Post-mortem activity of the glycogen debranching enzyme and change in the glycogen pools in porcine *M. longissimus dorsi* from carriers and non-carriers of the RN$^-$ gene

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Abstract

Glycogen debranching enzyme (GDE) is together with glycogen phosphorylase responsible for the degradation of glycogen. The present study compares the *post-mortem* activity of GDE and breakdown of the glycogen pools in *M. longissimus dorsi* of RN^- carrier pigs and in wild type animals. The activity of GDE (n=14) and pH (n=20) was measured 0.5, 3, 5, 24 and 48 h *post-mortem*. The change in pro-glycogen and in macro-glycogen content (n=20) was followed until 216 h *post-mortem* and the transcription level of GDE, glycogenin and glycogen synthase m-RNA (n=19) were measured 0.5 h *post-mortem*. Both the activity of GDE and the transcription level of GDE were found to be similar in RN^- carriers and wild type animals shortly after slaughter. However, the activity declined faster in wild type animals compared with RN^- carriers with increasing time *post-mortem*. The contents of both pro-glycogen and macro-glycogen were higher in RN^- carriers compared with wild type animals, and further, the proportion of macro-glycogen was higher in RN^- carriers compared with wild type animals. During the *post-mortem* period, only degradation of pro-glycogen was observed in both genotypes. The decrease in pH was faster and the ultimate pH lower in RN^- carriers than in wild type animals. It was suggested that the higher GDE activity in the late phase of the *post-mortem* period in muscles from RN^- carriers renders the extended pH decrease in these muscles.

Keywords: Gglycogen debranching enzyme; RN-genotype; Pig; Pro-glycogen; Macro-glycogen

1 Introduction

The increased introduction of the Hampshire breed within pig breeding has caused a widespread frequency of the RN^- gene in many countries, e.g. the frequency approach 70 % in Swedish Hampshire crossbreeds (Enfält, Lundström, Karlsson, & Hansson, 1997b). The RN^- allele in the PRKAG3 gene on chromosome 15 is dominant and associated with an increased glycogen content in porcine glycolytic muscles (Enfält et al., 1997b; Estrade, Vignon, Rock, & Monin, 1993; Fernandez, Tornberg, Naveau, Talmant, & Monin, 1992). This has been shown to result in a faster pH decrease (Josell, von Seth, & Tornberg, 2003a, b; Lindahl et al., 2004) and lower ultimate pH in meat from RN^- carriers compared with the wild type (Enfält et al., 1997b; Josell et al., 2003a; Le Roy et al., 2000; Lindahl et al., 2004).

Although the effects of the RN^- gene on the pH fall in *post-mortem* muscle and subsequent influence on meat quality have been studied extensively (Enfält et al., 1997b; Josell et al., 2003a; Lindahl et al., 2004), the understanding of the underlying mechanisms in the *post-*

mortem glycogen metabolism between carriers and non-carriers of the RN^- gene is almost absent. Considering that the differences in the *post-mortem* metabolism of glycogen between genotypes are highly important for the quality of meat (Bendall & Swatland, 1988; Briskey, 1964; Monin & Ouali, 1991), a thorough understanding of the physiological mechanisms giving rise to the fast rate of *post-mortem* pH decrease and low ultimate pH in meat of RN^- carriers may be crucial in the identification of biological markers of importance for future breeding strategies aiming at production of high quality meat.

The biosynthesis of glycogen is initiated by a self-glucosylating protein, glycogenin (Goldsmith, Sprang, & Fletterick, 1982; Gunja-Smith, Marshall, Mercier, Smith, & Whelan, 1970; Lomako, Lomako, & Whelan, 1988; Meléndez-Hevia, Waddell, & Shelton, 1993). Subsequently, glycogen synthase and glycogen branching enzyme then complete the glycogen molecules. Muscle glycogen exists in two forms: the acid-soluble, high molecular weight (10^7 Da) macro-glycogen rich in glucose molecules, and the acid-insoluble, low molecular weight (400,000 Da) pro-glycogen, which is characterised by a higher protein to glucose ratio (Lomako et al., 1991, 1993). These two glycogen pools seem to be metabolically distinct (Graham, Adamo, Shearer, Marchand, & Saltin, 2001; Shearer, Marchand, Tarnopolsky, Dyck, & Graham, 2001), although the physiological nature of the regulation of muscle glycogen stores is poorly understood (Roach, 2002). It seems that macro-glycogen is mobilised during aerobic exercise, while pro-glycogen is mobilised during anaerobic exercise (Essén-Gustavsson, Jensen-Waern, Jonasson, & Andersson, 2005; Graham et al., 2001; Shearer et al., 2001).

The degradation of glycogen progresses by cooperation of two enzymes: glycogen phosphorylase (phosphorylase) and glycogen debranching enzyme (GDE) (Brown & Illingworth-Brown, 1966). The phosphorylase breaks down linear glucose chains to the so-called limit dextrin state whereupon GDE takes over and breaks down the branching point, which enables the further action of phosphorylase (Brown & Illingworth-Brown, 1966; Nelson, Kolb, & Larner, 1969). It has been suggested that GDE catalyses the rate-limiting step in *post-mortem* glycogenolysis and thus glycolysis (Kylä-Puhju, Ruusunen, & Puolanne, 2005; Taylor, Cox, Kernohan, & Cohen, 1975; Ylä-Ajos, Ruusunen, & Puolanne, 2006).

The aim of this study was to further elucidate basic factors of importance for *post-mortem* glycogen metabolism in relation to the pH fall by comparing the transcription levels of central genes (GDE, glycogenin and glycogen synthase) involved in the glycogen metabolism, and

measuring the activity of GDE and degradation of pro-glycogen and macro-glycogen in *M. longissimus dorsi* from Hampshire crossbred animals with and without presence of the RN^- allele.

2 Materials and Methods

2.1 Animals and sampling

This study was part of a larger project studying the effect of the RN-gene on meat colour stability and glycogen metabolism, described by Lindahl, Enfält, Andersen and Lundström (in preparation). For the present study, twenty crossbred slaughter pigs, Hampshire x (Swedish Landrace x Yorkshire) from four herds were stunned in CO_2 and slaughtered at a commercial slaughterhouse in Sweden. Small muscle samples (*M. longissimus dorsi* and *M. semimembranosus*) were obtained 0.5, 3, 5, 24 and 48 hours after bleeding. After 48 hours the whole muscles were excised from the carcasses, vacuum-packed and stored at 5°C for another 2, 5 or 7 days before sampling. The collected samples were frozen in liquid nitrogen immediately after sampling and stored at -80ºC. The pH was measured (Knick portable pH-meter equipped with a combination gel electrode, SE104, Knick Berlin, Germany) in *M. longissimus dorsi* at the last thoracic vertebra and in the middle of *M. semimembranosus* at 0.5, 3, 5, 24 and 48 hours after bleeding. The pH electrode was calibrated in pH 4.01 and 7.00 buffers (Radiometer, Denmark) at ambient temperature when pH was measured 30 min *post-mortem*, and at 4°C at the other time points.

2.2 Genotyping

The PRKAG3 alleles were identified with a DNA test using the polymerase chain reaction (PCR) method described by Milan et al. (2000). Three alleles were designated as follows: RN^- (199V-200Q), rn^+ (199V-200R) and rn^* (199I-200R). RN^- carriers had the combinations: RN^-/rn^+, RN^-/rn^*, and the wild type animals had: rn^+/rn^+, rn^+/rn^*, rn^*/rn^*.

2.3 The activity of GDE

The *post-mortem* activity of GDE was determined from *M. longissimus dorsi* (n=14) and *M. semimembranosus* (n=6) using the method of Nelson, Palmer & Larner (1970) with minor modifications (Kylä-Puhju et al., 2005). The method is a colorimetric assay based on a shift in the absorbance at 525 nm upon GDE-catalysed conversion of limit dextrin to glycogen. The GDE activity measurements were made at 39°C in triplicate at three time points and the activity was calculated from the slope of the linear phase of the absorbance curve. In addition, the absorption spectra between 375 nm and 800 nm of the blank and the reaction mixtures

were obtained to ensure the conversion of limit dextrin to glycogen (results not shown). The assay measures the combined activity of GDE, i.e. glycan transferase (EC 2.4.1.25) and amylo-1,6-glucosidase (EC 3.2.1.33) activities.

The reproducibility of the assay was determined as follows: a large muscle sample was ground in liquid nitrogen, aliquoted, stored at -80ºC, and used as an internal standard. The coefficient of variation (CV) for the internal standard was ±18.3% for the GDE activity.

2.4 Glycogen content

Pro-glycogen and macro-glycogen were separated as described by Adamo and Graham (1998). In short, the pro-glycogen was precipitated by perchloric acid. After centrifugation pro-glycogen in the pellet and macro-glycogen in the supernatant were hydrolysed for 2 h in 1M HCl. Glucose from the hydrolysed homogenates were analysed according to the procedure of Passonneau & Lowry, 1990. The analysis method does not distinguish the origin of the glucose molecules, thus glycogen, glucose and glucose-6-phosphate all contribute to the total glucose amount in the two fractions. Since pro-glycogen is precipitated in the pellet, the free glucose and glucose-6-phosphate will be included in the macro-glycogen fraction. Thus, in the present study "macro-glycogen" = macro-glycogen + free glucose + glucose-phosphates.

2.5 Gene expressions

The transcription levels of three genes (GDE, glycogen synthase and glycogenin) and two housekeeping genes (the structural protein β-Actin and the glycolytic enzyme glyceraldehyd-3-phosphate dehydrogenase i.e. GAPDH) were quantified in *M. longissimus dorsi* of RN^- carriers (n=9) and wild type animals (n=10). However, the housekeeping gene GAPDH was excluded from the analyses because its transcription differed significantly between genotypes. The samples for gene expression analyses were taken 0.5 h after slaughter and frozen in liquid nitrogen. The RNA was purified from 30 mg of muscle sample using the method described by Chomczynski and Mackey (1995). In short, muscle tissue was homogenised in TriReagent (Molecular Research Center Inc. Cincinnati, Ohio), the phases were separated with 1-bromo-3-chloropropane and finally the RNA was precipitated with isopropanol. The dry pellet, containing RNA was solubilised in H_2O and stored at -80°C. Total RNA was determined by measuring the absorbance at 260 nm. Equal amounts of RNA were reverse transcribed with oligo-dT and Superscript II RNase H reverse transcriptase kit (Invitrogen, Taastrup, Denmark) and ultrapure dNTPSet (Pharmacia Amersham) as described by Theil, Sørensen,

Therkildsen and Oksbjerg (2006). Reverse transcribed material (1 μl) was amplified with TaqMan Universal PCR Master Mix (Applied Biosystems, Stockholm, Sweden) using primers specific for each gene. The signal was detected quantitatively by gene specific probes labelled with FAM™ fluorophore in the 5'end. Primers and probes were designed by using Primer Express (Applied Biosystem, Stockholm, Sweden) version 2.0 software, and either a primer or a probe was designed to anneal to a splice site to avoid amplification of genomic DNA. The primer pairs predicted 78, 74, 80, 113 and 76 bp fragments for GDE, glycogen synthase, glycogenin, β-Actin and GAPDH, respectively. For real time RT-PCR, 40 cycles at 95°C for 15 s and 60°C for 60 s were applied to amplify the PCR products. The number of PCR cycles required to reach the threshold for a gene of interest (Ct value) was used in the statistical analysis. Serial dilutions for obtaining a standard curve were analysed in triplicate, whereas unknown samples were analysed in duplicates using ABI 7900HT Sequence Detection System (Applied Biosystems, Stockholm, Sweden).

The sequences of forward primers, MGB probes and reverse primers were as follows:

GDE: 5'-TGTTCTTTCTCGACATTATGTTCATCT-3', 5'-AGCGATCCCCTTGGAAAGGACTTCCA-3', 5'-TGTCCATTCTCGTTGGTCAGTT-3'

Glycogen synthase: 5'-CCGGCTTCGGCTGCTT-3', 5'-CGCAGACCCCTCGGCTTACGGTATC-3', 5'-CCGCCGGTCCAGAATG-3'

Glycogenin: 5'-ATCAGCTGTTGCACCTTGCTT-3', 5'-TGAGCAAGGTAGTTTTGATGGTGG-3', 5'-GCTGCTAAAAAATGTGTTCAGTAAACC-3'

β-Actin: 5'-ACCCAGATCATGTTCGAGACCTT-3', 5'-CTGTATGCCTCTGGCCGCACCA-3', 5'-TCACCGGAGTCCATCACGAT-3'

GAPDH: 5'-GTCGGAGTGAACGGATTTGG-3', 5'-CGCCTGGTCACCAGGGCTGCT-3', 5'-CAATGTCCACTTTGCCAGAGTTAA-3'

2.6 Statistical analysis

The statistical analysis was carried out using the Statistical Analysis System version 8.02 (SAS Institute Inc., 1999). The MIXED procedure was applied when testing the differences in the activity of GDE, pH and in the pro-glycogen and "macro-glycogen" content between the genotypes. The model included the fixed effects of genotype, sex, time and interaction between genotype and time and a random animal effect to account for repeated measurements performed within the same animal. The correlation between pro-glycogen content and pH was tested by using the GLM procedure with the factors sex, slaughter date and herd. Also the differences in transcription levels between RN-genotypes were tested by using the GLM

procedure. The model included the following factors: RN-genotype, sex, slaughter date and interaction between RN-genotype and sex. The normalisation of transcriptional data was obtained with the transcription of the housekeeping gene β-actin by calculating ΔCt values (ΔCt = Ct of the target gene - Ct of the housekeeping gene). The calculations were made as described by Theil et al. (2006). The difference between the genotypes was considered to be significant when $P<0.05$.

3 Results

The total muscle glucose content 0.5 h *post-mortem* was 77% higher in RN^- carriers compared with wild type animals, 101.9 and 57.6 mmol/kg w.w., respectively (Table 1). Pro-glycogen corresponded to 57% of the total muscle glucose content in RN^- carriers and to 66% in wild type animals. Independent of genotype, only pro-glycogen was broken down *post-mortem*, while no degradation of "macro-glycogen" was registered.

The degradation of muscle pro-glycogen continued until 96 h *post-mortem* in RN^- carriers, while it was already abated 48 h *post-mortem* in muscles from wild type animals. Interestingly, an increase in "macro-glycogen" content simultaneously with the decrease in pro-glycogen content was observed in muscles from RN^- carriers in the period 48 h to 96 h *post-mortem*. The "macro-glycogen" fraction, however, includes also glucose-6-phosphate and free glucose and the observed increase in this fraction could be due to an increase in content of these compounds.

In *M. longissimus dorsi*, the activity of GDE decreased slowly as a function of time *post-mortem*, and the decrease was statistically significant in all animals until after 5 hours *post-mortem*. The activity of GDE was similar in both RN-genotypes up to 5 hours after slaughter (Figure 1). However, 24 h *post-mortem* the activity of GDE was significantly higher in RN^- carriers compared with wild type animals, and this difference was maintained up to 48 h *post-mortem*.

In *M. semimembranosus*, no significant difference in the *post-mortem* activity of GDE between the two genotypes was found (Figure 2). There was a tendency to a decrease in activity of GDE with increasing time *post-mortem*, but it became significant only in the muscles from wild type animals after 48 h.

234 The transcription levels of GDE and glycogen synthase were similar in both genotypes, while
235 a greater transcription of glycogenin was found in RN^- carriers. Thus, the glycogenin m-RNA
236 was more abundant in RN^- carriers compared with wild type animals (Table 2).
237
238 The pH 0.5 h *post-mortem* was not significantly different between the two genotypes
239 (Table 3). However, the rate of pH decrease was faster, and the ultimate pH was lower in the
240 *M. longissimus dorsi* from RN^- carriers compared with wild type animals. The correlation
241 between the degradation of pro-glycogen and pH decrease was high in RN^- carriers (r=0.89)
242 and in wild type animals (r=0.93). The pro-glycogen content 0.5 h *post-mortem* explained 44
243 and 48% of the variation in ultimate pH in RN^- carriers and in wild type animals, respectively.
244 No significant differences in pH in *M. semimembranosus* were found between the two
245 genotypes.
246
247 **4 Discussion**
248 The present study showed in accordance with a vast number of earlier studies that RN^-
249 carriers possess a higher glycolytic potential, a faster initial pH decrease and a lower ultimate
250 pH than wild type animals. This results in a larger drip loss and cooking loss, a lower Napole
251 Yield, but improved tenderness in most cases (Enfält 1997a, b; Josell et al. 2003b; Lindahl et
252 al., 2004).
253
254 Glycogen exists in two forms in skeletal muscle: acid-soluble, high molecular weight macro-
255 glycogen and acid-insoluble, low molecular weight pro-glycogen (Lomako et al., 1991, 1993).
256 In the present study, the high glycolytic potential in RN^- carriers was found to result from
257 accumulation of both glycogen types, pro-glycogen and "macro-glycogen", in the muscle. In
258 contrast, Essén-Gustavsson et al. (2005) reported that high glycogen content in RN^- carriers is
259 exclusively due to increased macro-glycogen content. The pigs were, however, younger and
260 lighter than in the present study. In RN^- carriers the proportion of "macro-glycogen" was
261 higher than in the wild type animals, which is consistent with several studies showing that the
262 macro-glycogen fraction increases with increasing muscle glycogen content (Adamo &
263 Graham, 1998; Adamo, Tarnopolsky, & Graham, 1998; Asp, Daugaard, Rohde, Adamo, &
264 Graham, 1999; Derave, Gao, & Richter, 2000; Shearer, Marchand, Sathasivam, Tarnopolsky,
265 & Graham, 2000). The pro-glycogen to "macro-glycogen" ratio in wild type animals was
266 similar as reported for pigs by Rosenvold, Essén-Gustavsson and Andersen (2003).
267

Only pro-glycogen was used for muscle energy production *post-mortem* (from 0.5 to 48 h), which is consistent with the results of Rosenvold et al. (2003) who observed only pro-glycogen degradation during the first 45 minutes *post-mortem*. Essén-Gustavsson et al. (2005) showed recently that the breakdown of macro-glycogen takes place during aerobic, low intensity exercise in both RN-genotypes. In the present study, the analytical method for measuring "macro-glycogen" fraction included also glucose-6-phosphate and free glucose. Thus, it could be possible that also some of the macro-glycogen has broken down *post-mortem* although the analytical method does not detect that. The "macro-glycogen" content in *M. longissimus dorsi* in RN^- carriers increased in the period from 48 h to 96 h *post-mortem*. The increase might be explained by accumulation of glucose-6-phosphate and free glucose as a function of pro-glycogen degradation. This is supported by the results from Monin and Sellier (1985), who found that the glucose-6-phosphate content, a day after slaughter, was significantly higher in the Hampshire breed pigs (11 mmol/kg), which were very likely RN^- carriers, compared with pigs from Large White (6 mmol/kg) and Pietrain (6 mmolg/kg) breeds. In pig muscle an hour after slaughter, the content of free glucose is 3-6 mmol/kg (Monin and Sellier, 1985). Furthermore, the action of GDE results in liberation of free glucose (Brown & Illingworth-Brown, 1966). GDE, and probably also phosphorylase, is still active 48 h *post-mortem* and hereby enables the accumulation of glucose-6-phosphate and free glucose. Thus, this suggests that in RN^- carriers muscle glycolysis is ceased due to inhibition of phosphofructokinase, which is generally considered a rate-limiting enzyme in glycolysis (Stryer, 1988). The rate and quantity of muscle H^+ production are consequences of ATP hydrolysis coupled with glycolysis (Bendall, 1973; Hamm, 1977; Robergs, Ghiasvand & Parker, 2004). Lactate production coincides with pH decrease, but against general belief this is not a causal relationship, since lactate production retards, not causes, muscle acidosis (Bendall, 1973; Robergs et al., 2004). The proposed hypothesis on inhibition of phosphofructokinase in RN^- carriers agrees with studies reporting similar ultimate lactate contents in RN^- carriers and in wild type animals despite differences in ultimate pH (Enfält, Lundström, Hansson, Johansen & Nyström, 1997a; Lundström, Enfält, Tornberg & Agerhem, 1998). In wild type animals no change in the "macro-glycogen" fraction was observed.

The decrease in pH was faster, and the ultimate pH was lower in *M. longissimus dorsi* of RN^- carriers than in wild type animals, which agrees with earlier findings (Enfält et al., 1997b; Josell et al., 2003a; Lindahl et al., 2004). The extended pH decrease in RN^- carriers is possibly a result of longer maintenance of the high activity of GDE in these animals. The activity of GDE was similar for RN^- carriers and wild type animals during the first five hours *post-*

303 *mortem*, which is in agreement with Estrade, Ayoub, Talmant & Monin (1994) who reported
304 an uniformity in the activity of GDE and of glycogen phosphorylase between the
305 RN-genotypes five minutes after slaughter. In both genotypes, the activity of GDE remained
306 at the level found shortly after slaughter for several hours, but was reduced significantly after
307 24 h *post-mortem*. However, the decrease was more pronounced in wild type animals. It is
308 possible that high muscle glycogen content protects the GDE from denaturation, since binding
309 on glycogen limit dextrin or to lesser extent on glycogen serves as protection from denaturing
310 agents (Gillard, White, Zingaro, & Nelson, 1980; Scraba, Bradley, Fitzgerald, & Madsen,
311 1988). Then again, the RN^- mutation is located in the gene coding for adenosine
312 monophosphate-activated protein kinase (AMPK), which is a key metabolic enzyme (Milan et
313 al., 2000). Activated AMPK inhibits the ATP-consuming pathways, stimulates ATP-
314 generating pathways (Hardie & Carling, 1997) and as reported recently by Shen and Du
315 (2005) is important for maintaining the activity of glycogen phosphorylase in *post-mortem*
316 muscle. Thus the mutation in the AMPK gene might explain why RN^- carriers exhibit a
317 prolonged high GDE activity, as found in the present study. The pH itself has only a minor
318 effect on the activity of GDE when the pH ranges between 5.5 and 7 (Kylä-Puhju et al.,
319 2005).
320
321 The differences between the studied muscles offer indirect support to the importance of the
322 activity of GDE in the development of ultimate pH between RN-genotypes. The change in the
323 activity of GDE was followed from 0.5 h to 48 h *post-mortem*. The activity was stable,
324 particularly in *M. semimembranosus* of RN^- carriers, where no significant decrease in the
325 activity of GDE with increasing time was found. Furthermore, in *M. semimembranosus* (n=6),
326 unlike in *M. longissimus dorsi*, the activity of GDE, the rate of pH decrease or ultimate pH
327 did not differ between the RN-genotypes. It seems that the activity of GDE might be related to
328 the formation of ultimate pH, and it remains to be investigated if a difference in the activity of
329 GDE late *post-mortem* between the RN-genotypes would lead to differences in ultimate pH
330 also in *M. semimembranosus*. Lindahl et al. (in preparation) found, with a larger number of
331 pigs from the same project, a faster pH decline and lower pH 3 and 5 h *post-mortem* in the *M.*
332 *semimembranosus* of the RN^- carriers compared with wild type animals, however, no
333 difference in ultimate pH. Although *M. longissimus dorsi* and *M. semimembranosus* are very
334 similar to each other in glycolytic potential, the activity of glycogenolytic enzymes (Fischer &
335 Dobrowolski, 2002; Ylä-Ajos et al., 2006) and fibre type composition (Ruusunen & Puolanne,
336 2004), the pH decrease is slower in *M. semimembranosus* (Henckel, Karlsson, Oksbjerg, &

337 Petersen, 2000). However, combining the data obtained from the present study concerning the
338 studied muscles, indicate that the activity of GDE affects the pH decline *post-mortem*.
339
340 It seems that immediately post slaughter, the glycogenolytic enzymes are not more active in
341 RN^- carriers than in wild type animals despite the higher glycogen content in RN^- carriers.
342 Besides the activity of GDE, also the transcription level of GDE was similar between the
343 genotypes. Thus, the differences in the activity of GDE between the genotypes do not explain
344 the faster pH decrease early *post-mortem* in RN^- carriers compared with wild type animals.
345 Hedegaard et al. (2004) found indications of an up-regulation of phosphofructokinase enzyme
346 in RN^- carriers, which is the rate-limiting enzyme in glycolysis, and this would explain the
347 fast glycolysis during early *post-mortem* in RN^- carriers.
348
349 The mechanisms behind the high glycogen content in the glycolytic muscles of RN^- carriers
350 are not well described. Estrade et al. (1993) reported that the localization and ultrastructural
351 pattern of glycogen was similar in both genotypes, but the density of the glycogen particles
352 was higher in muscle fibres from RN^- carriers, which may indicate higher glycogenin activity.
353 The present study showed a slightly higher expression of glycogenin in RN^- carriers compared
354 with wild type animals. Glycogenin is an autocatalytic protein serving as substrate for
355 glycogen synthase, and theoretically, the number of glycogenin molecules available within
356 skeletal muscle would dictate the number of glycogen particles and hence the amount of
357 glycogen stored. Therefore the production of active glycogenin primer in the muscle fibre
358 could be the overall rate-limiting process in glycogen formation, capable of overriding the
359 role of the activity of glycogen synthase (Alonso, Lomako, Lomako, & Whelan, 1995).
360 Furthermore, Shearer et al. (2000) found a positive correlation in human muscle between
361 glycogenin activity and total glycogen content, pro-glycogen content as well as macro-
362 glycogen content.
363
364 Alternatively, the hyperaccumulation of glycogen in RN^- carriers could originate from the
365 higher expression of UDP-glucose pyrophosphorylase protein and high activity of the enzyme
366 reported by Hedegaard et al. (2004). Furthermore, Hedegaard et al. (2004) suggested that the
367 synthesis of glycogen in the muscles of RN^- carriers was increased due to increased influx of
368 glucose into the muscle fibres. Estrade et al. (1994) reported a tendency of higher glycogen
369 synthase activity in RN^- carriers compared with wild type animals. In the present study, no
370 difference in the transcription of the glycogen synthase between genotypes was found,
371 however, the transcription of a gene does not necessarily correspond to the activity of the

enzyme for which it is coding. In the present study, the activities of glycogenin and glycogen synthase were not analysed.

5 Conclusions

In *M. longissimus dorsi*, the decrease in the activity of GDE with increasing time *post-mortem* is slower and less pronounced in RN^- carriers than in wild type animals. At the same time the decrease in pH is faster and the ultimate pH lower in RN^- carriers than in wild type animals. The long period of high GDE activity in RN^- carriers may enable the extended pH decrease. The transcription of the glycogenin gene is slightly higher, and both pro-glycogen and macro-glycogen are more abundant in RN^- carriers than in wild type animals, however, only the pro-glycogen is degraded *post-mortem* irrespective of genotype.

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References

Adamo, K. B., & Graham, T. E. (1998). Comparison of traditional measurements with macroglycogen and proglycogen analysis of muscle glycogen. *Journal of applied physiology: respiratory, environmental and exercise physiology, 84*(3), 908-913.

Adamo, K. B., Tarnopolsky, M. A., & Graham, T. E. (1998). Dietary carbohydrate and postexercise synthesis of proglycogen and macroglycogen in human skeletal muscle. *The American Journal of Physiology, 275*(2 Pt 1), E229-34.

Alonso, M. D., Lomako, J., Lomako, W. M., & Whelan, W. J. (1995). A new look at the biogenesis of glycogen. *The FASEB journal, 9*(12), 1126-1137 (review).

Asp, S., Daugaard, J. R., Rohde, T., Adamo, K., & Graham, T. (1999). Muscle glycogen accumulation after a marathon: roles of fiber type and pro- and macroglycogen. *Journal of Applied Physiology, 86*(2), 474-478.

Bendall, J. R. (1973). Postmortem changes in muscle. In H. G. Bourne, *The structure and function of muscle* (Vol II) (pp. 243-309). New York: Academic Press.

409 Bendall, J. R., & Swatland, H. J. (1988). A review of the relationships of pH with physical
410 aspects of pork quality. *Meat Science, 24*(2), 85-126.

411 Briskey, E. J. (1964). Etiological status and associated studies of pale, soft, exudative porcine
412 musculature. *Advances in food research, 13*, 89-178.

413 Brown, D. H., & Illingworth-Brown, B. I. (1966). Enzymes of glycogen debranching: Amylo-
414 1,6-glucosidase (I) and oligo-1,4->1,4-glucantransferase (II). In S. P. Colowick, & N.O.
415 Kaplan, *Methods in Enzymology* (Vol 8) (pp. 515-524). New York: Academic Press.

416 Chomczynski, P., & Mackey, K. (1995). Substitution of chloroform by bromo-chloropropane
417 in the single-step method of RNA isolation. *Analytical Biochemistry, 225*(1), 163-164.

418 Derave, W., Gao, S., & Richter, E. A. (2000). Pro- and macroglycogenolysis in contracting rat
419 skeletal muscle. *Acta Physiologica Scandinavica, 169*(4), 291-296.

420 Enfält, A., Lundström, K., Hansson, I., Johansen, S., & Nyström, P. (1997a). Comparison of
421 non-carriers and heterozygous carriers of the RN^- allele for carcass composition, muscle
422 distribution and technological meat quality in Hampshire-sired pigs. *Livestock*
423 *Production Science, 47*(3), 221-229.

424 Enfält, A. C., Lundström, K., Karlsson, A., & Hansson, I. (1997b). Estimated frequency of the
425 RN allele in Swedish Hampshire pigs and comparison of glycolytic potential, carcass
426 composition, and technological meat quality among Swedish Hampshire, Landrace, and
427 Yorkshire pigs. *Journal of animal science, 75*(11), 2924-2935.

428 Essén-Gustavsson, B., Jensen-Waern, M., Jonasson, R., & Andersson, L. (2005). Effect of
429 exercise on proglycogen and macroglycogen content in skeletal muscles of pigs with the
430 Rendement Napole mutation. *American Journal of Veterinary Research, 66*(7), 1197-
431 1201.

432 Estrade, M., Vignon, X., Rock, E., & Monin, G. (1993). Glycogen hyperaccumulation in
433 white muscle fibres of RN- carrier pigs. A biochemical and ultrastructural study.
434 *Comparative biochemistry and physiology. B: Comparative biochemistry, 104*(2), 321-
435 326.

436 Estrade, M., Ayoub, S., Talmant, A., & Monin, G. (1994). Enzyme activities of glycogen
437 metabolism and mitochondrial characteristics in muscles of RN- carrier pigs (Sus scrofa
438 domesticus). *Comparative biochemistry and physiology. Biochemistry and molecular*
439 *biology, 108*(3), 295-301.

440 Fernandez, X., Tornberg, E., Naveau, J., Talmant, A., & Monin, G. (1992). Bimodal
441 distribution of the muscle glycolytic potential in french and swedish populations of
442 hampshire crossbred pigs. *Journal of Science in Food and Agriculture, 59*, 307-311.

443 Fischer, K., & Dobrowolski, A. (2002). Topographic variation of the glycolytic potential in
444 the muscles of slaughter pigs. *Fleischwirtschaft, 82*(2), 78-82. (In German).

445 Gillard, B. K., White, R. C., Zingaro, R. A., & Nelson, T. E. (1980). Amylo-1,6-
446 glucosidase/4-alpha-glucanotransferase. Reaction of rabbit muscle debranching enzyme
447 with an active site-directed irreversible inhibitor, 1-S-dimethylarsino-1-thio-beta-D-
448 glucopyranoside. *Journal of Biological Chemistry, 255*(18), 8451-8457.

449 Goldsmith, E., Sprang, S., & Fletterick, R. (1982). Structure of maltoheptaose by difference
450 Fourier methods and a model for glycogen. *Journal of Molecular Biology, 156*(2), 411-
451 427.

452 Graham, T. E., Adamo, K. B., Shearer, J., Marchand, I., & Saltin, B. (2001). Pro- and
453 macroglycogenolysis: relationship with exercise intensity and duration. *Journal of*
454 *applied physiology: respiratory, environmental and exercise physiology, 90*(3), 873-879.

455 Gunja-Smith, Z., Marshall, J. J., Mercier, C., Smith, E. E., & Whelan, W. J. (1970). A
456 revision of the Meyer-Bernfeld model of glycogen and amylopectin. *FEBS letters, 12*(2),
457 101-104.

458 Hamm, R. (1977). Postmortem breakdown of ATP and glycogen in ground muscle: A review.
459 *Meat Science, 1*(1), 15-39.

460 Hardie, D. G., & Carling, D. (1997). The AMP-activated protein kinase--fuel gauge of the
461 mammalian cell? *European journal of biochemistry / FEBS, 246*(2), 259-273.

462 Hedegaard, J. et al. (2004). UDP-glucose pyrophosphorylase is upregulated in carriers of the
463 porcine RN- mutation in the AMP-activated protein kinase. *Proteomics, 4*(8), 2448-2454.

464 Henckel, P., Karlsson, A., Oksbjerg, N., & Petersen, J. S. (2000). Control of post mortem pH
465 decrease in pig muscles: experimental design and testing of animal models. *Meat Science,*
466 *55*(1), 131-138.

467 Josell, A., Martinsson, L., & Tornberg, E. (2003a). Possible mechanism for the effect of the
468 RN^- allele on pork tenderness. *Meat Science, 64*(4), 341-350.

469 Josell, A., von Seth, G., & Tornberg, E. (2003b). Sensory and meat quality traits of pork in
470 relation to post-slaughter treatment and RN genotype. *Meat Science, 66*(1), 113-124.

471 Kylä-Puhju, M., Ruusunen, M., & Puolanne, E. (2005). Activity of porcine muscle glycogen
472 debranching enzyme in relation to pH and temperature. *Meat Science, 69*(1), 143-149.

473 Le Roy, P. et al. (2000). Comparison between the three porcine RN genotypes for growth,
474 carcass composition and meat quality traits. *Genetics, selection, evolution. : GSE, 32*(2),
475 165-186.

476 Lindahl, G. et al. (2004). A second mutant allele (V199I) at the PRKAG3 (RN) locus. I.
477 Effect on technological meat quality of pork loin. *Meat Science, 66*(3), 609-619.

478 Lomako, J., Lomako, W. M., & Whelan, W. J. (1988). A self-glucosylating protein is the
479 primer for rabbit muscle glycogen biosynthesis. *The FASEB journal, 2*(15), 3097-3103.

480 Lomako, J., Lomako, W. M., & Whelan, W. J. (1991). Proglycogen: a low-molecular-weight
481 form of muscle glycogen. *FEBS letters, 279*(2), 223-228.

482 Lomako, J., Lomako, W. M., Whelan, W. J., Dombro, R. S., Neary, J. T., & Norenberg, M. D.
483 (1993). Glycogen synthesis in the astrocyte: from glycogenin to proglycogen to glycogen.
484 *The FASEB journal, 7*(14), 1386-1393.

485 Lundström, K., Enfält, A. C., Tornberg, E., & Agerhem, H. (1998). Sensory and technological
486 meat quality in carriers and non-carriers of the RN- allele in Hampshire crosses and in
487 purebred Yorkshire pigs. *Meat Science, 48*(1/2), 115-124.

Meléndez-Hevia, E., Waddell, T. G., & Shelton, E. D. (1993). Optimization of molecular design in the evolution of metabolism: the glycogen molecule. *The Biochemical journal, 295*(2), 477-483.

Milan, D. et al. (2000). A mutation in PRKAG3 associated with excess glycogen content in pig skeletal muscle. *Science, 288*, 1248-1251.

Monin, G., & Sellier, P. (1985). Pork of low technological quality with a normal rate of muscle pH fall in the immediate post-mortem period: The case of the Hampshire breed. *Meat Science, 13*, 49-63.

Monin, G., & Ouali, A. (1991). Muscle differentiation and meat quality. In R. A. Lawrie, *Developments in Meat Science-5*(pp. 89-157). Essex, England: Elsevier Science Publishers LTD.

Nelson, T. E., Kolb, E., & Larner, J. (1969). Purification and properties of rabbit muscle amylo-1,6-glucosidase-oligo-1,4->1,4-transferase. *Biochemistry, 8*(4), 1419-1428.

Nelson, T. E., Palmer, D. H., & Larner, J. (1970). An investigation of the properties of rabbit muscle oligo-1,4->1,4-glucantransferase. *Biochimica et Biophysica Acta, 212*(2), 269-280.

Passonneau, J. V., & Lowry, O. H. (1993). *Enzymatic Analysis: a Practical Guide.* New Jersey, USA, The Humana Press.

Roach, P. J. (2002). Glycogen and its metabolism. *Current Molecular Medicine, 2*(2), 101-120.

Robergs, R. A., Ghiasvand, F., & Parker, D. (2004). Biochemistry of exercise-induced metabolic acidosis. *American Journal of Physiology. Regulatory, Integrative and Comparative Physiology, 287*(3), R502-16.

Rosenvold, K., Essén-Gustavsson, B., & Andersen, H. J. (2003). Dietary manipulation of pro- and macroglycogen in porcine skeletal muscle. *Journal of Animal Science, 81*(1), 130-134.

Ruusunen, M., & Puolanne, E. (2004). Histochemical properties of fibre types in muscles of wild and domestic pigs and the effect of growth rate on muscle fibre properties. *Meat Science, 67*(3), 533-539.

SAS Institute Inc. (1999). *SAS OnlineDoc®, Version 8.* Cary, NC, SAS Institute Inc.

Scraba, D. G., Bradley, R. D., Fitzgerald, P. M., & Madsen, N. B. (1988). Electron microscopy of glycogen degrading enzymes. *FEBS letters, 240*(1-2), 133-138.

Shearer, J., Marchand, I., Sathasivam, P., Tarnopolsky, M. A., & Graham, T. E. (2000). Glycogenin activity in human skeletal muscle is proportional to muscle glycogen concentration. *American Journal of Physiology. Endocrinology and Metabolism, 278*(1), E177-80.

Shearer, J., Marchand, I., Tarnopolsky, M. A., Dyck, D. J., & Graham, T. E. (2001). Pro- and macroglycogenolysis during repeated exercise: roles of glycogen content and phosphorylase activation. *Journal of applied physiology: respiratory, environmental and exercise physiology, 90*(3), 880-888.

528 Shen, Q., W., & Du, M. (2005). Role of AMP -activated protein kinase in the glycolysis of
529 postmortem muscle. *Journal of the Science of Food and Agriculture, 85*(14), 2401-2406.

530 Stryer, L. (1988). *Biochemistry.* 3rd edn. New York, Freeman.

531 Taylor, C., Cox, A. J., Kernohan, J. C., & Cohen, P. (1975). Debranching enzyme from rabbit
532 skeletal muscle. Purification, properties and physiological role. *European Journal of*
533 *Biochemistry, 51*(1), 105-115.

534 Theil, P. K., Sørensen, I. L., Therkildsen, M., & Oksbjerg, N. (2006). Changes in proteolytic
535 enzyme mRNAs relevant for meat quality during myogenesis of primary porcine satellite
536 cells. *Meat Science, 73*(2), 335-343.

537 Ylä-Ajos, M., Ruusunen, M., & Puolanne, E. (2006). The significance of the activity of
538 glycogen debranching enzyme in glycolysis in porcine and bovine muscles. *Meat*
539 *Science, 72*(3), 532-538.
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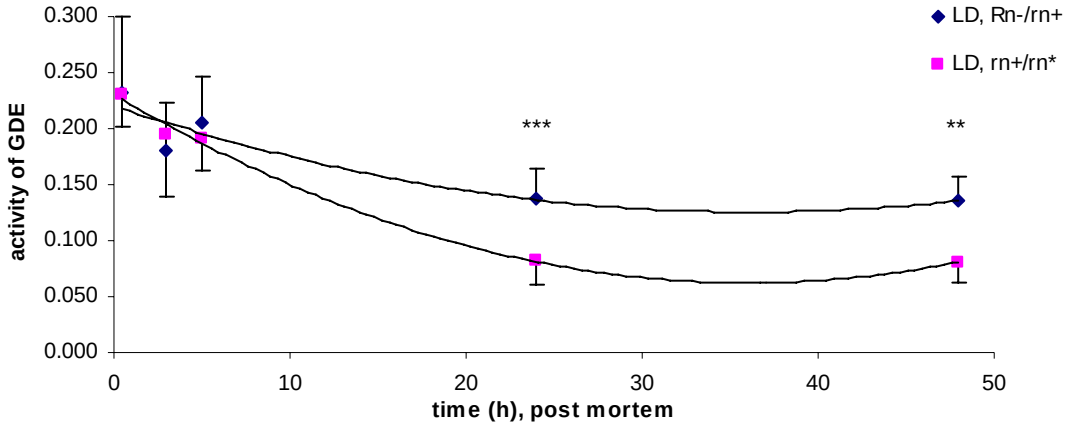
Figure 1. Activity of GDE (Δabs/Δmin, means and standard deviations) in *M. longissimus dorsi* of different RN-genotypes (♦ RN^- carriers, n=7, ■ wild type (rn^+), n=7). Asterisks indicate significant difference between the two genotypes (*** P<0.001, ** P<0.01).



549 Figure 2. Activity of GDE (Δabs/Δmin, means and standard deviations) in *M.*
550 *semimembranosus* of different RN-genotypes (♦ RN^- carriers, n=3, ■ wild type (rn^+), n=3).
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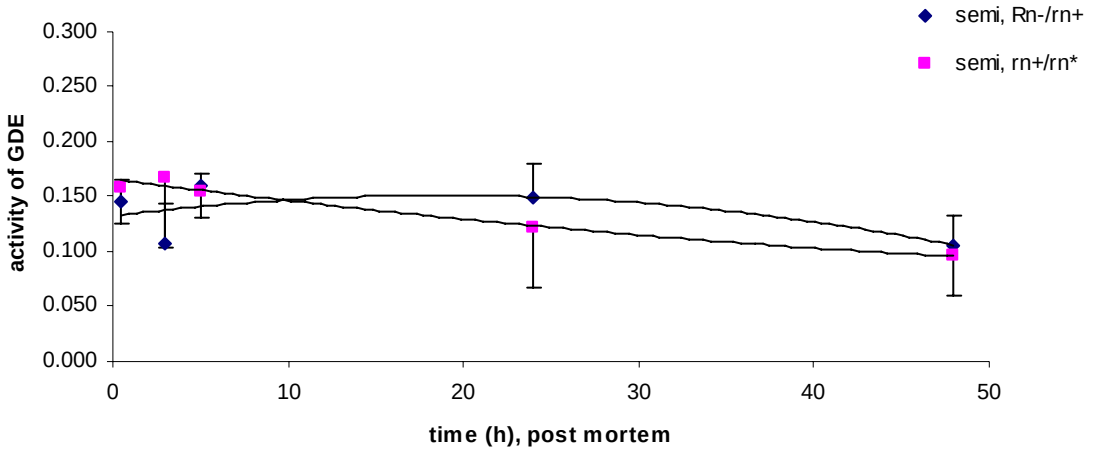

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557 Table 1. LSMeans and SEM for pro-glycogen, "macro-glycogen" and total glucose contents
558 in *M. longissimus dorsi* of RN^- carriers and wild type (rn^+) animals during the *post-mortem*
559 period

	Pro-glycogen, mmol/kg		"Macro-glycogen", mmol/kg		Total glucose content, mmol/kg	
Time *post-mortem*	RN^-	rn^+	RN^-	rn^+	RN^-	rn^+
0.5 h	57.8±2.5a	38.2±2.7a	44.2±2.7a	19.3±2.9	101.9±3.9a	57.6±4.1a
5 h	46.8±3.8 b	30.0±3.7b	46.1±2.5ab	16.9±2.7	92.8±4.6b	47.0±4.9b
24 h	34.2±1.7c	9.4±1.8c	44.3±1.8a	15.7±1.9	78.4±2.4c	25.1±2.5c
48 h	22.9±1.1d	2.7±1.2d	49.0±1.8b	17.6±1.9	71.8±2.7d	20.4±2.8d
96 h	7.3±0.4e	1.6±0.4d	58.3±1.9c	15.4±2.0	65.5±2.0e	17.2±2.1d
168 h	6.8±0.5e	1.6±0.5d	60.6±2.4c	15.2±2.5	67.3±2.5de	17.0±2.7d
216 h	6.8±0.4e	1.5±0.4d	59.8±2.0c	14.5±2.1	66.5±2.3e	16.1±2.4d

560 When comparing the genotypes, both pro-glycogen content and "macro-glycogen" content as well as total
561 glucose content differed significantly ($p < 0.001$) between the genotypes at each stage.
562 Different letter within a column indicates significant ($P<0.05$) difference between values.
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567 Table 2. Relative transcription of GDE, glycogen synthase and glycogenin genes in
568 *M. longissimus dorsi* of RN^-carriers and wild type (rn^+) animals.[a]

Gene	RN^-	rn^+	p-value
GDE	0.75	1	0.142
glycogen synthase	1.14	1	0.330
glycogenin	1.62	1	0.0361

569 [a] Data are normalised according to the transcription level observed in wild type animals.
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574 Table 3. Decrease in pH (LSMeans±SE) in RN^-carriers and wild type (rn^+) animals. For
575 *M. longissimus dorsi* n=20 and for *M. semimembranosus* n=6.

	M. longissimus dorsi			*M. semimembranosus*		
Time *post-mortem*	RN^-	rn^+	p-value	RN^-	rn^+	p-value
0.5 h	6.53±0.06a	6.65±0.06a	0.223	6.59±0.09a	6.58±0.09a	0.979
3 h	6.14±0.08b	6.48±0.08a	0.007	6.11±0.09b	6.18±0.09b	0.595
5 h	5.96±0.07c	6.25±0.07b	0.004	6.21±0.09b	6.04±0.09b	0.169
24 h	5.38±0.03d	5.51±0.03c	0.017	5.36±0.09c	5.37±0.09c	0.894
48 h	5.21±0.03e	5.36±0.03d	0.002	5.29±0.09c	5.33±0.09c	0.709

576 Different letter within a column indicates significant ($P<0.05$) difference between values