponin degradation 5h pm was greater in HI than LI heifers. At 48h pm, 80 kDa calpain-1, metavinculin and troponin levels did not differ by grazing intensity or horn status.

3.3. Relationships between measurements

Heart rates showed many correlations amongst different pre-

Table 3
Raw means, standard errors and effects of pre and post mortem measurements related to physiology, energy metabolism and meat quality by grazing intensity and horn status.

		HI				LI				P values		
		H+		H-		H+		H-		Grazing intensity	Horn status	Grazing intensity x Horn status
		mean	SE	mean	SE	mean	SE	mean	SE			
Heart rate [bpm]												
	Loading	122.3	7.0	125.3	6.6	107.9	4.9	107.8	4.2	**0.010**	0.72	-
	0-10 min of transport	99.4	4.3	93.7	3.9	100.3	5.5	92.4	5.6	0.92	*0.058*	-
	Unloading	106.6	8.6	96.0	7.9	110.1	11.6	99.1	3.4	0.84	**0.007***	-
	From unloading to stunning	90.6	4.8	90.6	4.7	109.9	5.7	107.7	6.8	**0.001**	0.72	-
	Last 10 min before stunning	89.8^{ab}	4.0	83.1^{a}	3.7	106.9^{b}	4.7	85.3^{a}	3.5	0.99	0.24	*0.061*
	Prebox	104.7	5.5	108.7	5.4	116.0	7.9	106.8	6.1	0.73	**0.003***	-
	Stunning	132.3	6.4	121.8	3.5	146.2	7.8	121.3	8.6	0.62	**<0.001**	-
Stress hormones												
	Salivary cortisol before loading (ng/ml)	0.36	0.08	0.11	0.02	0.31	0.04	0.31	0.04	0.25	**<0.001**	-
	Salivary cortisol after stunning (ng/ml)	4.74	0.95	3.73	0.46	5.64	0.63	5.64	0.63	**0.046**	0.21	-
Indicators related to post mortem energy metabolism												
	pH_{4h}	5.82^{a}	0.03	5.94^{b}	0.03	5.94^{b}	0.02	5.83^{a}	0.02	**0.001**	**0.004**	**0.0001**
	pH_{6h}	5.69^{ab}	0.03	5.77^{b}	0.03	5.75^{b}	0.02	5.70^{a}	0.02	**0.006**	**0.021**	**0.008**
	pH_{48h}	5.54	0.03	5.55	0.04	5.57	0.04	5.52	0.02	0.49	0.56	-
	$Temperature_{4h}$ (C°)	18.81	0.28	18.73	0.41	18.96	0.53	18.19	0.26	0.65	*0.059**	-
Indicators related to water holding capacity (%)												
	Thawing $loss_{48h}$	4.68	0.28	5.49	0.35	6.19	0.47	5.74	0.27	**0.002**	0.12	-
	Thawing $loss_{14d}$	4.30^{a}	0.15	4.90^{a}	0.21	5.11^{a}	0.27	4.73^{a}	0.19	0.66	*0.075*	**0.036**
	Cooking $loss_{48h}$	19.4	0.54	20.9	0.44	19.6	0.46	20.2	0.62	0.84	**0.032**	-
	Cooking $loss_{14d}$	20.3^{a}	0.68	19.5^{a}	0.39	20.2^{a}	0.43	21.3^{a}	0.48	*0.012*	0.16	*0.068*
Table 3 continued												
Indicators related to meat tenderness												
	$WBSF_{14d}$ (N/cm3)	29.1	0.93	28.9	0.82	30.3	1.05	30.6	0.73	*0.077*	0.98	-
Indicators related to post mortem proteolysis												
	80 kDa $Calpain\text{-}1_{3h}$	98.6^{ab}	1.37	101.8^{b}	0.65	99.0^{b}	1.98	95.8^{a}	1.68	**0.002**	**0.072**	**0.011**
	80 kDa $Calpain\text{-}1_{5h}$	96.5^{a}	1.54	97.9^{a}	0.79	96.3^{a}	2.44	92.2^{a}	1.94	**0.037**	0.65	*0.05*
	$Troponin_{3h}$	107.2^{ab}	5.62	86.1^{a}	5.09	100.5^{ab}	9.03	121.4^{b}	5.73	**<0.0001**	**0.009**	**<0.0001**
	$Troponin_{5h}$	94.4	5.81	88.4	6.05	100.1	5.97	115.2	7.69	**0.010**	0.80	-
Sensory analysis												
	Juiciness	6.46	0.23	6.94	0.20	6.31	0.29	6.69	0.21	0.23	**0.030**	-

* Effect only in one replicate present Bold and italic letters represent statistical significance and tendency, respectively
[a,b]Means within a row with different superscripts are statistically different (Tukey's post-hoc test).
GI - Grazing intensity; HI and LI - high and low grazing intensity, respectively; H+ and H- - horned and disbudded heifers
Means, standard errors and effects of all pre and post mortem measurements are presented in Table S1.

slaughter stages, for each of the subgroups (r between 0.54 and 0.96, mean r-value 0.72, considering the 71 r-values with $p < 0.05$, data not shown). Correlations were stronger between a given period and the preceding and subsequent period than when comparing periods separated by longer intervals. Excluding HR data (to avoid bias given their many correlations amongst themselves), the z-score correlation matrix across all animals found 198 significant correlations, representing 17.6 % of all calculated correlation coefficients. Twenty robust correlations were found for three or four treatment groups (Table 4, top section). Slower heart rates during unloading and greater pre-slaughter glycogen potential were associated with greater drip loss 48h pm. ANCOVA analysis found that both heart rate at unloading, glycolytic potential, and the presence of horns explained 35.1 % of the variability in drip loss between individuals (Table S3). Heart rate at unloading was further associated with pH 48h pm, with positive correlations for horned heifers, but a negative correlation for disbudded heifers on low grazing intensity (Table 4). These inverse relationships were best described by a second order algorithm (Fig. 3a; Table S3). Considering all experimental heifers with HR during unloading below and above 110 bpm separately, and using additionally early pm pH (between 1 and 6h), glycolytic potential and residual glycogen as explanatory factors, only the regression model for the group with fast heart rates kept HR as explanatory variable (Fig. 3b; Table S3).

Many of the other robust correlations reflect relationships among indicators of pm energy metabolism including glycogen levels, lactate production and pm pH, and among various indicators of proteolysis and water holding capacity (WHC) including drip, thawing and ageing losses (Table 4, top section). Different attributes of sensory analysis were correlated amongst each other (Table 4, top section).

Forty-two correlations were found for coherent subgroups, that is, for the LI, the HI, the horned or the disbudded groups only (Table 4, middle and bottom sections). Correlations found for the HI heifers concern the same categories as mentioned above, as well as correlations between indicators of pm energy metabolism and T-bars and carcass temperature at 6h pm. Thawing loss 48h pm, cooking loss 48h pm and Warner-Bratzler shear force 48h pm were negatively correlated with

Table 4
Relationships among physiological, post-mortem muscle and sensory measurements: correlation coefficients by treatment group (untransformed data), and several groups combined (on z-scores calculated over the relevant groups). Bold r-values: $p<0.05$

More than 2 treatment groups			Pearson correlation coefficients* HI H+	z-scores r-value HI H-	LI H+	LI H-	across the 4 groups
	HR at unloading	Drip loss$_{48h}$	**-0.61**		**-0.67**	**-0.52**	**-0.44**
	pH$_{6h}$	Lactate$_{48h}$	**-0.51**	**-0.53**	**-0.53**		**-0.41**
	pH$_{48h}$	Lactate$_{48h}$	**-0.87**	**-0.86**	**-0.62**	-0.49	**-0.73**
	pH$_{48h}$	Glycolytic potential	**-0.88**	**-0.90**	**-0.60**	**-0.65**	**-0.73**
	pH$_{48h}$	Residual glycogen	**0.65**	**-0.70**		**-0.54**	**-0.50**
	pH$_{48h}$	Thawing loss$_{48h}$	**-0.65**	**-0.63**	**-0.57**		**-0.49**
	Residual glycogen	Glycolytic potential	**0.85**	**0.88**	**0.91**	**0.97**	**0.89**
	Glycolytic potential	Lactate$_{48h}$	**0.83**	**0.81**	**0.61**		**0.68**
	Glycolytic potential	Drip loss$_{48h}$	**0.67**	**0.52**	**0.63**		**0.43**
	Drip loss$_{48h}$	Maturation loss during 14d	**0.66**	**0.69**	**0.77**	**0.55**	**0.63**
	Drip loss$_{48h}$	Thawing loss$_{48h}$	**0.70**	**0.78**	**0.55**	**0.64**	**0.61**
	Cooking loss$_{48h}$	WBSF$_{48h}$	**0.52**	**0.59**	**0.80**	**0.52**	**0.59**
	Thawing loss$_{48h}$	Maturation loss during 14d	**0.71**	**0.74**	**0.76**		**0.64**
	Metavinculin$_{48h}$	80 kDa Calpain-1$_{48h}$	**0.74**		**0.65**	**0.59**	**0.42**
	Metavinculin$_{168h}$	80 kDa Calpain-1$_{48h}$	**0.63**	0.50	**0.54**	**0.56**	**0.54**
	Troponin T$_{3h}$	Troponin T$_{5h}$	**0.61**	**0.64**	0.41	**0.68**	**0.61**
	Tbars$_{48h}$	Tbars$_{14d}$	**0.89**	**0.79**	**0.62**	**0.64**	**0.76**
	Global flavor	Tenderness	**0.53**	**0.66**	**0.88**		**0.57**
	Overall liking	Juiciness	**0.75**	**0.78**	**0.67**		**0.67**
	Overall liking	Tenderness	**0.52**	**0.69**	**0.61**	**0.56**	**0.56**
Table 4 continued							
High grazing intensity		**HI H+**	**HI H-**			across the HI groups	
	pH$_{48h}$	Thawing loss$_{14d}$	**-0.52**	**-0.54**	-0.40		**-0.48**
	Glycolytic potential	Thawing loss$_{48h}$	**0.69**	**0.54**	0.51		**0.63**
	Tbars$_{14d}$	Thawing loss$_{48h}$	**-0.54**	**-0.70**			**-0.62**
	Lactate$_{48h}$	Thawing loss$_{48h}$	**0.68**	**0.63**	0.49		**0.63**
	Lactate$_{48h}$	T$_{6h}$	**0.73**	**0.61**			**0.59**
	Lactate$_{48h}$	Tbars$_{48h}$	**-0.63**	**-0.59**			**-0.61**
	Lactate$_{48h}$	Tbars$_{14d}$	**-0.55**	**-0.69**	0.44		**-0.61**
	T$_{6h}$	Tbars$_{48h}$	**-0.55**	**-0.64**	0.47		**-0.53**
	Glycolytic potential	T$_{6h}$	**0.63**	**0.55**	-0.45		**0.50**
	Tbars$_{14d}$	Cooking loss$_{48h}$	**-0.55**	**-0.52**			**-0.57**
	Tbars$_{14d}$	WBSF$_{48h}$	**-0.59**	**-0.64**			**-0.62**
	Métavinculin$_{48h}$	Troponin T$_{48h}$	**0.78**	**0.60**	0.48		**0.68**
	Times counted as intermediate (leadership test)	WBSF$_{14d}$	**-0.50**	**-0.50**			**-0.50**
	Global flavor	Overall liking	**0.80**	**0.75**	0.46	0.40	**0.78**
Low grazing intensity				**LI H+**	**LI H-**	across the LI groups	
	HR from waiting in lorry until stunning	Metavinculin$_{3h}$			**-0.57**	**-0.50**	**-0.62**
	HR from waiting in lorry until stunning	Metavinculin$_{5h}$			**-0.55**	**-0.64**	**-0.43**
	Cooking loss$_{14d}$	WBSF$_{14d}$		0.42	**0.56**	**0.61**	**0.55**
	Tbars$_{14d}$	80 kDa Calpain-1$_{5h}$			**0.53**	**0.53**	**0.55**
	Tbars$_{14d}$	Overall liking			**0.53**	**0.68**	**0.49**
Horned		**HI H+**		**LI H+**		across the H+ groups	
	HR at unloading	pH$_{48h}$	**0.57**		**0.53**	**-0.64**	-
	HR last 10 min in the lorry before unloading	Thawing loss$_{48h}$	**-0.53**		**-0.58**		**-0.57**
Table 4 continued							
	Residual glycogen	Drip loss$_{48h}$	**0.59**	0.45	**0.66**		**0.62**
	Residual glycogen	Maturation loss during 14d	**0.53**		**0.77**		**0.67**
	Cooking loss$_{14d}$	Tbars$_{14d}$	**-0.56**		**-0.55**		**-0.55**
	Glycolytic potential	80 kDa Calpain-1$_{3h}$	**0.62**		**0.72**		**0.63**
	Lactate$_{48h}$	80 kDa Calpain-1$_{3h}$	**0.71**		**0.55**		**0.58**
	Metavinculin$_{168h}$	Troponin T$_{48h}$	**0.67**		**0.63**		**0.65**
	WBSF$_{48h}$	Troponin T$_{48h}$	**0.55**		**0.56**		**0.55**
	WBSF$_{48h}$	80 kDa Calpain-1$_{48h}$	**0.79**		**0.64**		**0.65**
	Metavinculin$_{48h}$	Juiciness	-0.51	-0.43	-0.45	-0.41	**-0.48**
	Metavinculin$_{168h}$	Juiciness	**-0.68**		**-0.70**		**-0.68**
	pH$_{48h}$	Global flavor	**-0.62**		**-0.60**		**-0.61**
	Lactate$_{48h}$	Global flavor	**0.65**		**0.79**		**0.67**
	Glycolytic potential	Global flavor	**0.52**		**0.70**		**0.58**

(*continued on next page*)

Table 4 (*continued*)

More than 2 treatment groups			Pearson correlation coefficients* HI H+	z-scores r-value HI H-	LI H+	LI H-	across the 4 groups
Disbudded			**HI H-**		**LI H-**	across the H-groups	
	Mean HR during transport	T_{48h}		**-0.78**		**-0.66**	**-0.76**
	HR last 10 min before stunning	pH_{2h}		**-0.53**		**-0.56**	**-0.55**
	HR last 10 min before stunning	pH_{6h}		**-0.57**		**-0.67**	**-0.59**
	HR slaughter corridor	$Métavinculin_{48h}$		**-0.60**		**-0.59**	**-0.59**
	Times counted as leader (leadership test)	Troponin T_{168h}		**0.55**		**0.60**	**0.57**
	Movement score (crush test)	80 kDa Calpain-1_{5h}		**-0.62**	-0.43	**-0.75**	**-0.56**
	Thawing $loss_{14d}$	$WBSF_{48h}$		**0.59**		**0.59**	**0.57**
	pH_{4h}	$Tbars_{48h}$		**0.66**		**0.53**	**0.55**
	Maturation loss during 14d	$Tbars_{14d}$		**-0.67**		**-0.51**	**-0.59**

*for subgroups:
r-values > 0.51: p< 0.05; r-values > 0.50: p< 0.06; r-values > 0.43: p< 0.10

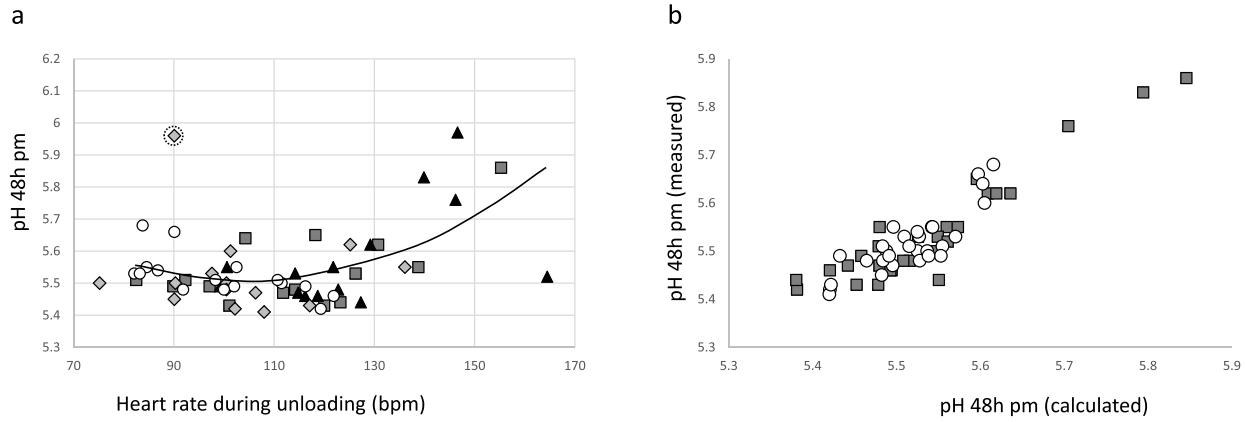

Fig. 3. Left: Correlation between heart rate during unloading and pH at 48h. H- HI, H- LI, H+ HI and H+ LI are indicated with rhombuses, circles, squares and triangles, respectively, in black and white and different shades of grey. After removal of one outlier (in the circle), a second order polynomial algorithm was a good fit: pH48h = 0.00009*(HR)^2 - 0.0194*HR + 6.52 (r=0.55; p=0.0001). Right: correlations between calculated and measured pH48h values for animals with heart rates at unloading above (squares) and below (circles) 110 bpm, using the following algorithms: pH48h = 2.0 + 0.002 * HR at unloading + 0.6 * pH 6h – 0.002 * Glycolytic Potential (r=0.93; p<0.00001) and pH48h = 4.3 + 0.3 * pH 6h – 0.003 * Glycolytic Potential (r=0.83; p=0.0001), respectively.

Tbars at 14d pm (Table 4). The more often an animal was counted as "intermediate" in the leadership test, the less tender was its meat at 14 d pm. Liveweight gain during summer grazing was not robustly correlated with meat quality indicators, it was only positively correlated with drip loss (HI H+: r = 0.49, P > 0.05; HI H-: r= 0.49, P > 0.05; LI H-: r = 0.59, P < 0.05) and thawing loss 48h pm (HI H+: r = 0.65, P < 0.05; LI H-: r = 0.57, P < 0.05).

Specific correlations found for the LI heifers (Table 4) concern negative relationships between HR averaged over the period between waiting in the lorry and stunning and metavinculin at 3h and 5h pm. T-bars at 14d pm had positive relationships with calpain-1 5h pm and overall liking.

For horned heifers (Table 4), HR while waiting in the lorry until unloading and thawing loss 48h pm were negatively correlated, and indicators of pm energy metabolism and calpain-1 levels at 3h pm were positively correlated. WBSF at 48h pm was positively correlated with calpain-1 at 48h pm, and troponin levels at 48h pm. Global flavour was correlated with pm energy metabolism indicators and juiciness with metavinculin at 48h pm and 168h pm. For disbudded heifers (Table 4), greater pre-slaughter HR in the last 10 min before stunning were associated with lower pH at 2h pm and pH at 6h pm and greater HR at stunning with lower metavinculin at 48h pm levels. Greater HR during transport was associated with colder carcasses 48h pm. Greater move scores (crush test) were associated with lower calpain-1 at 5h pm. Greater levels of Tbars at 48h pm and Tbars at 14d pm were associated with higher pH at 4h pm and lower ageing losses during 14d pm, respectively. The more often an animal was counted as "leader" in the leadership test, the greater were its troponin levels 168h pm.

Considering the experimental heifers slaughtered at the first and second slaughter day, slaughter order was positively correlated with the HR during unloading (Table S4). An ANCOVA analysis found that the slaughter order and slaughter day explained 36.7 % of the variability in the heart rate at unloading. For heifers slaughtered at the second and fourth slaughter day, slaughter order was negatively correlated with thawing loss 48h pm. Together with the slaughter day, slaughter order explained 41.6% of the variability in thawing loss at 48h pm (Table S4).

All PCAs, across all animals or for separate groups, found that the 1st axis was correlated with metabolic processes and markers of oxidation (Table 5). This same axis was further correlated with water holding capacity for all but the LI group and with markers of proteolysis for all but the PCA across all animals. The 2nd axis was correlated with sensory attributes, amongst others, for all PCAs (Table 5, Table S5).

The PCA on tenderness and related variables found that the 1st axis (29.1% of the variation) was correlated with instrumental measurements obtained after 2 or more days, and the 2nd axis (18.9% of the variation) was correlated with tenderness and instrumental measurements obtained 3 and 5h pm (Table S5).

Using Graphia cluster analysis for each of the treatment groups separately, it was found that cluster 1 contained six variables common to each of the treatment groups (Fig. 4). They were related to glycolytic

Table 5
Main tendencies based on variables loadings on the principal axes.

PCA	1st axis	2nd axis
Across all animals		
All variables	**Metabolism, oxidation**, WHC	**Sensory**
Tenderness, WBSF, indicators of proteolysis	Calpain 48h pm, troponin 48h pm, metavinculin 48 and 168h pm, WBSF 48h and 14d pm	Tenderness, calpain 3h and 5h pm
HI	**Metabolism, oxidation**, proteolysis, WHC	**Sensory**, proteolysis, sensory
LI	**Metabolism, oxidation**, proteolysis, sensory	**Sensory**, oxidation, WHC
H+	**Metabolism, oxidation**, proteolysis, sensory, WHC	**Sensory**, proteolysis
H-	**Metabolism, oxidation**, proteolysis, WHC	**Sensory**

HI and LI: high and low grazing intensity, respectively; H+ and H-: horned and disbudded heifers, respectively; WBSF: Warner-Bratzler shear force; WHC: water holding capacity

activity (glycolytic potential, glycogen consumption, residual glycogen, lactate) and calpain-1 at 3 and 5h pm. Eleven variables were common to 3 groups, both HI and the horned LI groups. These were related to occupation of space during period 1 of the NOT (time spent in the 4 zones, frequency of changing zones), muscle pH at 1, 2, 4 and 48h pm, and temperature 1h pm. Three variables, metavinculin, WBSF and cooking loss 14 days pm, were common to both LI and the horned HI groups. Three more variables, cooking, drip and thawing losses 48h pm were common to both HI and the disbudded LI groups.

The cluster representation combining all treatment groups (Fig. 5) found 7 clusters. Cluster 1 was related to proteolysis (calpain-1 at 3 and 5h pm, metavinculin at 3, 5 and 48h pm), WHC (thawing and cooking losses at 48h and 14d, drip loss), glycolysis, tenderness (sensory tenderness and WBSF at 48h and 14d pm) and other sensory attributes (overall liking, global flavor, juiciness). Clusters 2 and 3 are related to pre-slaughter heart rate and pm (1, 2, 4, 6, and 48h pm) pH and temperature, respectively. The other clusters are related to various behavioural classes in the NOT.

4. Discussion

4.1. Effects of rearing conditions and grazing intensity on performance, behaviour and pre-slaughter stress levels

The heifers' live weight gains during summer grazing were poor (mean: 0.18 kg/d) compared to Velik et al. (2013) and Morel et al. (2016) and are likely explained by poor herbage quality. The absence of an influence of grazing intensity is in line with other studies comparing continuous to rotational grazing at similar stocking rates (Briske et al.,

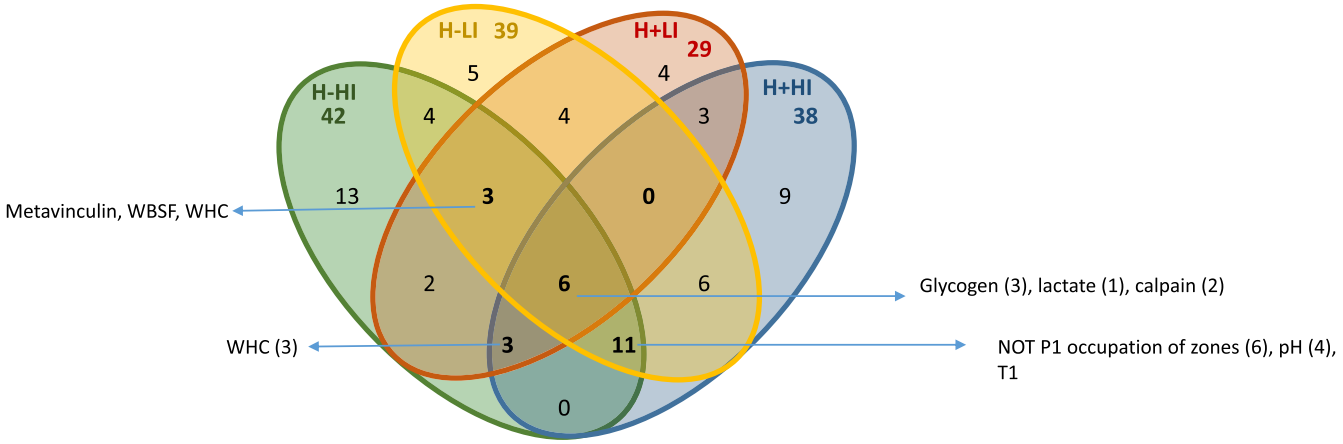

Fig. 4. Venn diagram of overlapping variables of cluster 1 of the correlation graph made by Graphia. Bold coloured figures indicate the total amount of variables retained in cluster 1 for each group. Bold black figures indicate similar variables retained for at least 3 groups. Chosen minimal Pearson r-values were 0.69; 0.67; 0.61 and 0.63 and chosen cluster granularity 1.30; 1.28; 1.34 and 1.23 for H-HI; H-LI; H+LI and H+HI, respectively. These parameters created 4 (H-HI; H-LI; H+LI) or 3 (H+HI) clusters. A total of 73 different variables were retained from the 135 variables submitted to the analysis, 23 of them were common to 3 or 4 of the treatment groups. The Venn diagram shows that the 4 groups were very similar, with only 13, 5, 4 and 9 uniquely retained variables for H-HI, H-LI, H+LI and H+HI, respectively. All other variables were also retained for at least one of the other groups.

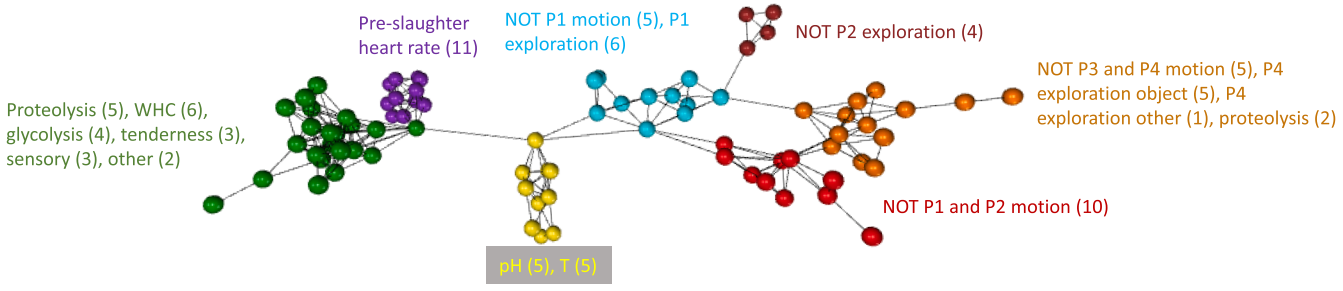

Fig. 5. Correlation graph made by Graphia of the combined 4 treatment groups (H-HI; H-LI; H+LI and H+HI) using the 135 variables. Chosen minimal Pearson r-value was 0.49 and cluster granularity was 1.43. The different clusters identified are indicated using different colours. The categories of variables are indicated for each cluster (number of variables for each category between brackets).

2008).

The greater physical activity of LI heifers, as indicated by their greater walking times and strides, than HI heifers is in accordance with previous work, in which compared to rotational grazing, continuous grazing increased travelled distances for water or to select grass, as larger paddocks and lower stocking density increased grass heterogeneity (Hepworth et al., 1991; Hart et al., 1993). Within the LI condition, the horned heifers spent more time standing immobile than the disbudded heifers. This is in line with previous indoor-observations of these same animals at 4 and 7 mo of age and may simply reflect behavioural tendencies of these LI heifers (Reiche et al., 2020b).

The moderate to high correlations between leadership scores found in the present and earlier works indicate that the order of animals in a situation is not random, but fairly consistent (Syme, 1981; Reinhardt, 1983). The groups of disbudded heifers showed a more stable leadership order, indicated by their greater correlation coefficients among leadership observations compared to horned groups. Albeit a different aspect of social hierarchy, an earlier study found that different rules govern dominance in hornless and horned heifers (Bouissou, 1972). In sheep, consistency in leadership order during forced movements was related to sociability with greater consistency reflecting stronger social relationships (Syme, 1981). This would suggest that disbudded heifers had stronger social relationships. The order of an animal during a forced group movement may also be motivated by its nutritional status (Rands et al., 2003) or its affinity to humans (D'Souza et al., 1998), which may have varied more in horned than in disbudded heifers in our study.

Horn status and rearing conditions influenced the heifers' physiological pre-slaughter reactions. Compared to disbudded heifers, horned heifers had greater cortisol concentrations before loading, faster heart rates at the beginning of transport and in the abattoir and a tendency to greater carcass temperatures 4h pm, all indicative of greater physiological stress levels. One reason for elevated stress reactions at slaughter may be a greater reactivity to certain psychological stressors such as novelty, social disturbances or sudden events (Bourguet et al., 2015). At 11 mo of age, i.e. 6 mo before slaughter, these horned heifers showed greater fear-associated behaviour during Novel-Object-Tests than the disbudded (Reiche et al., 2020). In agreement, another study found that greater fear of novelty during rearing predicted greater stress reactions at slaughter in cows (Bourguet et al., 2010). The relatively lower stress levels of the disbudded heifers in the slaughter context may also be due to stronger social relationships. An earlier study, where similarly, the animals were maintained in their rearing groups during the slaughter period, found that stronger social coherence within the group lowers stress at slaughter (Mounier et al., 2006).

HI heifers showed slower heart rates than LI heifers from unloading to stunning and lower cortisol concentrations at stunning. These are slaughter stages where humans interact with the animals. On pasture, HI heifers were in closer contact to the stockworkers, as they were driven three times more often between paddocks than LI heifers. This has likely habituated HI heifers to the presence of humans, which may make cattle calmer and reduce stress reactions to humans including at slaughter (Lensink et al., 2000; Probst et al., 2012; Ceballos et al., 2016). During loading and transport, HI showed in contrast faster heart rates than LI heifers. These earlier stages represent more specifically novelty and social disturbances (Bourguet et al., 2010), which would thus appear to be more stressful for the HI than LI heifers.

4.2. Coherent correlations amongst meat quality indicators across treatment groups

The correlations, and the PCAs, showed associations between indicators of energy metabolism, water holding capacity and oxidation, similar for the different treatment groups. The correlation graphs showed also good coherence between the different treatment groups, both among meat quality variables and between stress reactivity and meat quality. The correlations are coherent with existing knowledge, for example, lower glycolytic potential is generally related to lower lactate production, and higher ultimate pH (Warriss, 1990; Fernandez and Tornberg, 1991; Immonen and Puolanne, 2000). The reasons for this are well described. After slaughter, in the absence of respiration and blood circulation, the muscle cell lacks oxygen. As a result, the cell generates lactate through the anaerobic breakdown of glycogen and hydrogen ions through the hydrolysis of ATP. The formation and accumulation of hydrogen ions during this process causes the post-mortem pH to decline (Robergs et al., 2004). At lower muscle glycogen reserves, generally, higher ultimate pH values are obtained.

Indicators of water holding capacity (thawing, cooking and ageing losses) were also often correlated amongst each other and with those of post-mortem energy metabolism, and generally associated with the first axis in the PCAs. The associations between greater ultimate pH and greater water holding capacity are also well known. At higher ultimate pH, microfibrillar proteins have a greater negative charge and maintain therefore greater distances between them thereby increasing their water holding capacity (Warner, 2023). Indicators of proteolysis were generally correlated amongst each other and so were sensory attributes, in line with earlier studies (Gagaoua et al., 2016; Gangnat et al., 2017b). In the PCA on tenderness and indicators of proteolysis, increased tenderness was associated with greater early pm calpain-1 levels, which is consistent with the role of early pm calpain-1 in the tenderization process (Hwang and Thompson, 2001). Increased WBSF was correlated with greater levels of calpain-1, troponin and metavinculin levels measured 2 days pm or later. This is also consistent with existing knowledge as greater levels of intact calpain-1 indicate lesser degrees of autolysis and thus, of proteolysis, which is in line with the associated greater amounts of intact structural proteins and greater toughness observed (Taylor et al., 1995; Hwang and Thompson, 2001). The positive correlations between thawing and cooking losses and WBSF are in keeping with studies showing that reduced degradation of structural proteins is associated with decreased water holding capacity (Huff-Lonergan and Lonergan, 2005).

Negative correlations between lactate levels and the formation of Tbars existed only for the HI groups, but the same associations were found in the PCAs of all treatment groups and may be explained by differences in fibre type composition of the muscles. Greater lactate levels and/or a faster post-mortem metabolism may be indicative of greater proportions of fast contracting glycolytic fibres and greater Tbars formation may be indicative of greater proportions of slow contracting oxidative fibres (Choi et al., 2007; Bai et al., 2022).

4.3. Differences between treatment groups in meat quality indicators and relationships with stress reactions at slaughter

Most of the values of meat quality indicators fell within the range of previous studies (Gagaoua et al., 2016; Gangnat et al., 2017a; Reiche et al., 2019). Live weight gain and carcass weights showed no robust correlations with stress reactions or meat quality indicators, suggesting that grazing performance has not influenced meat quality. Disbudded heifers produced juicier meat with greater cooking losses at 48h. Greater cooking losses were also found in disbudded compared to horned bulls (Reiche et al., 2019). In these bulls, horn status influenced abundances of various muscle proteins, which may be a new research line to understand these effects (Ben Mbarek et al., 2022). Effects of horn status on meat quality are in line with the knowledge that the presence of horns influences the physiological and metabolic status of the animals. Horn growth is energetically costly and depends on the presence of various hormones involved in the use of energy, such as growth and thyroid hormones, prolactin, and testosterone (Bubenik and Bubeník, 1990). Various studies indicate that at lower resource availability, horn and antler growth decrease (Picard et al., 1996; Toïgo et al., 1999; Festa--Bianchet et al., 2004; Mysterud et al., 2005; Douhard et al., 2017). Furthermore, bovid horns consist of a highly vascularized bony core covered by an outer keratin sheath (Taylor, 1966; Bubenik and Bubeník,

1990). In temperate regions, during winter, heat loss from the horn's vascularized bony core in cold winters may impose an energetic cost (Picard et al., 1994; Picard et al., 1996; Baars et al., 2019). In goats, horns may selectively control the temperature of the brain via an exchange of heat between arterial blood of the carotid rete and cooled venous blood returning from the horn via the cavernous sinus (Taylor, 1966). These studies indicate that the presence of horns likely influences not only behaviour but also the overall functioning of the organism, including of the muscles.

Disbudded heifers with faster pre-slaughter heart rates had lower early pm pH. Heart rate is controlled by the autonomic nervous system and faster heart rate is associated with higher adrenaline and noradrenaline levels. In addition, heart rate increases with physical exercise. Adrenaline stimulates glycogen breakdown, particularly in the exercising muscle (Watt et al., 2001), which explains the faster early post-mortem pH decline observed in the disbudded heifers with faster heart rates. Other studies have found similar correlations (Bourguet et al., 2010; 2015; Reiche et al., 2019).

Grazing intensity influenced more meat quality traits than horn status. Compared to its counterpart, low grazing intensity was associated with greater thawing losses 48h pm, greater WBSF 14 days pm, greater intact Troponin levels 3 and 5h pm, and lower 80 kDa calpain-1 levels 3 h pm. Although often correlated in other studies (Ertbjerg et al., 1999; Reiche et al., 2019; Onopiuk et al., 2022) in the present study these influenced meat characteristics were not, including in the PCAs. The increased thawing loss and particularly, the slower proteolysis is possibly related to the greater pre-slaughter stress levels of the LI heifers. Greater pre-slaughter stress may lead to tougher meat as shown earlier (Warner et al., 2007; Gruber et al., 2010; Terlouw et al., 2021). Alternatively, or in addition, these effects may be due to greater physical activity of the LI heifers, as physically more active beef calves (increased strides and less time lying) also produced meat showing greater water losses and shear force and lower tenderness than less active calves (Gangnat et al., 2017a).

The greater amounts of Tbars in high grazing intensity heifers with faster heart rates at stunning may be caused by a faster metabolism associated with the faster heart rates. Elevated pre-slaughter stress levels, induced by physical exercise and transportation stress, stimulated the formation of free radicals and consequently lipid oxidation in cattle and pigs (Young et al., 2003; Delosière et al., 2020; Deters and Hansen, 2020). The negative association between lactate production and Tbars formation, related to fibre type composition as described above, and the positive association between a faster post-mortem metabolism and Tbars formation, described here, may coexist, as they are based on different mechanisms.

The associations between faster pre-slaughter heart rates and lower amounts of metavinculin levels, indicative of faster proteolysis, 3h (low grazing intensity groups) and 48h pm (disbudded groups) are difficult to explain. Greater pre-slaughter stress and/or faster post-mortem metabolism are usually associated with tougher meat, due to reduced or slowed proteolytic processes (Geesink and Koohmaraie, 1999; Gruber et al., 2010; Kim et al., 2012; Terlouw et al., 2021). Often, inversions of correlations indicate that additional factors play a role (Terlouw et al., 2021). In the present case, this additional factor may be related to carcass temperature. Faster early post-mortem pH decline was associated with more tender meat if muscle temperature was below 35°C when entering rigor (pH=6; Strydom and Rosenvold (2014). This was the case in the present study where carcass temperature at pH=6 was 22.5 ± 0.6°C. Another example of an inversion of a correlation is the relationship between heart rate at unloading and pH 48 pm observed in the present study. Only relatively fast heart rates (above 110 bpm in the present example) resulted in a reduced overall pH decline (higher pH 48h pm). An earlier study found also a positive correlation between pre-slaughter heart rate and ultimate pH (Reiche et al., 2019). Higher heart rates are generally associated with increased adrenaline levels and it was hypothesized that adrenaline-stimulated AMP deamination (England et al., 2015; Goodman and Lowenstein, 1977) may limit post-mortem glycolysis, leading to a higher ultimate pH compared to animals with lower heart rates (Reiche et al., 2019). The weak negative slope indicates a limited effect of heart rates during unloading below 110 bpm on ultimate pH. The effect is possibly related to a faster very early post-mortem pH decline, but this would need further investigation to be confirmed. Hence, ultimate pH was higher at lower glycolytic potential and at higher pH 6h pm, and at higher heart rates during unloading as long as heart rates were above 110 bpm. The decreased drip and thawing losses 48h pm observed in heifers with faster heart rates before or during unloading is in agreement with their higher ultimate pH and consequently, greater water holding capacity, as explained above (section 4.1).

The correlation graphs found also good coherence for the 4 treatment groups, with much overlap in the variables retained in the first cluster. Combining the 4 groups, united into a single cluster of indicators of post-mortem biochemical processes, including proteolytic and metabolic processes and water holding capacity, and sensory attributes. This cluster was associated with pre-slaughter heart rate on the one hand, and with indicators of rate of metabolism (pH, carcass temperature) on the other. The other clusters were related to behaviour in the novel object test and relatively removed from cluster 1, indicating the absence of strong correlations. This shows that reactions during this test were not strong predictors for the rate of biochemical processes post-mortem. This is probably related to the relatively long delay (6 months) between the dates of testing and of slaughter, compared to the 3 to 4 weeks in studies where behaviour during tests and stress reactions at slaughter and meat quality showed relatively strong relationships (Bourguet et al., 2010; 2015). This suggests that to investigate the relationships between meat quality and stress reactivity, animals should be tested close in time to slaughter. In agreement, results indicated that heart rate and thawing loss were influenced by day and order of slaughter, suggesting that in addition to the treatments, events close to stunning and bleeding had a significant impact on stress and meat variables.

Finally, while the use of the crossbreed reflects the local rearing practices, the use of F1 animals with different dam breeds increases the variability between animals and may have limited the detection of significant treatment effects.

5. Conclusion

The present study shows that horn status and grazing intensity influence activity levels during rearing, physiological pre-slaughter reactions and meat quality, including sensory attributes. Presence of horns was associated with decreased juiciness of the meat, but overall, grazing intensity had stronger influences on meat quality than horn status. A greater grazing intensity had mostly positive effects on stress levels at slaughter and meat quality, likely largely attributed to the greater contact with stockpeople during frequent pasture rotation. The different groups showed overall good coherence in correlations amongst meat quality indicators. These robust correlations were in keeping with knowledge of biochemical processes in the post-mortem muscle influencing meat quality and confirm the interdependence of such processes, including in quantitative terms. Specifically, lower pre-slaughter glycogen levels and faster heart rates, provided they were above 110 bpm, caused greater ultimate pH, associated with lower water holding capacity. Greater pre-slaughter stress, as indicated by faster heart rates, were further associated with faster early post-mortem pH decline.

CRediT authorship contribution statement

Anna-Maria Reiche: Writing – original draft, Visualization, Investigation, Formal analysis, Data curation. **Paolo Silacci:** Methodology, Investigation. **Frigga Dohme-Meier:** Supervision, Project administration. **E.M. Claudia Terlouw:** Writing – original draft, Visualization, Supervision, Methodology, Formal analysis, Conceptualization.

Declaration of competing interest

None.

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Supplementary materials

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