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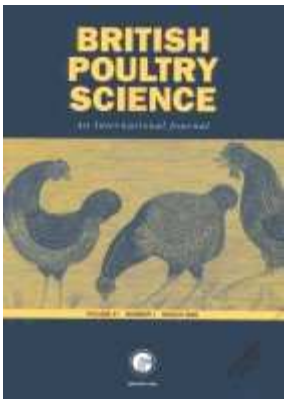
Alexandre Péron, Birger Svihus, Irène Gabriel, Serge Bérot, Daniel Tanguy, Brigitte Bouchet, Joëlle Gomez, Bernard Carré



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Journal:	*British Poultry Science*
Manuscript ID:	CBPS-2005-060.R2
Manuscript Type:	Original Manuscript
Date Submitted by the Author:	17-Jan-2007
Complete List of Authors:	Péron, Alexandre; INRA, Unité de Recherches Avicoles Svihus, Birger; Agricultural University of Norway, Animal and Aquacultural Sciences Gabriel, Irène; INRA, Unité de Recherches Avicoles Bérot, Serge; INRA, Unité de Recherche sur les Protéines Végétales et leurs Interactions Tanguy, Daniel; INRA, UMR Physiologie de la Reproduction et Comportement Bouchet, Brigitte; INRA, Unité de Recherche sur les Polysaccharides, leurs Organisations et leurs Interactions Gomez, Joëlle; INRA, Unité de Recherches Avicoles Carré, Bernard; INRA, Unité de Recherches Avicoles
Keywords:	Digestion, Broilers, wheat, grain hardness, Genetics

CBPS-2006-060
edited M G MacLeod, March 2007

Effects of two wheat cultivars on physico-chemical properties of wheat flours and digesta from two broiler chicken lines (D^+ and D^-) differing in digestion capacity

A. PÉRON, B. SVIHUS[1], I. GABRIEL, S. BÉROT[2], D. TANGUY[3], B. BOUCHET[4], J. GOMEZ AND B. CARRÉ*

Unité de Recherches Avicoles, Institut National de la Recherche Agronomique (INRA), 37380 Nouzilly, France, [1]Department of Animal and Aquacultural Sciences, Agricultural University of Norway, P.O. Box 5003, 1432 Ås, Norway, [2]Unité de Recherche sur les Protéines Végétales et leurs Interactions, INRA, 44316 Nantes, France, [3]UMR Physiologie de la Reproduction et du Comportement, INRA, 37380 Nouzilly, France and [4]Unité de Recherche sur les Polysaccharides, leurs Organisations et leurs Interactions, INRA, 44316 Nantes, France

DIGESTION OF HARD WHEAT

* Corresponding author: carre@tours.inra.fr

Accepted for publication 16th February 2007

Abstract 1. The current experiment is the second part of a study about the effects of wheat quality on digestibility of pelleted diets for broiler chickens. In the first part, it was shown that a hard cultivar resulted in a negative effect on starch digestibility in two divergent lines of chickens (D^+ and D^-) selected for digestion capacity. The aim of this second part was to investigate the reasons for this negative effect of a hard cultivar (Baltimor) compared to a soft one (Scipion) in D^+ and D^- lines.

2. Proventriculus pepsin activity and pancreas proteolytic and amylolytic activities were estimated in 4 pools of birds: "D^+ line (Baltimor fed)", "D^+ line (Scipion fed)", "D^- line (Baltimor fed)" and "D^- line (Scipion fed)". Results suggested greatest amount of pepsin units per g BW for D^+ birds and lowest amount of pancreas proteolytic units per g BW for D^+ birds fed Scipion wheat. Pancreas showed very similar α-amylase activities among treatments.

3. *In vitro* hydrolyses of wheat gluten proteins with proventriculus extracts from pools of D^+ and D^- birds did not show any differences between hard and soft cultivars, whatever the origin of pools.

4. Pepsin hydrolysis of fine (300 to 425 µm) and coarse (1180 to 1600 µm) fractions from wheat flours (Baltimor or Scipion) showed that the 30 min proteolysis rate was highest for the fine fraction in both cultivars. No difference was observed with extended hydrolysis time.

5. *In vitro* digestion simulation of whole wheat flours confirmed the results previously obtained *in vivo*, with a negative effect of hard cultivar on starch digestion rate and no effect on protein digestion.

6. Laser particle size analyses showed that ileum digesta from birds fed with hard wheat cultivar showed highest proportion of coarse particles.

7. Microscopic analyses of D^+ ileum digesta revealed that the concentration of undigested starch granules in sub-aleurone area of wheat bran particles was the highest with hard cultivar.

8. The results suggested that physical entrapment of starch granules in coarse particles was a major explanation for decreased starch digestibility values in chickens fed hard wheat diets.

INTRODUCTION

Hardness is a mechanical characteristic of wheat grain (*Triticum aestivum*) which is thought to be due to interactions between starch granules and their surrounding protein matrix (Barlow *et al*., 1973; Simmonds *et al*., 1973). Puroindolines a and b are major proteins involved in this interaction (Giroux and Morris, 1998). High hardness value results in less friable wheat endosperm and increased particle size after grain milling (Abécassis *et al*., 1997). Recent studies have demonstrated the negative effect of hard wheat cultivar on starch digestibility in pelleted diets for broiler chickens (Carré *et al*., 2002, 2005; Péron *et al*., 2006). Some hypotheses have been proposed to explain this negative effect, most of them involving an accessibility problem. Hard cultivar, high particle size after grinding (Abécassis *et al*., 1997; Carré *et al*., 2002; Péron *et al*., 2006) and/or strong starch-protein interactions (Barlow *et al*., 1973; Kim *et al*., 2004) could decrease physical access to starch granules and limit enzymatic digestion. As previously shown by Péron *et al*. (2005), fine grinding of a hard wheat cultivar before pelleting resulted in starch digestibility and AME_N improvements, indicating that particle size could be an important factor affecting starch digestion of hard wheat. Concerning other factors, it could also be supposed that protein matrix from hard cultivar is less susceptible to proteases than protein matrix from soft cultivar.

This work is the second part of an experiment (Péron *et al*., 2006) studying the effect of wheat quality on digestibility values in two lines (D^+ and D^-) of broiler chickens selected for divergent digestion capacity (Mignon-Grasteau *et al*., 2004). The first part of this experiment (Péron *et al*., 2006) confirmed that strong wheat hardness was negative for starch digestibility in broiler chickens: soft (Scipion cultivar) instead of hard (Baltimor cultivar) wheat resulted in an improvement of about 6% in both D^+ and D^- lines. The aim of the current paper was to investigate the causes for decreased starch digestibility with hard wheat. Laser particle size and microscopic analyses in ileum contents were used in order to observe possible differences in starch accessibility between hard and soft wheat. *In vitro* hydrolyses and digestion simulation processes were used to investigate hydrolysis susceptibility of hard and soft wheat gluten proteins and wheat flours. In order to test possible interactions with genetical origin of birds, digesta and enzyme extracts were taken from D^+ and D^- chickens.

MATERIALS AND METHODS

Animals, wheat cultivars and experimental diets

Chickens, housing conditions, composition of the experimental diets, pelleting parameters, particle size in diets, and *in vivo* digestibility measurements were described in the first part of the study (Péron *et al*., 2006). Briefly, the experiment was performed according to a 2x2 factorial design testing two chicken genetic lines (D^+ and D^-) and two wheat cultivars (Baltimor and Scipion). These cultivars were chosen because of their great difference in hardness value (Table 1). Wheat samples were obtained from plant breeders and were stored at ambient temperature before use in diets and *in vitro* studies. Chemical and physical compositions of wheat samples are given in Table 1. Before inclusion in diets, both wheat samples were ground using a hammer mill fitted with a 6 mm screen. Particle size distribution of wheat flours is shown in Table 2. All

diets were pelleted. From 7 to 26d of age, birds were fed with diets containing 546 g/kg wheat (Baltimor or Scipion), 353 g/kg soybean meal and 55 g/kg rapeseed oil (Péron *et al*., 2006). Digestibility measurements were performed from 20 to 23d. At 26 d, two new pelleted diets were offered to birds. They were just given for 1 d before killing chickens and sampling their digestive organs and ileum contents. These new diets contained the same wheat samples as previous diets, and differed from previous ones by the high wheat inclusion rate (942 g/kg). Other ingredients of these diets were rapeseed oil (20g/kg) and a mixture (38g/kg) of lysine, methionine, minerals and vitamins (Péron *et al*., 2006). Laser particle size distributions of the wheat (942 g/kg) pelleted diets are shown in Figure 2.

Tables 1, 2; Figures 1, 2 near here

Digestive organ and ileum content sampling

Sampling procedure for digestive organs and their contents was described in Péron *et al*. (2006). Briefly, at 27 d, 12 birds per treatment were weighed and killed by intracardiac injection of 1 ml of sodium pentobarbital (Sanofi, Marne la Coquette, France) for collection of digestive organs and ileum contents. Proventriculus and pancreas were quickly frozen in liquid nitrogen, stored at -70°C, ground by ball-milling in liquid nitrogen and stored again at -70°C. Total ileum content was homogenised and divided into two fractions F1 and F2: fraction F1 was frozen in liquid nitrogen and stored at -70°C for further laser particle size analyses, and fraction F2 was frozen with isopentane (cooled with liquid nitrogen) and stored at -70°C for further microscopic analyses. For enzymatic studies (*in vitro* hydrolyses and digestion simulation), one pool of 6 proventriculi and one pool of 4 pancreases were carried out for each treatment. Pools were constituted by mixing equal quantities of tissue powder from each bird. Pool origins were designated as: "D^+ line (Baltimor fed)", "D^+ line (Scipion fed)", "D^- line (Baltimor fed)" and "D^- line (Scipion fed)".

Analytical methods

In order to prepare *in vitro* hydrolyses and digestion simulation, some activities of digestive enzymes of D^+ and D^- lines were investigated. Pepsin activity (EC 3.4.23.1) in proventriculus tissue was assayed at different pH with haemoglobin as a substrate. Two analytical replicates were performed for each proventriculus pool. Anson's method (1938) modified by Crévieu-Gabriel *et al*. (1999) was used with some modifications as follows: 100 mg tissue powder was homogenised in 2 ml of 0.01 M phosphate buffer pH=7.4, then centrifuged at 10000 *g* (4°C, 15 min). Supernatants (50 µl) were diluted with 950 µl of 0.01 M phosphate buffer pH=7.4. Then, 500 µl of diluted extract were activated with 100 µl HCl 300 mM for 15 minutes and pepsin activity was measured by adding 200 µl of the activated extract in 1.35 ml of a haemoglobin solution (20 g/l). Pepsin activity of proventriculus was expressed as units/mg tissue. According to Crévieu-Gabriel *et al*. (1999), one pepsin unit (U) was defined as the amount of enzyme that increased optical density (280 ηm) by 0.001 per min under the assay conditions. Proteolytic activity of pancreas was measured (with 4 replicates per pancreas pool) at different pH values using a casein substrate, as described by Susbilla *et al*. (2003). Pancreas proteolytic activity was expressed as units/g tissue, with 1 proteolytic unit (U) being equivalent to 1 µmole tyrosine released per min under the assay conditions. Amylolytic activity of pancreas was estimated (with 2 replicates per pancreas pool) using 50 mg purified maize starch (Roquette, France) suspended in 1 ml of sodium-phosphate buffer 0.05 M (pH=6.75) and placed in a thermostatically controlled water bath (40°C). Pancreatic extract (490 µl from 150 mg homogenised tissue in 2.5 ml Ringer solution pH=7.4) was added to the suspension. At various times of the hydrolysis process (t=30 min, 1 h, 2 h, 3 h or 4 h), an aliquot was kept and centrifuged

at 10000 g. Released dextrins in the supernatant were determined using amyloglucosidase treatment followed by glucose determination (Carré *et al*., 2002).

In vitro protein hydrolyses with proventriculus extracts were also performed on wheat gluten suspensions with 3 replicates per pool extract. Gluten was obtained by lixiviation (Bérot and Davin, 1996) from the wheat samples (Baltimor and Scipion cultivars) given to birds. Hydrolyses were performed in Erlenmeyer flasks closed with synthetic rubber bungs, placed in a thermostatically controlled water bath (40°C), under magnetic stirring (180 rpm). To each Erlenmeyer, 10 ml of HCl (pH=3.0) were added. When the correct temperature was reached, 50 mg of wheat gluten were also added. They were suspended for 10 min and the proventriculus extracts (414 pepsin U activated with 100 µl HCl 300 mM during 15 min), were added to the suspension. Hydrolyses were allowed to proceed for 10 min at 40°C. Reactions were stopped by adding TCA for a final concentration of 100 g/l. Blanks were made by adding TCA before the proventriculus extract. Then, suspensions were left at 4°C for 30 min. TCA precipitates were discarded after centrifugation at 10000 *g* for 10 min at 4°C. TCA-soluble products were measured by Landry and Delhaye's method (1996) modified by Crévieu-Gabriel *et al*. (1999).

Kinetics of protein hydrolysis by proventriculus extracts were performed on two fractions (fine or coarse particles) of Baltimor and Scipion wheat flours, as described above for gluten, with various times of hydrolysis from 30 min to 4 h, using the proventriculus extract (414 pepsin U activated with 100 µl HCl 300 mM for 15 min) from the "D^+ line (Baltimor fed)" pool and flour samples equivalent to 50 mg protein. Coarse and fine flour fractions were obtained by dry sieving of the whole flour included in diets for chicken experiment (particle size distribution is shown in Table 2). Two replicates per pool and per hydrolysis time were carried out.

With whole wheat flours from hard and soft cultivars (particle size distribution is shown in Table 2), *in vitro* digestion simulation was also performed. Whole wheat flours were the same as those included in diets. The physiological parameters of the intestinal tract used for this study, such as temperature, pH, or digesta retention time, were simulated by an *in vitro* method described by Tervilä-Wilo *et al*. (1996), with some modifications. 200 mg of whole wheat flour were hydrolysed using enzymatic extracts from the "D^+ line (Baltimor fed)" pool. Proventriculus and pancreas enzymatic activities added were respectively 1000 pepsin U (activated with 100 µl HCl 300 mM during 15 min) and 2.3 proteolytic U (activated with 6.25 µg enterokinase in 25 µl Tris-HCl buffer pH=7.4 for 2 h). In order to obtain noticeable starch hydrolysis, the pancreatic step was longer (6h 00) than in the method of Terwilä-Wilo *et al*. (1996) (1h 00). After centrifugation, supernatants were assayed for protein and starch contents, according to Lowry *et al*. (1951) and Carré *et al*. (2002), respectively.

Particle size distributions in ileum contents and in the wheat (942 g/kg) pelleted diets (Baltimor and Scipion cultivars) were determined (12 birds replicates for D^+ Baltimor and D^- Scipion, and 11 for D^+ Scipion and D^- Baltimor) using a Malvern Mastersizer S instrument (Malvern Instruments Ltd., Worcestershire, UK), as described by Hetland *et al*. (2002). Particle diameters were detected in the range from 0.02 to 2000 µm. Results were analysed using the Malvern 2000 Software (version 5.22).

Fluorescence microscopy was performed on an ileum sample from a D^+ bird fed on Baltimor, as follows: sections (16 µm) obtained from a portion frozen at -25°C were allowed to dry for one week. They were then stained for 5 min with 100 mg/l Calcofluor (White M2R; Sigma) and quickly rinsed with distilled water. The fluorescence section was examined with a Zeiss Axioplan 2 microscope, fitted with an appropriate filter set (Zeiss, n°18) and phase interference (H/DIC II). Bright-field

microscopy was preferred to fluorescence because the staining process to be used with the latter observation technique resulted in starch granule losses. These losses were observed by comparing successive sections treated either with the bright-field technique or with the fluorescence one. Bright-field microscopy analyses were made on ileum contents of D^+ birds only (n=11 birds per diet). For bright-field microscopy, the sections (16 µm) were not stained and were observed with no preliminary treatment. Bright-field sections were examined with a Zeiss Axioplan 2 microscope, fitted with phase interference (H/DIC II). Photomicrographs were obtained using a Kappa camera DX30C and analysed with Visilog Software (version 6.3). The software was programmed in order to measure the length (µm) of particles. It was also able to measure the concentration of starch granules in a defined area as the mean optical density (OD) of this area. Observations were made of the subaleurone area of bran particles. The subaleurone area was defined as described in Figure 3, with 75 µm width for the considered area. An enlarged view of the subaleurone area is also shown in Figure 4. About 10 particles per animal were observed.

Statistical analysis

ANOVA analyses with Statview Software (SAS Institute) were used to test the effects of "line" and "cultivar" for *in vitro* hydrolyses, digestion simulation procedure and laser particle size analyses. For image analysis of ileum sections, GLM analyses with SAS Software were used to test the effects of "cultivar", and "individual chicken nested in a cultivar", on the length of bran particles and on the estimated starch concentration in the subaleurone area of bran particles.

RESULTS

Proventriculus and pancreas enzymatic activities

Pepsin activities of proventriculus on haemoglobin at various pH are shown in Table 3. Optimum pH level of the activity was found to be around 3.0 for each treatment. Pepsin activities expressed as U/g BW (Table 4) suggested higher activity in D^+ than in D^- birds. Proteolytic activities of pancreas on casein at various pH are shown in Table 3. Activity increased from pH=6.15 to 7.50. Pancreatic proteolytic activities expressed as U/g BW (Table 4) suggested lowest activity for D^+ birds fed the Scipion wheat diet. Kinetic of starch hydrolysis with pancreatic extracts suggested very similar α-amylase concentration in pancreas among treatments (Table 3). Tables 3, 4 near here

Gluten hydrolyses with proventriculus extracts

Hydrolyses of gluten from Baltimor or Scipion wheats with proventriculus extracts are shown in Table 5. There were no significant differences between hard and soft wheat cultivars, whatever the origin of proventriculus extract.

Hydrolyses of fine and coarse wheat fractions with proventriculus extracts

Results for the kinetic hydrolyses of fine (300 to 425 µm) or coarse (1180 to 1600 µm) fractions from wheats (Baltimor or Scipion) with proventriculus extract from the "D^+ line (Baltimor fed)" pool are shown in Figure 1. There were no significant effects except at t=30 min. At this time, protein hydrolysis was significantly higher (P=0.016) in fine than in coarse particles. Figure 1 near here

***In vitro* digestion simulation**

Results of *in vitro* digestion simulation on whole wheat flours (Baltimor *versus* Scipion) are shown in Table 6. Starch hydrolysis values were low (around 0.30). Hard cultivar instead of soft one resulted in a negative effect (P<0.001) on starch digestibility. There were no differences in protein hydrolysis between cultivars. Tables 5, 6 near here

Laser particle size analysis of ileum contents and wheat (942 g/kg) pelleted diets

Laser particle size distributions of the wheat (942 g/kg) pelleted diets (Baltimor or Scipion cultivars) and ileum contents of birds fed these diets are shown in Figure 2. Statistical analyses for particle size analysis of chicken ileum contents are shown in Table 7. It was observed that the pattern of the distributions was not very different between diets and related ileum contents. However, distributions in ileum contents of birds were shifted towards lower sizes, compared with those of the wheat (942 g/kg) pelleted diets (Figure 2). Moreover, the soft cultivar resulted in an increased proportion of small particles in ileum compared to the diet, while the reverse was observed with the hard cultivar (Figure 2). Particle size histograms exhibited one mode (main mode) or two mode (bimodal) response types. For the main mode response type, ileum particles of D^+ chickens tended (P=0.062) to show smaller single peak position than those of D^- chickens. Proportion of ileum particles at this single peak position did not differ between treatments. With bimodal response type, ileum particles from soft wheat showed lower peak 1 position than hard one (18.8 µm *versus* 20.7 µm) (P<0.001), and the proportion of ileum particles at this peak 1 position was higher (P=0.003) for Scipion than for Baltimor. Peak 2 position did not differ between treatments.

Figure 2, Table 7 near here

In order to study all animal responses together, two peak positions (µm) were investigated: a small one at 17.4 µm (mean peak 1 position of mean particle size histograms shown in Figure 2) and a coarse one at 478.6 µm (mean peak 2 position of mean particle size histograms shown in Figure 2; Table 7). At 17.4 µm, proportions of particles were higher for Scipion than for Baltimor (P=0.005; Table 7), and at 478.6 µm, particle proportions were lowest for Scipion (P=0.045; Table 7). No significant effect of chicken line was observed on particle proportion at these two positions.

Microscopy

Results for microscopic analyses are shown in Table 8 and Figures 3, 4 and 5. It was decided to analyze only D^+ line ileum digesta because these birds have less variable responses, which could improve detection of differences. With the bright-field observation technic, the visible structures were essentially starch granules and cell walls from aleurone and/or bran layers (Figure 3). Few diffuse structures, maybe proteins, were also visible. All these structures appeared as grey, with different intensity according to their concentration. Concerning endosperm cell walls, they were only visible using fluorescence (Figure 5). Image analyses revealed that the concentration of starch granules remaining in the sub-aleurone area of ileum bran particles was higher for hard than for soft wheat ($P<0.001$; Table 8).

Figures 3,4,5, Table 8 near here

DISCUSSION

Particle size distribution was measured in the current experiment using a high performance laser apparatus, which resulted in high precision in the data and a very large range in particle size recording (0.02 µm to 2000 µm). The small peak (peak 1) appearing in the particle size distributions of ileum digesta probably corresponded to starch granules, especially because the variability of its position was very low in bimodal responses. The effect of hardness on the position of this small peak probably came directly from the diet that exhibited also the same effect. This probably reflected a difference in the size of starch granules between Scipion and Baltimor cultivars. This would be in agreement with previous observations (Bechtel *et al.*, 1993) showing difference in starch granule size between cultivars. The pattern of particle size distributions in ileum digesta of chickens was shifted towards the left (decreased particle size) compared to those of diets. This probably came from the erosive and grinding activity of the digestive tract. The variability in particle size distribution in ileum digesta was high, especially in D^- birds fed on the Scipion diet. It remains to be

known whether this variability not only reflected variability between birds, but also variability in the digestion process of a bird.

The current experiment gave the opportunity to test the differences between D^+ and D^- lines in terms of digestive enzymatic activities. The high potential of D^+ birds for pepsin production may be one of the reasons explaining their high digestion capacity. Potential of D^+ birds fed Scipion diet for the production of pancreatic proteolytic enzymes seemed rather low. This group also exhibited the highest protein digestibility (Péron *et al.*, 2006). Thus, it is probable that pancreatic enzyme syntheses were not limiting and changed in order to counterbalance digestion levels resulting from some unknown limiting factors. In general, it was observed that organ weight variation was the main factor at the origin of the variation in the potential for enzyme production expressed per gram of body weight.

In relation to the wheat quality effect, Péron *et al.* (2006) observed decreased starch digestibility with hard wheat cultivar compared to soft wheat cultivar in both chicken lines D^+ and D^-. This could come from various factors in hard wheat such as starch accessibility problems due to great amount of coarse particles, strong starch-protein interaction, or resistance of matrix proteins.

In vitro digestion simulation of whole wheat flours showed a negative effect of hard cultivar on starch hydrolysis, as previously shown *in vivo* (Péron *et al.*, 2006). However, *in vitro* starch hydrolysis values were low (around 0.30). This may have been due to an insufficient addition of pancreatic enzymes compared with the amount secreted *in vivo* by chickens. It could also be supposed that a part of the observed difference in *in vitro* starch hydrolysis could be due to different concentrations of endogenous α-amylase or α-amylase inhibitors between cultivars. These values were not measured in this experiment.

Observation of particle size distribution in ileum digesta allowed us to estimate the location of undigested starch granules among particles of ileum digesta. High amount of undigested free starch granules should result in high proportion of small particles (<30 µm) in ileum digesta. This was not observed in ileum digesta of birds fed hard wheat, despite low starch digestion with this kind of wheat. Thus, for hard wheat, the major part of undigested starch granules was probably located in coarse particles, which suggests an accessibility problem in coarse particles for the starch digestion of hard wheat. The reverse was observed with soft wheat: despite high starch digestion, a large amount of small particles was found. Thus, the limiting factor for starch digestion probably did not concern free starch granules digestion. Moreover, in the present experiment, *in vitro* proteolysis of fine and coarse particles from wheat flours with pepsin extract gave higher hydrolysis values at 30 minutes for the finest particles in both cultivars. This observation reinforce the hypothesis of an access problem in coarse particles of ground wheat. Rather similar observations were made, *in vitro*, by Weiguo *et al*. (2003) with ground maize or ground wheat bran. However, after 4 h of hydrolysis, there was no significant difference in proteolysis value between treatments, indicating that the access problem acted essentially by creating a lag time in the digestion process. It can also be noted that protein hydrolysis values for *in vitro* hydrolysis of fine and coarse flour fractions were low (about 0.20) compared with those obtained with *in vitro* digestion simulation of whole flours (about 0.80). This may have been due to the fact that, for the simulation procedure, added pepsin activity was higher and flour amount was lower, resulting in a better enzyme/substrate ratio. Higher protein hydrolysis values in the simulation digestion procedure may also have been related to the presence of pancreatic proteases in addition to pepsin

It has previously been shown that, in the human small intestine, undigested starch granules of barley were mainly located close to the aleurone and bran layers (Livesey *et al*., 1995). In the current experiment, a large amount of undigested starch granules was also observed in the endosperm subaleurone area of bran particles found in the ileum. Microscopic analyses of these particles showed that the amount of starch in the endosperm subaleurone area was higher for the hard than for the soft wheat ileum samples. This suggests that the problem of access with hard wheat mainly concerned the endosperm subaleurone area. Subaleurone cells from hard wheat may be more resistant and then, after grinding, starch granules may be more protected from enzyme hydrolysis. Such a difference in resistance might be related to the difference in composition of subaleurone cell walls between hard and soft wheats (Barron *et al*., 2005).

A previous study showed that adding protease (such as pepsin) could increase *in vitro* starch digestibility of cereals (Aura *et al*., 1999), indicating that the resistance of the protein matrix surrounding starch granules may affect starch digestion. Gluten is the major component of the protein matrix. Gluten hydrolyses with proventriculus extracts showed that there were no differences between hard and soft cultivar origins of gluten, which suggests that gluten was not involved. It might be supposed that 10 minutes of hydrolysis was too short to result in observation of notable differences. However, with the hydrolysis of fine and coarse particles from Baltimor and Scipion wheat flours, or with the *in vitro* digestion simulation of whole flours, protein hydrolysis was longer (4 h or 6.75 h, respectively) and no significant differences were observed between hard and soft wheat flours. Thus, differences in starch digestibility probably did not result from differences in protein susceptibility to enzyme hydrolysis.

CONCLUSION

In conclusion, the current experiment seemed to confirm *in vitro* the negative effect of a hard cultivar on the *in vivo* digestion of wheat starch observed by Péron *et al*. (2006). However, other factors such as endogenous α-amylase or α-amylase inhibitors in grain may have been involved in this observation. The use of isogenic lines of wheat might be an interesting solution to understand more precisely the role of hardness independently of the other factors. Concerning explanations for the negative effect of hard wheat cultivar on starch digestibility, most observations were in favour of the hypothesis of an access problem in coarse particles of hard wheat, as shown by *in vitro* hydrolyses of wheat flours, and examinations of chicken ileum digesta using laser particle size determinations or light microscope analyses.

AKNOWLEDGMENTS

The authors are grateful to INZO° (Château-Thierry, France) for financial support. They also thank Ms M. Leconte (INRA, Nouzilly, France) for advice in the *in vitro* digestion procedures, Mr F. Sunby (Agricultural University of Norway) for laser particle size measurements, Mr K. Gérard (INRA, Nouzilly, France) for helpful assistance in the management of birds, Ms I. Grasseau and Mr J.P. Brillard (INRA, Nouzilly, France) for advice in microscopy, and Mr C. Blassel (INRA, Nantes, France) for preparation of gluten from wheat flours.

REFERENCES

ABÉCASSIS, J., MABILLE, F., HADDAD, Y., AUTRAN, J.-C. & BENET, J.-C. (1997) La dureté des blés : état des connaissances actuelles. *Industrie des céréales*, **101:** 11-18.

ANSON, M.L. (1938) The estimation of pepsin, trypsin, papain, and cathepsin with haemoglobin. *Journal of General Physiology*, **22**: 79-89.

ASAE (1983) Method of determining and expressing fineness of feed materials by sieving. ASAE standard S319, in: *Agricultural Engineers Yearbook of Standards*, p325 (American Society of Agricultural Engineers).

AURA, A.M., HÄRKÖNEN, H., FABRITIUS, M. & POUTANEN, K. (1999) Development of an in vitro enzymatic digestion method for removal of starch and protein and assessment of its performance using rye and wheat breads. *Journal of Cereal Science*, **29:** 139-152.

BARLOW, K.K., BUTTROSE, M.S., SIMMONDS, D.H. & VESK, M. (1973) The nature of the starch-protein interface in wheat endosperm. *Cereal Chemistry*, **50:** 443-454.

BARRON, C., PARKER, M.L., MILLS, E.N.C. & ROUAU, X. (2005) FTIR imaging of wheat endosperm cell walls in situ reveals compositional and architectural heterogeneity related to grain hardness. *Planta*, **220:** 667-677.

BECHTEL, D.B., ZAYAS, I., DEMPSTER, R. & WILSON, J.D. (1993) Size-distribution of starch granules isolated from hard red winter and soft red winter wheats. *Cereal Chemistry*, **70**: 238-240.

BÉROT, S. & DAVIN, A. (1996) Technologie d'extraction et de purification des matières protéiques végétales, in: Lavoisier Tech. Doc. (Eds) *Les protéines végétales*, pp 279-363.

CARRÉ, B. & BRILLOUET, J.M. (1989) Determination of water-insoluble cell walls in feeds : interlaboratory study. *Journal of the Association of Official Analytical Chemists*, **72**: 463-467.

CARRÉ, B., BEAUFILS, E. & MELCION, J.P. (1991) Evaluation of protein and starch digestibilities and energy value of pelleted or unpelleted pea seeds from winter or spring cultivars in adult and young chickens. *Journal of Agricultural and*

Food Chemistry, **39**: 468-472.

CARRÉ, B., GOMEZ, J., MELCION, J.P. & GIBOULOT, B. (1994) La viscosité des aliments destinés à l'aviculture. Utilisation pour prédire la consommation et l'excrétion d'eau. *Productions Animales*, **7**: 369-379.

CARRÉ, B., IDI, A., MAISONNIER, S., MELCION, J.P., OURY, F.X., GOMEZ, J. & PLUCHARD, P. (2002) Relationships between digestibilities of food components and characteristics of wheats (*Triticum aestivum*) introduced as the only cereal source in a broiler chicken diet. *British Poultry Science*, **43:** 404-415.

CARRÉ, B., MULEY, N., GOMEZ, J., OURY, F.X., LAFFITTE, E., GUILLOU, D. & SIGNORET, C. (2005) Soft wheat instead of hard wheat in pelleted diets results in high starch digestibility in broiler chickens. *British Poultry Science*, **46:** 66-74.

CRÉVIEU-GABRIEL, I., GOMEZ, J., CAFFIN, J.P. & CARRÉ, B. (1999) Comparison of pig and chicken pepsins for protein hydrolysis. *Reproduction Nutrition and Development*, **39:** 443-454.

GIROUX, M.J. & MORRIS, C.F. (1998) Wheat grain hardness results from highly conserved mutations in the friabilin components puroindoline a and b. *Proceedings of the National Academy of Science of the USA*, **95:** 6262-6266.

HETLAND, H., SVIHUS, B. & OLAISEN, V. (2002) Effect of feeding whole cereals on performance, starch digestibility and duodenal particle size distribution in broiler chickens. *British Poultry Science*, **43:** 416-423.

KIM, W., CHOI, S.G., KERR, W.L., JOHNSON, J.W. & GAINES, C.S. (2004) Effect of temperature on particle size distribution in hard and soft wheat flour. *Journal of Cereal Science*, **40:** 9-16.

LANDRY, J. & DELHAYE, S. (1996) A simple and rapid procedure for hydrolysing minute amounts of proteins with alkali. *Analytical Biochemistry*, **243:** 191-194.

LIVESEY, G., WILKINSON, J.A., ROE, M., FAULKS, R., CLARK, S., BROWN, J.C., KENNEDY, H. & ELIA, M. (1995) Influence of the physical form of barley grain on the digestion of its starch in the human small intestine and implications for health. *American Journal of Clinical Nutrition*, **61:** 75-81.

LOWRY, O.H., ROSEBROUGH, N.J., FARR, A.L. & RANDALL, R.J. (1951) Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry*, **193:** 265-275.

MIGNON-GRASTEAU, S., MULEY, N., BASTIANELLI, D., GOMEZ, J., PÉRON, A., SELLIER, N., MILLET, N., BESNARD, J., HALLOUIS, J.M. & CARRÉ, B. (2004) Heritability of digestibilities and divergent selection for digestion ability in growing chicks fed on a wheat diet. *Poultry Science*, **83:** 860-866.

PÉRON, A., BASTIANELLI, D., OURY, F.X., GOMEZ, J. & CARRÉ, B. (2005) Effects of food deprivation and particle size of ground wheat on digestibility of food components in broilers fed a pelleted diet. *British Poultry Science*, **46:** 223-230.

PÉRON, A., GOMEZ, J., MIGNON-GRASTEAU, S., SELLIER, N., DEROUET, M., JUIN, H., CARRÉ, B. (2006) Effects of wheat quality on digestion differ between the D^+ and D^- chicken lines selected for divergent digestion capacity. *Poultry Science*, **85:** 462-469.

SIMMONDS, D.H., BARLOW, K.K. & WRIGLEY, C.W. (1973) The biochemical basis of grain hardness in wheat. *Cereal Chemistry*, **50:** 553-562.

SUSBILLA, J.P., TARVID, I., GOW, C.B. & FRANKEL, T.L. (2003) Quantitative feed restriction or meal-feeding of broiler chicks alter functional development of enzymes for protein digestion. *British Poultry Science*, **44:** 698-709.

TERVILÄ-WILO, A., PARKKONEN, T., MORGAN, A., HOPEAKOVSKI-NURMINEN, M., POUTANEN, K., HEIKKINEN, P. & AUTIO, K. (1996) In vitro digestion of wheat microstructure with xylanase and cellulase from trichoderma reesei. *Journal of Cereal Science*, **24:** 215-225.

WEIGUO, W., PING, L., JUNQING, W., YIQIANG, X., HUANLONG, L. (2003) Effect of particle sizes on in vitro protein digestibility and energy consumption of seven feed ingredients. *Chinese Journal of Animal Science*, **39**: 18-20.

Table 1. *Chemical and physical composition of wheat samples from Baltimor and Scipion cultivars*

	Baltimor	Scipion
Crude protein (g/kg)	101.9	111.5
Starch[1] (g/kg)	612.7	590.4
Water-insoluble cell walls[2] (g/kg)	103.0	93.1
Real Applied Viscosity[3] (mL/g)	1.73	2.18
Potential Applied Viscosity[3] (mL/g)	2.91	3.15
NIR hardness value	75	5

[1]: Starch according to Carré *et al.* (1991).
[2]: Water-insoluble cell walls (WICW) according to Carré and Brillouet (1989).
[3]: Viscosity determinations according to Carré *et al*. (1994), using the ethanol pre-treatment for the Potential Applied Viscosity value.

Table 2. *Particle size distribution (weight proportion (g/g) retained on sieves) of ground wheat (dry and wet sieving)*

Sieve openings (µm)		< 75	75	150	300	425	600	850	1180	1600	2000	2360	GMD[1] (µm)	Ratio[2] Coarse/Fine
Wheat flour (dry sieving)	Baltimor	0.002	0.038	0.038	0.048	0.052	0.089	0.103	0.244	0.247	0.084	0.054	908	1.10
	Scipion	0.005	0.034	0.041	0.073	0.071	0.093	0.093	0.191	0.297	0.062	0.038	854	0.65
Wheat flour (wet sieving)	Baltimor	0.232	0.025	0.017	0.016	0.028	0.091	0.110	0.158	0.158	0.165		481	1.18
	Scipion	0.283	0.086	0.033	0.027	0.033	0.085	0.099	0.122	0.118	0.115		320	0.58

[1] Geometrical Mean Diameter. calculated according to ASAE method (1983).
[2] Ratio of coarse to small particles.
For dry sieving: (2360+2000)/(<75+75+150+300).
For wet sieving: (2000+1600)/(<75+75+150).

Table 3. *Proteolytic enzyme activities in proventriculus[1] and pancreas[2] (expressed as U/mg tissue or U/g tissue) at 27 d, and kinetics of hydrolysis of pure maize starch with pancreatic extract[3]*

	Origin of pools			
	D+ line (Baltimor fed)	D+ line (Scipion fed)	D- line (Baltimor fed)	D- line (Scipion fed)
Proventriculus pepsic activity[4] (U/mg tissue) at pH=1	5.6	6.6	8.3	8.2
Proventriculus pepsic activity[4] (U/mg tissue) at pH=2	14.5	15.0	16.8	17.9
Proventriculus pepsic activity[4] (U/mg tissue) at pH=3	16.5	17.5	18.3	19.2
Proventriculus pepsic activity[4] (U/mg tissue) at pH=4	14.7	14.9	14.8	15.4
Proventriculus pepsic activity[4] (U/mg tissue) at pH=5	8.1	7.9	7.8	8.4
Pancreas proteolytic activity[5] (U/g tissue) at pH=6.15	17.5	13.2	16.7	17.3
Pancreas proteolytic activity[5] (U/g tissue) at pH=6.75	21.4	16.6	19.1	21.2
Pancreas proteolytic activity[5] (U/g tissue) at pH=7.15	22.7	17.6	19.5	21.7
Pancreas proteolytic activity[5] (U/g tissue) at pH=7.50	24.5	19.3	21.8	24.1
Starch hydrolysis[6] (g/g) at t=30 min	0.274	0.279	0.281	0.295
Starch hydrolysis[6] (g/g) at t=1h00	0.398	0.398	0.398	0.413
Starch hydrolysis[6] (g/g) at t=2h00	0.570	0.588	0.572	0.575
Starch hydrolysis[6] (g/g) at t=3h00	0.663	0.698	0.689	0.676
Starch hydrolysis[6] (g/g) at t=4h00	0.724	0.727	0.732	0.728

[1] One unit (U) was defined as the amount of enzyme which produces an absorbance increase of 0.001 per min at 280 ηm under the assay conditions (haemoglobin substrate).
[2] One unit (U) was defined as 1 µmole of tyrosine released per min under the assay conditions (casein substrate).
[3] 50 mg of pure maize starch hydrolysed with 29.4 mg of pancreas tissue at pH 7.4.
[4] Determined in one proventriculus pool of 6 chickens per treatment. with 2 analytical replicates.
[5] Determined in one pancreas pool of 4 chickens per treatment. with 4 analytical replicates.
[6] Determined in one pancreas pool of 4 chickens per treatment. with 2 analytical replicates.

Table 4. *Body weight (g), proventriculus and pancreas weights (g), and proteolytic enzyme activities in proventriculus[1] and pancreas[2] (expressed as U/mg tissue, U/g tissue, or U/g BW) at 27 d*

	Origin of pools				SEM	*P*-value		
	D+ line (Baltimor fed)	D+ line (Scipion fed)	D- line (Baltimor fed)	D- line (Scipion fed)		Line effect	Diet effect	Line x Diet effect
Mean body weight (g)	875.7	923.2	847.0	895.8	27.87 [3]	0.33	0.10	0.98
Mean proventriculus weight (g)	7.16	7.13	4.19	3.95	1.341 [3]	0.033	0.92	0.94
Proventriculus pepsin activity (U/mg tissue) [5]	16.5	17.5	18.3	19.2				
Proventriculus pepsin activity (U/g BW) [5]	134.6	134.9	90.7	84.8				
Mean body weight (g)	912.5	922.5	837.0	895.8	36.61 [4]	0.19	0.37	0.52
Mean pancreas weight (g)	1.95	1.84	2.27	1.98	0.178 [4]	0.22	0.28	0.65
Pancreas proteolytic activity (U/g tissue) [6]	24.5	19.3	21.8	24.1				
Pancreas proteolytic activity (U/g BW) [6]	0.052	0.038	0.059	0.053				

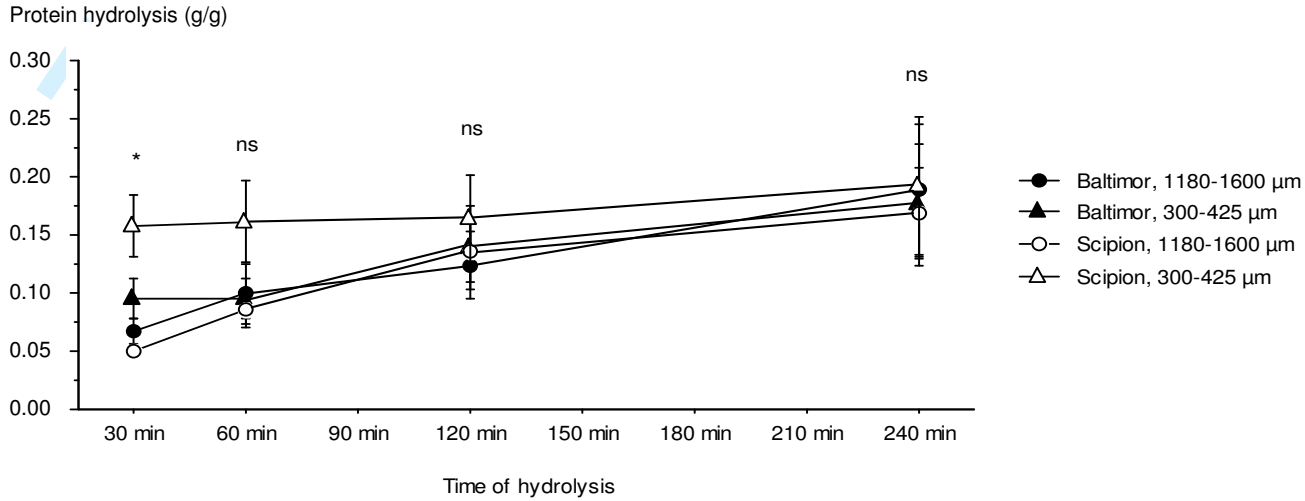
[1] One unit (U) was defined as the amount of enzyme which produces an increase in absorbance of 0.001 per min at 280 ηm under the assay conditions (pH=3.0).
[2] One unit (U) was defined as 1 µmole of tyrosine released per min under the assay conditions (pH=7.5).
[3] Pooled standard error for n=6 replicates.
[4] Pooled standard error for n=4 replicates.
[5] Determined in one proventriculus pool of 6 chickens per treatment, with 2 analytical replicates.
[6] Determined in one pancreas pool of 4 chickens per treatment, with 4 analytical replicates.

Table 5. *Gluten (from Baltimor or Scipion wheat cultivar) hydrolyses (g/g) with proventriculus extract from pools of 6 chickens (pH=3.0. T=40°C. time=10 minutes. Proventriculus proteolytic activity added=8.3 U per mg gluten)*

Origin of proventriculus extract pool	Wheat cultivar origin of gluten	
	Baltimor	Scipion
D^+ line (Baltimor fed)	0.109[a]	0.110[a]
D^+ line (Scipion fed)	0.103[a]	0.105[a]
D^- line (Baltimor fed)	0.113[a]	0.121[a]
D^- line (Scipion fed)	0.115[a]	0.117[a]
SEM[1]	0.0084	0.0083

[1] Pooled standard error for n=3 analytical replicates.
[a, b] Means on the same line with different letter are significantly different ($P<0.05$).

Figure 1. *Protein hydrolysis of fine (300 to 425 µm) and coarse (1180 to 1600 µm) fractions from wheat flours (Baltimor and Scipion) using a proventriculus extract from a pool of 6 chickens (D^+ line. Baltimor fed) (pH=3.0. T=40°C. Proventriculus proteolytic activity added=8.3 U per mg wheat protein) (Mean±SEM with 2 analytical replicates).*



* particle size effect: *P*=0.0159.

Table 6. *Protein and starch hydrolysis (g/g) after the in vitro digestion simulation (gizzard and pancreas steps according to Tervilä-Wilo et al., 1996) on the Baltimor and Scipion flours. Proventriculus and pancreas enzymatic extracts are from D^+ birds fed on Baltimor wheat*

	Baltimor flour	Scipion flour	SEM[1]	Cultivar effect[2]
Protein hydrolysis (g/g)	0.750	0.778	0.0334	0.5711
Starch hydrolysis (g/g)	0.269	0.348	0.0031	< 0.0001

[1] Pooled standard error for n=4 analytical replicates.
[2] *P*-value.

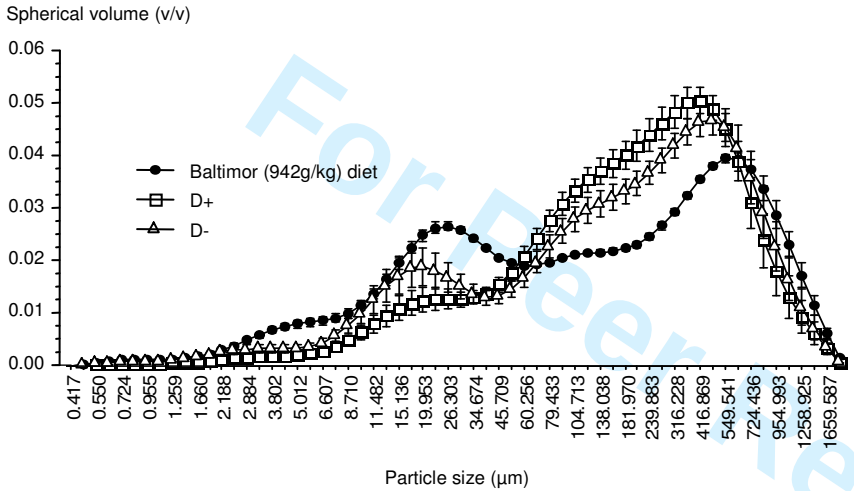
Table 7. *In vivo starch digestibility values (g/g) (Péron et al., 2006) and laser particle size analysis (spherical volume, v/v) of ileum contents in D^+ and D^- chicken lines fed with the wheat pelleted diets (942g wheat/kg diet; Baltimor or Scipion cultivar) (means and residual standard deviations)*

Line Wheat	D^+ Baltimor	D^+ Scipion	D^- Baltimor	D^- Scipion	RSD[1]	Line Effect[2]	Diet Effect[2]	Line x Diet Effect[2]
Starch digestibility (g/g) (n=18)	0.906	0.967	0.881	0.935	0.0347	0.001	<0.0001	0.732
n for particle size analysis	12	11	11	12	-	-	-	-
Main mode response type (n[3])	5	3	3	2	-	-	-	-
Bimodal response type (n[3])	7	8	8	10	-	-	-	-
Median diameter[4] of the ileum particles (µm)	233.9	198.3	222.5	158.9	109.75	0.4369	0.1334	0.6670
Main mode response type								
Peak position (µm)	362.7	416.8	457.8	553.5	93.18	0.0622	0.2025	0.7116
Proportion of particles (v/v) at peak position	0.0649	0.0627	0.0563	0.0755	0.0113	0.7516	0.2299	0.1392
Bimodal response type:								
Peak 1 position (µm)	20.8	18.8	20.6	18.9	0.75	0.8186	<0.0001	0.6301
Proportion of particles (v/v) at peak 1 position	0.0179	0.0352	0.0260	0.0417	0.0145	0.1597	0.0030	0.8758
Peak 2 position (µm)	480.8	520.1	491.1	422.6	162.78	0.4513	0.7999	0.3538
Proportion of particles (v/v) at peak 2 position	0.0554	0.0482	0.0531	0.0472	0.0108	0.6690	0.0947	0.8537
Proportion of particles (v/v) at the small peak position (17.4 µm)[5]	0.0120	0.0246	0.0186	0.0318	0.0147	0.1208	0.0051	0.9467
Proportion of particles (v/v) at the coarse peak position (478.6 µm)[5]	0.0491	0.0414	0.0470	0.0376	0.0141	0.4819	0.0451	0.8408

[1] Residual Standard Deviation.
[2] *P*-value.
[3] Number of birds.
[4] Half of particles are under this size (µm).
[5] Analysis with all birds (see Figures 2(a) and 2(b)) (n=11-12).

Figure 2. *Particle size distribution in the wheat (942 g wheat/kg) pelleted diets (Mean±SEM. n=6) and ileum contents of D^+ and D^- chickens fed on these diets (mean±SEM. n=11-12). (a) Baltimor wheat; (b) Scipion wheat.*

(a)



(b)

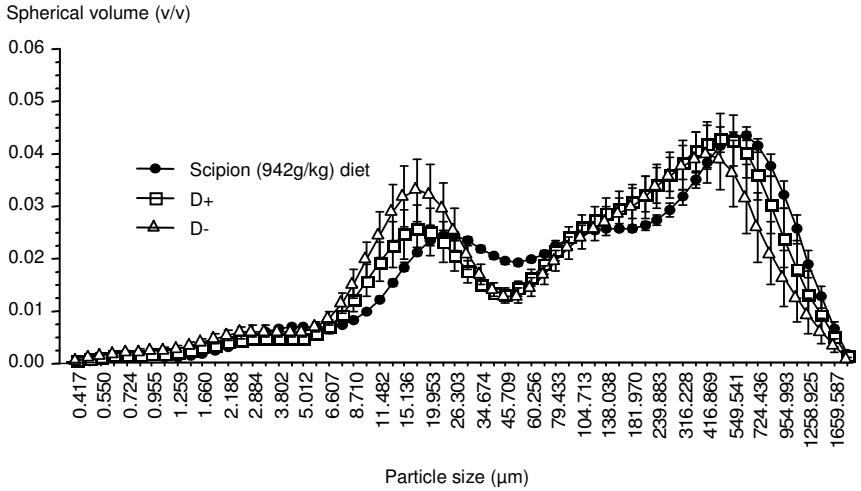


Figure 3. *Photomicrograph of an ileum content section. observed with bright-field microscopy and analysed with Visilog Software: starch granules (SG). aleurone+bran layer (ABL). example of an analysed subaleurone area (75 µm width) of a bran particle (AREA).*

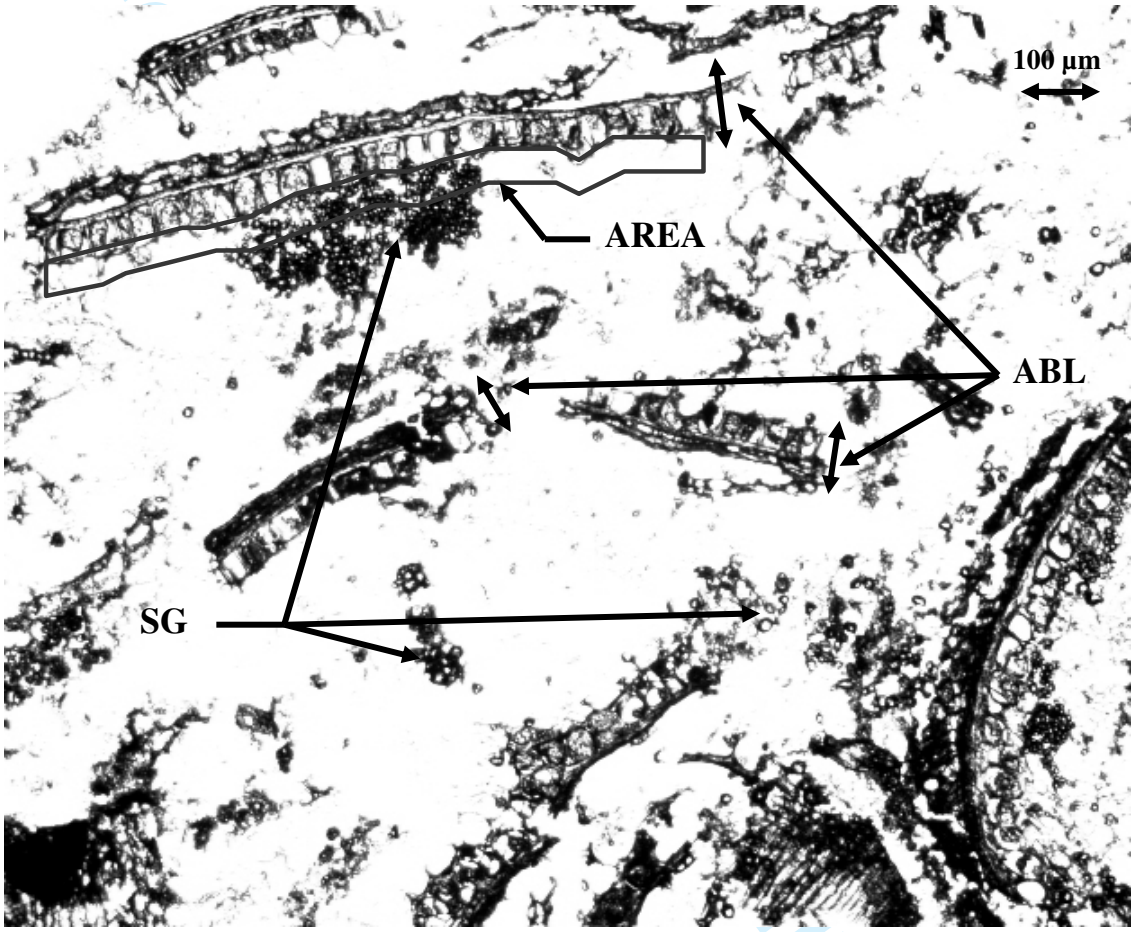

100 µm
AREA
ABL
SG

Figure 4. *Enlarged view of the subaleurone area observed with bright-field microscopy: aleurone cells (AC). starch granules (SG).*

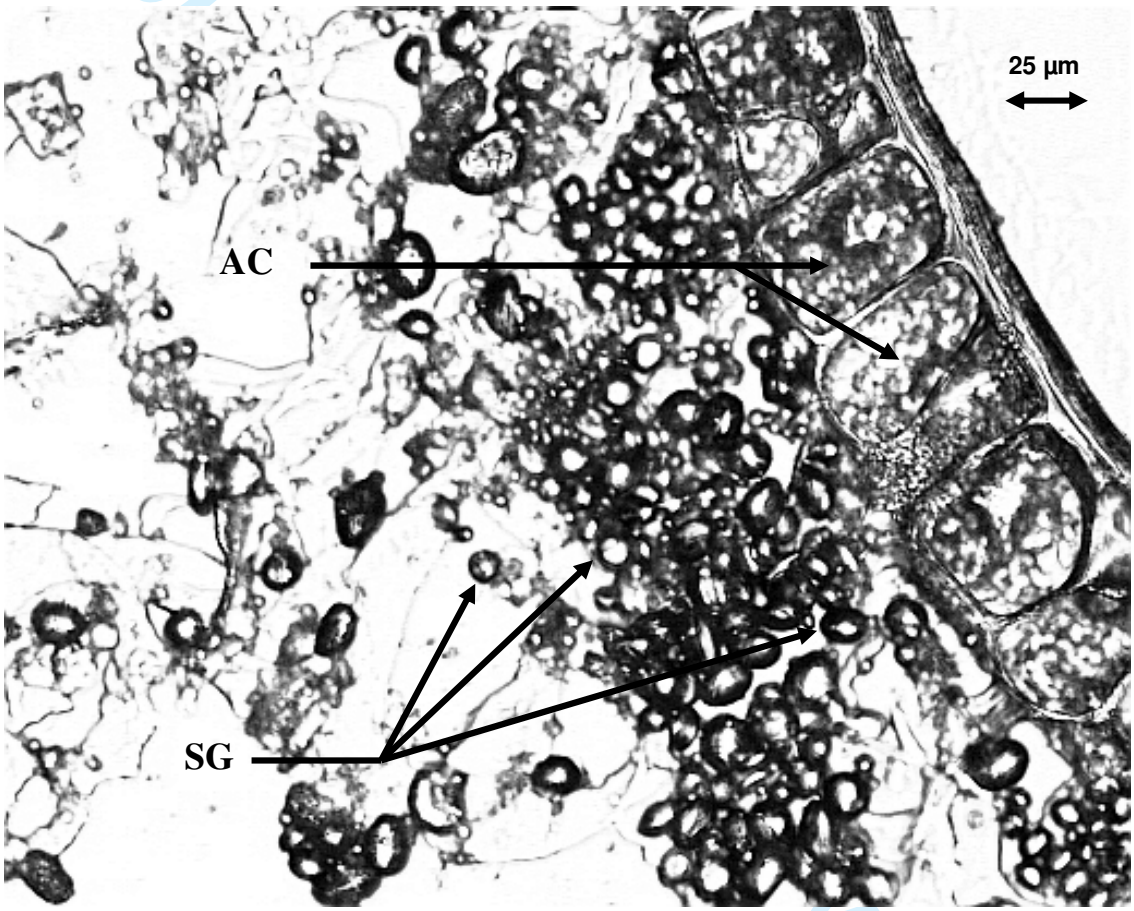

25 µm
AC
SG

Figure 5. *Photomicrograph of an ileum content section. observed with fluorescent microscopy: starch granules (SG). endosperm cell walls (ECW). aleurone+bran layer (ABL).*

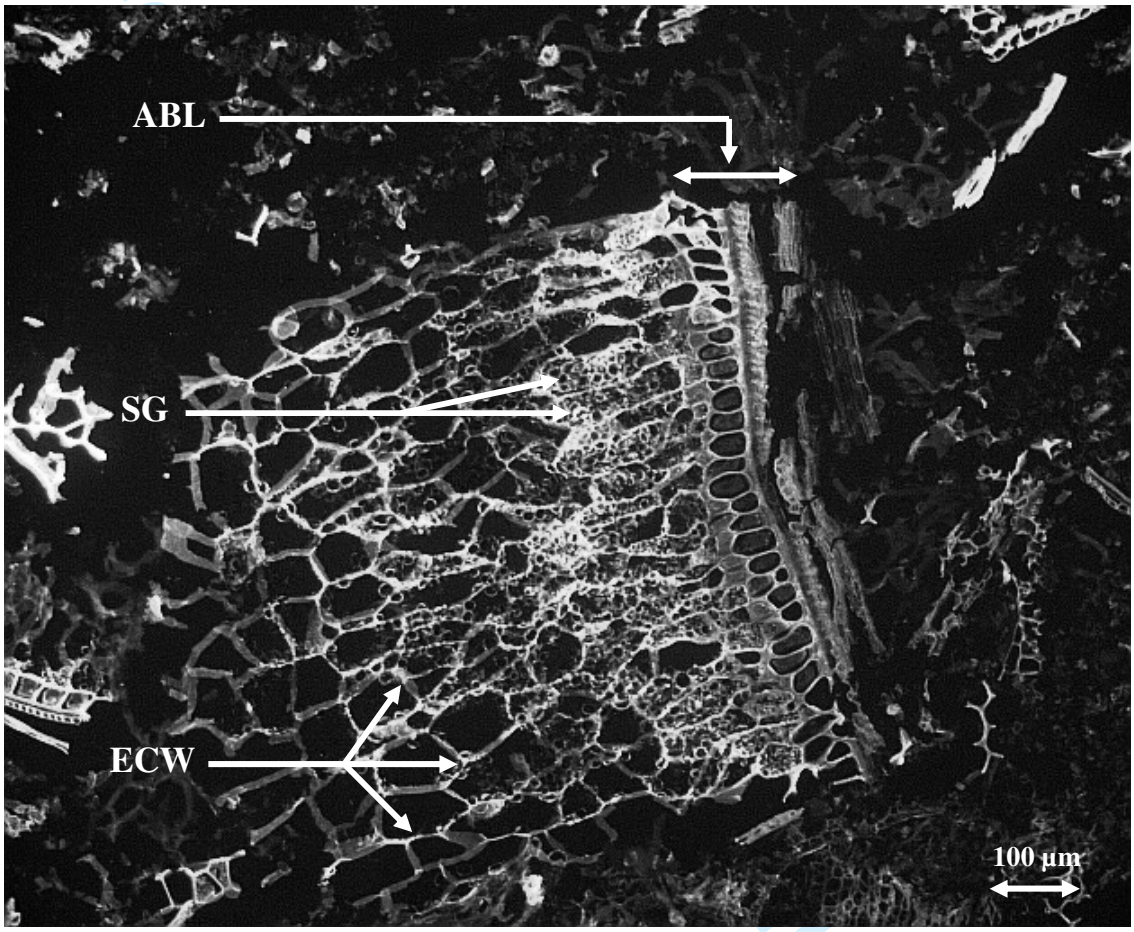

ABL
SG
ECW
100 µm

Table 8. *Image analyses of bran particles found in ileum contents of D^+ birds fed Baltimor or Scipion diets*

	Baltimor	Scipion	RSD[1]	Cultivar effect[2]
Lentgh of bran particles (µm)	1043.8	936.0	433.91	0.10
Estimated starch concentration in the subaleurone area (OD)	0.361	0.231	0.1937	< 0.001

[1] Residual standard deviation (df=180).
[2] *P*-value.
OD: Optical density. High values correspond to high starch concentration.