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Original Research Paper

ASSOCIATION OF PLASMA STRESS MARKERS AT SLAUGHTER WITH CARCASS OR MEAT QUALITY IN PIGS

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Summary: The association between plasma level of three stress markers at slaughter (cortisol, neopterin, Hsp70) and carcass and meat quality traits was studied in pigs. The study comprised data from 51 pigs fattened on the same farm (one crossbreed, no *RYR1* mutation) and slaughtered in one abattoir in five batches. At slaughter blood was collected for the analysis of neopterin, cortisol and HSP70 level using commercial enzyme immunoassay kits. A day after the slaughter, carcass traits (fat and muscle thickness, lean meat percentage, *longissimus dorsi* (LD) muscle and fat area, belly leanness and ham weight) and meat quality traits (pH_1 at 1 h, pH_U at 24 h *post-mortem*, Minolta L*a*b* and drip loss) were assessed. No significant differences between slaughter batches were observed for stress markers and meat quality. However, individual animal variation of stress markers within slaughter batch was very high. Correlation analysis demonstrated no association between plasma neopterin and Hsp70 levels with any of carcass or meat quality properties. On the contrary, significant correlations of cortisol level were observed with some carcass or meat quality properties. Higher cortisol levels were associated with thicker subcutaneous fat (r=0.30 to 0.33) and lower carcass meat percentage (r=–0.37 to 0.41), which indicates that higher cortisol levels on the long term basis increase body fatness. Regarding meat quality, higher cortisol levels were associated with higher pH_1 (r=0.36 to 0.58) and pH_U (r=0.36 to 0.32), and consequently with lower LD Minolta L* (r=–0.44), b* (r=–0.38) and drip loss after 24 (r=–0.44) and 48 hours (r=–0.39) suggesting that cortisol level at slaughter reflects more than just acute preslaughter stress.

Key words: stress; pig; carcass quality, meat quality; cortisol; neopterin; Hsp70

Introduction

It has been recognized that stress in meat animals, and in particular in pigs, has a marked impact on meat quality. Prior to being slaughtered pigs endure food withdrawal, change of environment, loading, transport, mixing with unfamiliar animals, crowd, noise, spurring, very often cold or heat. It is difficult to measure animal's stress condition, therefore many markers (behavioural, physiological) have been used like body temperature, blood pressure, heart rate (1, 2), movement and jiggling (3), vocalisation (4, 5), different stress markers in body fluids and tissues such as cortisol (6, 7, 8, 9, 10, 11, 12, 13), neopterin (11), creatine phosphokinase (4, 9, 10, 13), heat shock proteins (14, 15, 16, 17, 18), lysine vasopressin (6, 8), lactate dehydrogenase (10), beta-endorphin (6, 7, 8), ACTH (9), adrenalin and noradrenalin (12, 19). Among many different biomarkers of stress we decided for plasma cortisol, neopterin and Hsp70. Cortisol is the main hormone of HPA (hypothalamic-pituitary-adrenocortical) axis released by the adrenal cortex in response to stress. It influences feeding behaviour, pancreatic hormone secretion, energy expenditure and protein/lipid balance (20). Although the role of neopterin (pyrazino-[2,3-D]-pyrimidin) is not fully understood, clinical studies in human suggest it is a good marker of cellular immunity activation since higher neopterin concen-

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trations were associated with infections, autoimmune diseases, tumours (11). At a cellular level, the stress response includes a synthesis of heat shock proteins, which are classified according to their molecular weight (21). One of the most abundant and best characterized is the 70-kDa family (Hsp70). Hsp70 (stress-limiting factor) during stress prevents inappropriate protein aggregation, mediates the transport of proteins for degradation, helps proteins to maintain their conformation and even assists in their repair (22, 23).

In pigs the quality of meat largely depends on the nature of post-mortem muscle pH decline, which is strongly influenced by stress prior to slaughter. An acute slaughter stress can induce PSE condition in pork due to a rapid pH decline (24), while fasting, transport (25, 26), or exercise (27) prior to slaughter can lead to glycogen depletion resulting in higher ultimate pH and better water holding capacity (28). Thus slaughter batch has been recognized as a major factor of variation in meat quality as demonstrated by various studies dealing with pork quality. The individual response of the animal to preslaughter stress will be determined by a complex interaction with genetics and previous experience (29). Relationship between stress status of individual pig and its meat quality has seldom been established. The objective of the present experiment was to analyze slaughter batch effect on the level of selected plasma stress markers and meat quality and to determine the association between stress status of individual pigs at slaughter with carcass or meat quality traits.

Material and methods

Animals

Current study was performed as a collateral study within another project (30). Our experiment comprised 51 pigs (20 females and 31 castrates) commercial four-breed crosses known to be free of *RYR1* mutation. Pigs were reared in the experimental station of Faculty of Agriculture and Life Sciences of Maribor University, and slaughtered at 85±14 kg live weight in a commercial abattoir (Košaki d.d., Maribor, Slovenia) in five batches. Slaughter batch stands for a group of pigs that were reared in the same pen and slaughtered in one batch (no mixing with unknown pigs). All batches were submitted to similar preslaughter treatment. Pigs were fasted 24 h prior to slaughter, they were loaded on the truck between 6 and 8 hour in the morning, and transported for 20 minutes to the abattoir where they were slaughtered between 8 and 11 a.m. according to the routine abattoir procedure. However, all other possible stress factors could not be controlled.

Carcass quality measurements

At the end of the slaughter line, pigs were classified according to SEUROP by official classification body, using a method known as ZP, which consists of taking two measurements at carcass split line; DM fat (minimal fat thickness over the *m. gluteus medius*) and DM muscle (shortest distance between cranial end of *m. gluteus medius* and dorsal edge of vertebral canal). Additionally, the measurements of muscle (HGP muscle) and fat thickness (HGP fat) were performed using a HGP4 Hennessy grading probe (Hennessy Grading Systems Ltd., Auckland, New Zealand) with a puncture between the 2nd and the 3rd last rib 7 cm laterally from the carcass split line. The carcass lean meat percentage (DM meat %, HGP meat %) was calculated according to the formulas approved for Slovenia (31). A day following slaughter, additional carcass traits were assessed. The hind leg (without shank) was cut off the carcass between 6th and 7th lumbar vertebra. It was weighed prior and after the removal of subcutaneous fat and the ratio between them was calculated. A digital image of carcass cross section (last rib) was taken with digital photo camera (Canon PowerShot G3, Canon Inc., Tokyo, Japan). Loin eye area (LD area), corresponding fat area (fat over LD), their ratio (LD meat:fat ratio) and belly leanness were determined on images with LUCIA.NET 1.16.5 software (Laboratory Imaging s.r.o, Prague, Czech Republic).

Meat quality measurements

Values of pH were taken with MP120 Mettler Toledo pH meter (Mettler-Toledo, GmbH; 8603 Schwarzenbach, Switzerland) fitted with a combined glass electrode (InLab427) one hour (pH_1) and 24 h post-mortem (pH_U). Duplicate measurements were taken in *longissimus dorsi* (LD) muscle at the level of last rib and in *semimembranosus* (SM) muscle app. 4 cm laterally to the *os pubis*. The measurements of colour (Minolta L*a*b*) were taken a day after the slaughter on a freshly cut surface of LD (level of last rib) using Minolta Chroma Meter CR-300 (Minolta

Co. Ltd, Osaka, Japan) with an 11 mm diameter aperture, D_{65} illuminant, calibrated against a white tile. Colour of LD was also assessed using 1-6 colour scale (32). A 2.5 cm thick slice of LD was taken from the loin at the level of the last rib for drip loss (EZ drip loss) determination according to Christensen (33). Drip loss was determined after 24 and 48 hours storage at 4°C and expressed as a percentage of the initial sample weight.

Blood sampling

At slaughter, blood samples (app. 4 ml) were taken into plastic tubes containing EDTA (against blood coagulation). After blood collection the tubes were immediately placed on ice. Within one hour blood samples were taken to the laboratory where blood was centrifuged at 1800 rpm for 15 minutes. Supernatants (plasma) were collected and stored at –20°C until further analyses.

Determination of plasma stress markers

The levels of plasma stress markers were determined using commercial kits based on the enzyme immunoassays for the in-vitro diagnostic quantitative determination of cortisol (34), neopterin (35) and Hsp70 (36) in plasma samples according to manufacturer instructions. The intensity of the colour was read at 450 nm using spectrophotometer Varioscan Flash and SkanIt Software Version 2.4.3. RE (Thermo Fisher Scientific Inc. Waltham, MA, USA). Plasma concentrations were expressed in μgdl^{-1}, $nmoll^{-1}$ and $ngml^{-1}$ for cortisol, neopterin and Hsp70, respectively.

Statistical analysis

Statistical analysis was performed using a statistical package SAS (37). Basic statistic parameters for all studied traits were calculated using the MEANS procedure. Relationships between plasma stress markers and carcass or meat quality traits were calculated using the CORR procedure on raw experimental data (phenotypic correlations) and after the adjustment for the effects in the model (residual correlations). To calculate residual correlation coefficients, a GLM procedure was used (model with the effect of sex, slaughter batch and their interaction and additionally carcass weight as covariate in case of carcass traits). Significant differences ($P<0.05$) in least square means (LS means) between slaughter batches were evaluated using the PDIFF option, adjust=*Tukey*.

Results

Carcass traits, plasma stress markers and meat quality traits

The effect of slaughter batch on carcass traits has no practical (biological) significance in the context of the present study (Table 1). In any case, well known effect of animal sex on carcass traits was confirmed, as well as a significant relationship between carcass weight and the majority of carcass traits. However, we do not present or discuss this data since they were not the objective of the present study. On the other hand, analysing slaughter batch effect for plasma level of stress markers as related to meat quality traits (Table 1) was the main point of the present study. Despite notable differences in the level of plasma stress markers at slaughter among slaughter batches (in particular HSP70 and neopterin) none of them was significant (Figure 1), which could be due to high within batch variability and low number of pigs per batch. A significant effect of slaughter batch was found for some of meat quality traits (LD ultimate pH, LD colour and Minolta L*), however with no consequences for main technological meat quality - drip loss (batch effect insignificant). Here again, high within batch variability and low number of pigs could be the reason for non significant differences between slaughter batches.

Relationship between plasma stress markers and carcass or meat quality traits

Phenotypic and residual (accounted for the effects in the model) correlation coefficients between the level of plasma stress markers and carcass quality traits (Table 2) or meat quality traits (Table 3) are presented. There were no significant phenotypic or residual correlations between different plasma stress markers ($r<|0.20|$; data not shown). No significant correlation between the level of neopterin or HSP70 with carcass traits was observed, indicating no relationship between them. On the other hand significantly higher cortisol levels were associated with more subcutaneous fat (HGP fat and DM fat) and consequently lower carcass meatiness (HGP meat %, DM meat %, LD meat:fat ratio, and ham meat %), meaning that fattier pigs exhibit higher cortisol

Table 1: Basic statistics for carcass, meat quality traits and plasma stress markers and results of analysis of variance

n=51	mean	sd	EFFECT				R^2	rmse
			sex	slaughter batch	slaughter batch × sex	[1]carcass weight		
CARCASS QUALITY TRAITS								
HGP fat, mm	13.2	2.2	***	NS	†	0.10***	0.65	1.5
HGP muscle, mm	45.5	8.3	NS	NS	NS	0.61***	0.73	4.8
HGP meat, %	60.4	1.8	***	NS	†	-0.04^{NS}	0.55	1.4
DM fat, mm	14.0	3.9	***	†	NS	0.20***	0.67	2.5
DM muscle, mm	62.1	6.0	*	NS	NS	0.43***	0.72	3.5
DM meat, %	58.1	2.7	***	*	NS	−0.09**	0.59	1.9
LD area, cm^2	37.6	7.8	**	***	NS	0.52***	0.87	3.1
Fat area over LD, cm^2	11.7	2.9	***	NS	NS	0.17***	0.86	1.2
LD meat:fat ratio	3.30	0.63	***	†	NS	-0.003^{NS}	0.69	0.39
Belly leanness, %	53.7	5.1	NS	*	NS	−0.21**	0.40	4.4
Ham (muscle+bone), kg	7.1	1.2	**	**	NS	0.10***	0.96	0.3
Ham, kg	8.5	1.6	NS	NS	NS	0.13***	0.93	0.5
Ham meat, %	83.7	4.9	*	NS	NS	-0.05^{NS}	0.20	4.9
LEVEL OF PLASMA STRESS MARKERS AT SLAUGHTER								
Cortisol, μgdl^{-1}	11.0	4.3	NS	NS	NS	/	0.17	4.4
Neopterin, $nmoll^{-1}$	3.1	1.9	NS	NS	NS	/	0.20	1.8
HSP70, $ngml^{-1}$	5.0	6.1	NS	NS	NS	/	0.22	5.9
MEAT QUALITY TRAITS								
SM pH_1	6.39	0.33	NS	NS	NS	/	0.21	0.33
SM pH_U	5.71	0.16	NS	†	NS	/	0.28	0.15
LD pH_1	6.19	0.46	NS	†	NS	/	0.22	0.44
LD pH_U	5.59	0.10	NS	***	NS	/	0.62	0.07
LD Minolta L*	53.8	2.9	NS	*	NS	/	0.31	2.6
LD Minolta a*	6.4	1.2	NS	NS	NS	/	0.22	1.1
LD Minolta b*	2.8	0.9	NS	NS	NS	/	0.23	0.8
LD colour (1-6)	3.6	0.5	NS	***	NS	/	0.49	0.4
LD drip loss 24h, %	5.0	2.3	NS	NS	NS	/	0.16	2.3
LD drip loss 48h, %	7.4	2.4	NS	NS	NS	/	0.22	2.3

LD – muscle *longissimus dorsi*; SM – muscle *semimembranosus*; pH_1 – pH measured one hour after slaughter; pH_U – pH measured 24 hours after slaughter; [1] coefficient of regression; *** P<0.001; ** P<0.01; * P<0.05; † P<0.10; ns - P>0.10.

levels. After the adjustment for the influence of the experimental factors, the obtained residual correlations were mainly similar. Additionally, significant correlation of cortisol level with fat over LD muscle (r=0.41), cortisol level with ham (muscle+bone) (r=−0.31) and neopterin level with LD muscle thickness (r=−0.33) was detected. Hsp70 showed no significant residual correlation with any of carcass quality traits. Cortisol level was also significantly correlated to all meat quality traits except Minolta a*. Higher cortisol levels were associated with higher pH_1 (r=0.36 and 0.58 in LD and SM muscle, respectively) and higher pH_U (r=0.36 and 0.32 in LD and SM muscle, respectively). Consequently higher cortisol levels were related to lower *LD* Minolta L* (r=−0.44), b* (r=−0.38) and lower drip loss (r=−0.44

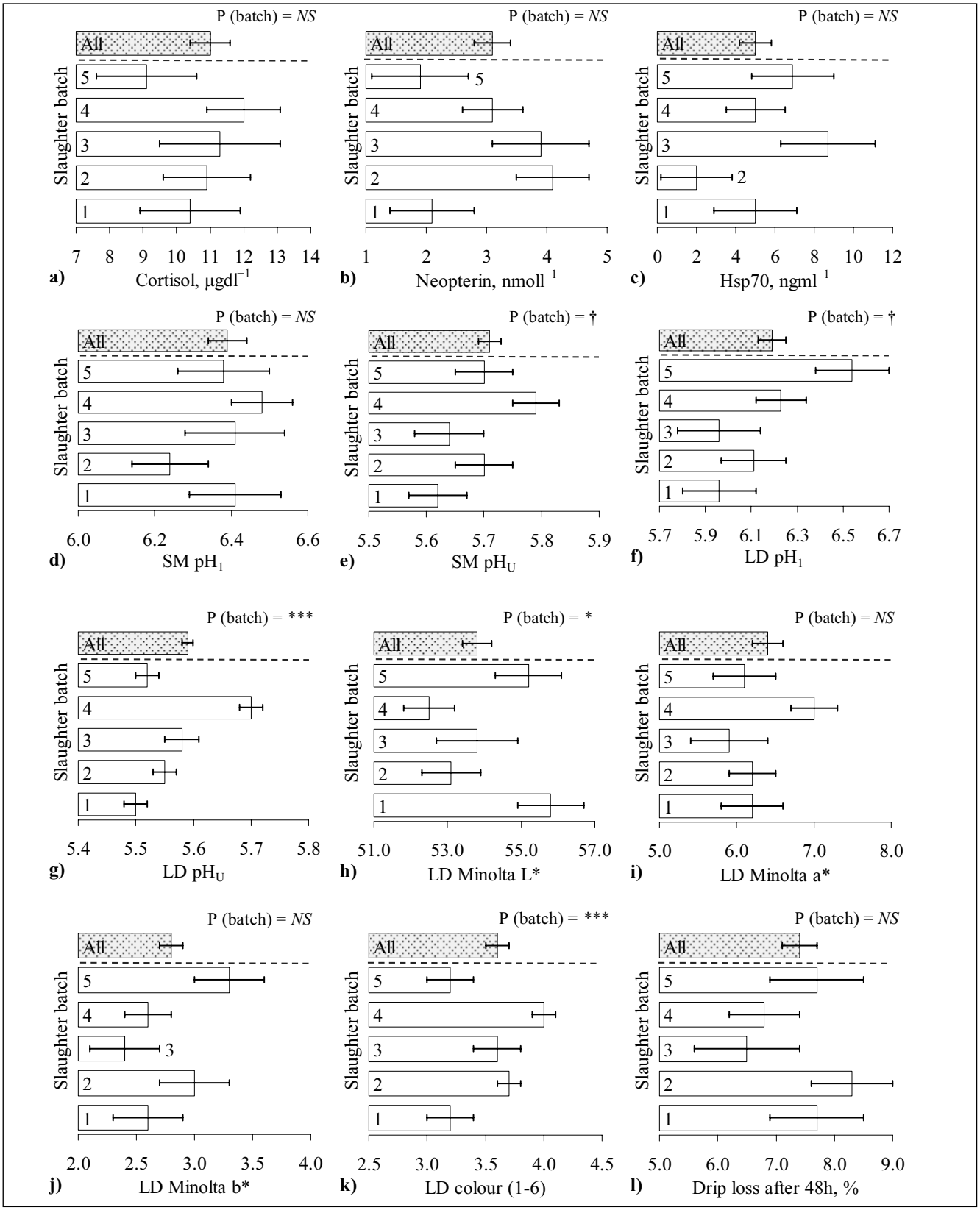

Figure 1: Effect of slaughter batch (LS means ± standard errors) on plasma stress markers and meat quality traits

LD – muscle *longissimus dorsi*; SM – muscle *semimembranosus*; pH$_1$ – pH measured one hour after slaughter; pH$_U$ – pH measured 24 hours after slaughter; *** - P<0.001; * - P<0.05; † - P<0.10; NS - P>0.10.

Table 2: Phenotypic and residual correlation coefficients between level of plasma cortisol and carcass quality traits[1]

N=51	Phenotypic correlations	Residual correlations
HGP fat, mm	**0.30**	**0.38**
HGP muscle, mm	–0.10	0.02
HGP meat, %	**–0.37**	**–0.36**
DM fat, mm	**0.33**	**0.53**
DM muscle, mm	–0.21	–0.15
DM meat, %	**–0.41**	**–0.54**
LD area, cm^2	–0.17	–0.15
Fat over LD, cm^2	0.14	**0.41**
LD meat:fat ratio	**–0.31**	**–0.32**
Belly leanness, %	–0.16	–0.16
Ham (muscle +bone), kg	–0.21	**–0.31**
Ham, kg	–0.09	0.07
Ham meat, %	**–0.32**	–0.26

LD – muscle *longissimus dorsi*;
DM – measurements of fat (minimal fat thickness over the *m. gluteus medius*) and muscle (the shortest distance between cranial end of *m. gluteus medius* and dorsal edge of vertebral canal) taken within 45 minutes *p.m.*;
HGP – measurements of fat and muscle taken within 45 minutes *p.m.* with HGP4 probe with puncture between 2nd and 3rd last rib;
[1]Correlation coefficients between carcass quality traits and neopterin or Hsp70 were statistically insignificant (p>0.05). Values in bold are statistically significant (p<0.05)

Table 3: Phenotypic and residual correlation coefficients among meat quality traits and level of plasma cortisol[1]

n=51	Phenotypic correlations	Residual correlations
SM pH_1	**0.58**	**0.53**
SM pH_U	**0.32**	**0.32**
LD pH_1	**0.36**	**0.49**
LD pH_U	**0.36**	**0.35**
LD Minolta L*	**–0.44**	**–0.39**
LD Minolta a*	–0.27	**–0.30**
LD Minolta b*	**–0.38**	**–0.30**
LD colour	**0.33**	0.17
LD drip loss 24	**–0.44**	**–0.39**
LD drip loss 48	**–0.39**	**–0.32**

LD – muscle *longissimus dorsi*; SM – muscle *semimembranosus*; pH_1 – pH measured one hour after slaughter; pH_U – pH measured 24 hours after slaughter;
[1]Correlation coefficients between meat quality traits and neopterin or Hsp70 were statistically insignificant (p>0.05). Values in bold are statistically significant (p<0.05)

and –0.39 for drip loss after 24 and 48 hours, respectively). After the adjustment for the influence of the experimental factors, the obtained residual correlations between plasma stress markers and meat quality traits remained similar, only correlation of cortisol level with Minolta a* became significant. No significant phenotypic or residual correlations were found for neopterin and Hsp70 with any of meat quality traits.

Discussion

Reported values for concentration of plasma stress markers in blood samples vary widely (Table 1, Figure 1). Despite notable differences in the average level of plasma stress markers (Figure 1) a lack of significance in the present study to our opinion is mainly due to high within batch variation and small number of pigs per batch. Stress endured by farm animals can be physical (hunger, thirst, fatigue, illness, injuries, exercise, thermal extremes), or psychological (fear related to restraint, handling, novelty), short term or long term, the later having much more complex impact (29). In fact, individual response of the animal to preslaughter stress is a result of a complex interaction with genetics and previous experience (29) and can explain why under similar stress conditions animals react differently. Regarding cortisol level, there are numerous experiments in pigs which demonstrate increased cortisol level due to preslaughter stress (38), slaughter day (19), exercise (39). An advantage of measuring cortisol is that the act of stunning and ticking has negligible effect on the level of plasma cortisol concentration (40), contrary to catecholamines. However, the interpretation of plasma cortisol levels remains difficult and has been a subject of debate (41). When considering plasma cortisol levels different factors (time elapsed between stress and sample taking, diurnal fluctuation, genetics, effect of chronic stress) must be taken into account. The concentration of cortisol increases rapidly (<30 minutes) after the

exposure to stress but then it diminishes or recovers in few hours (6). Another aspect to consider is the chronic stress. It is well known, that animal's social status within group can affect immune status (42), access to feed (43), and increase physiological stress (44). Low social status also increases reaction to acute stress (45). Circadian variation in cortisol level and genetic factors can be considered less influential in our study. Namely, blood samples were always collected between 9-10 a.m., and animals were free of stress susceptibility gene (RYR1), which moreover according to the literature, does not seem to increase cortisol level (11, 41). Our results demonstrate positive relationship between plasma cortisol levels with carcass fatness, in agreement with available studies (12, 19, 41) and general metabolic effect of cortisol favouring the accretion of fat at the expense of proteins (46, 47). In humans, many clinical studies suggest that chronic stress causes metabolic disorders like hyperglycemia, hypertension, hyperlipidemia, obesity (48, 49) with prolonged excessive cortisol elevation leading to symptoms resembling Cushing's syndrome. It could be speculated that the correlations observed in our study might have been higher if fat depot had been measured in different location (e.g. abdominal or neck area). Studies dealing with neopterin and HSP70 as related to preslaughter stress in pigs are rare (11, 14, 16). A significant increase of neopterin or cortisol plasma level was shown to be affected by 30 minutes transport prior to slaughter (11). In agreement with our results, no effect of preslaughter stress was also reported (14, 16). It is possible that experienced stress in our as well as in their studies was not intense enough, since elevation of HSP70 with exercise in rats has been shown to be intensity dependent (50). Correlation analysis demonstrated no association between plasma neopterin and Hsp70 levels with any of carcass or meat quality traits. To our knowledge few studies were made on relationship between meat quality in pigs and the level of heat shock proteins (14, 16, 51) and as in the present study, none of them showed any significant relationship.

Published studies show comparable results regarding the association between plasma cortisol and meat quality. From our results it would seem, that cortisol level is indicative of long term rather than acute stress associated with slaughter. Namely, we found positive correlation between plasma cortisol level and pH_1, an indicator of the intensity of post-mortem glycolysis leading to PSE meat, known to be enhanced by stress (24). Moreover, positive correlation of cortisol level with pH_U indicates that muscle glycogen stores were more depleted prior to slaughter in pigs with higher pH_U. Consequently higher plasma cortisol levels were associated with darker meat (higher values of Minolta L* and LD colour) and better water-holding capacity (lower drip). These findings are in accordance with other studies (41) which demonstrated elevated cortisol levels with occurrence of DFD meat, resulting from glycogen depletion due to long term stress (28). However, there are also studies that report no significant relationship between cortisol levels and meat quality (8, 19).

Conclusions

Among the studied stress markers, only cortisol levels were associated with carcass and meat quality traits. Higher cortisol levels were associated with higher carcass fatness, supporting the theory that higher cortisol levels on the long term basis increase body fatness. Higher cortisol level was associated with higher muscle pH (pH_1 and pH_U) and lower drip loss suggesting that cortisol level at slaughter reflects more than just acute preslaughter stress.

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POVEZANOST MED VSEBNOSTJO STRESNIH OZNAČEVALCEV (KORTIZOL, NEOPTERIN, HSP70) V KRVNI PLAZMI OB ZAKOLU IN LASTNOSTMI KAKOVOSTI KLAVNEGA TRUPA OZ. MESA

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Povzetek: Cilj raziskave je bil analiza povezanosti različnih stresnih označevalcev v krvni plazmi ob zakolu (kortizola, neopterina in Hsp70) s klavno kakovostjo in kakovostjo mesa pri prašičih. V raziskavo je bilo vključenih 51 prašičev (isto križanje, odsotnost mutacije RYR1), ki so bili vzrejeni pri istem rejcu in zaklani v isti klavnici v petih serijah zakola. Ob zakolu so bili odvzeti vzorci krvi za analizo stresnih označevalcev, ki smo jih določili s komercialnimi kiti. Dan po zakolu smo izmerili/ocenili lastnosti klavne kakovosti (debelina mišice in slanine, mesnatost trupa, površina mišice *longissimus dorsi* (LD) in maščobe nad mišico LD, mesnatost potrebušine, masa stegna) in lastnosti kakovosti mesa (pH vrednost eno (pH_1) in 24 ur (pH_U) po zakolu, barvni parametri Minolta L*a*b* in izceja vode). Med serijami zakola ni bilo značilnih razlik v vsebnosti stresnih označevalcev in kakovosti mesa, ugotovili pa smo veliko variabilnost znotraj serije zakola. Analiza korelacij je pokazala, da vsebnosti neopterina in Hsp70 v plazmi nista povezani z nobeno lastnostjo kakovosti klavnega trupa oz. mesa. Nasprotno pa smo ugotovili značilne korelacije med vsebnostjo kortizola v plazmi in nekaterimi lastnostmi kakovosti klavnega trupa oz. mesa. Višja vsebnost kortizola je bila povezana z debelejšo hrbtno slanino (r=0,30 do 0,33) in manjšim deležem mesa v trupu (r=–0,37 do –0,41), kar kaže na to, da višje vrednosti kortizola v daljšem obdobju povečujejo zamaščenost. Glede kakovosti mesa smo ugotovili, da so višje vsebnosti kortizola povezane z višjim pH_1 (r=0,36 do 0,58) in pH_U (r=0,32 do 0,36) ter posledično z nižjimi vrednostmi za Minolta L* (r=–0,44), b* (r=–0,38) in izcejenostjo vode po 24ih (r=–0,44) in 48ih urah (r=–0,39), kar kaže na to, da vsebnost kortizola v krvi ob zakolu izraža več kot samo akutni predklavni stres.

Ključne besede: stres; prašič; klavna kakovost, kakovost mesa; kortizol; neopterin; Hsp70