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Alexandre Péron, Birger Svihus, Irène Gabriel, Serge Bérot, Daniel Tanguy, et al.. 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.. *British Poultry Science*, 2007, 48 (03), pp.370-380. 10.1080/00071660701341963 . hal-00545311

HAL Id: hal-00545311

<https://hal.science/hal-00545311v1>

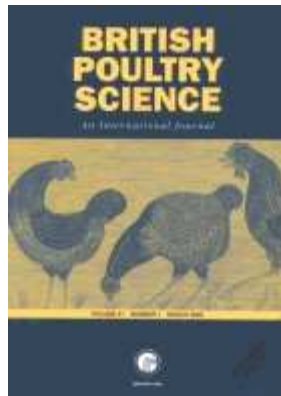
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Journal:	<i>British Poultry Science</i>
Manuscript ID:	CBPS-2005-060.R2
Manuscript Type:	Original Manuscript
Date Submitted by the Author:	17-Jan-2007
Complete List of Authors:	Péron, Alexandre; INRA, Unité de Recherches Avicoles Svihus, Birger; Agricultural 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

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DIGESTION OF HARD WHEAT

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Accepted for publication 16th February 2007

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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.

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3 1 7. Microscopic analyses of D⁺ ileum digesta revealed that the concentration of
4
5
6 2 undigested starch granules in sub-aleurone area of wheat bran particles was the highest
7
8 3 with hard cultivar.

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10 4 8. The results suggested that physical entrapment of starch granules in coarse particles
11
12 5 was a major explanation for decreased starch digestibility values in chickens fed hard
13
14 6 wheat diets.

7 INTRODUCTION

8 Hardness is a mechanical characteristic of wheat grain (*Triticum aestivum*) which is
9 thought to be due to interactions between starch granules and their surrounding protein
10 matrix (Barlow *et al.*, 1973; Simmonds *et al.*, 1973). Puroindolines a and b are major
11 proteins involved in this interaction (Giroux and Morris, 1998). High hardness value
12 results in less friable wheat endosperm and increased particle size after grain milling
13 (Abécassis *et al.*, 1997). Recent studies have demonstrated the negative effect of hard
14 wheat cultivar on starch digestibility in pelleted diets for broiler chickens (Carré *et al.*,
15 2002, 2005; Péron *et al.*, 2006). Some hypotheses have been proposed to explain this
16 negative effect, most of them involving an accessibility problem. Hard cultivar, high
17 particle size after grinding (Abécassis *et al.*, 1997; Carré *et al.*, 2002; Péron *et al.*, 2006)
18 and/or strong starch-protein interactions (Barlow *et al.*, 1973; Kim *et al.*, 2004) could
19 decrease physical access to starch granules and limit enzymatic digestion. As previously
20 shown by Péron *et al.* (2005), fine grinding of a hard wheat cultivar before pelleting
21 resulted in starch digestibility and AME_N improvements, indicating that particle size
22 could be an important factor affecting starch digestion of hard wheat. Concerning other
23 factors, it could also be supposed that protein matrix from hard cultivar is less
24 susceptible to proteases than protein matrix from soft cultivar.

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

14 MATERIALS AND METHODS

15 **Animals, wheat cultivars and experimental diets**

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

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

Tables 1, 2; Figures 1, 2 near here

11 **Digestive organ and ileum content sampling**

12 Sampling procedure for digestive organs and their contents was described in Péron *et al.*
13 (2006). Briefly, at 27 d, 12 birds per treatment were weighed and killed by intracardiac
14 injection of 1 ml of sodium pentobarbital (Sanofi, Marne la Coquette, France) for
15 collection of digestive organs and ileum contents. Proventriculus and pancreas were
16 quickly frozen in liquid nitrogen, stored at -70°C, ground by ball-milling in liquid
17 nitrogen and stored again at -70°C. Total ileum content was homogenised and divided
18 into two fractions F1 and F2: fraction F1 was frozen in liquid nitrogen and stored at -
19 70°C for further laser particle size analyses, and fraction F2 was frozen with isopentane
20 (cooled with liquid nitrogen) and stored at -70°C for further microscopic analyses. For
21 enzymatic studies (*in vitro* hydrolyses and digestion simulation), one pool of 6
22 proventriculi and one pool of 4 pancreases were carried out for each treatment. Pools
23 were constituted by mixing equal quantities of tissue powder from each bird. Pool
24 origins were designated as: "D⁺ line (Baltimor fed)", "D⁺ line (Scipion fed)", "D⁻ line
25 (Baltimor fed)" and "D⁻ line (Scipion fed)".

1 Analytical methods

2 In order to prepare *in vitro* hydrolyses and digestion simulation, some activities of
3 digestive enzymes of D⁺ and D⁻ lines were investigated. Pepsin activity (EC 3.4.23.1) in
4 proventriculus tissue was assayed at different pH with haemoglobin as a substrate. Two
5 analytical replicates were performed for each proventriculus pool. Anson's method
6 (1938) modified by Créviu-Gabriel *et al.* (1999) was used with some modifications as
7 follows: 100 mg tissue powder was homogenised in 2 ml of 0.01 M phosphate buffer
8 pH=7.4, then centrifuged at 10000 g (4°C, 15 min). Supernatants (50 µl) were diluted
9 with 950 µl of 0.01 M phosphate buffer pH=7.4. Then, 500 µl of diluted extract were
10 activated with 100 µl HCl 300 mM for 15 minutes and pepsin activity was measured by
11 adding 200 µl of the activated extract in 1.35 ml of a haemoglobin solution (20 g/l).
12 Pepsin activity of proventriculus was expressed as units/mg tissue. According to
13 Créviu-Gabriel *et al.* (1999), one pepsin unit (U) was defined as the amount of enzyme
14 that increased optical density (280 nm) by 0.001 per min under the assay conditions.
15 Proteolytic activity of pancreas was measured (with 4 replicates per pancreas pool) at
16 different pH values using a casein substrate, as described by Susbilla *et al.* (2003).
17 Pancreas proteolytic activity was expressed as units/g tissue, with 1 proteolytic unit (U)
18 being equivalent to 1 µmole tyrosine released per min under the assay conditions.
19 Amylolytic activity of pancreas was estimated (with 2 replicates per pancreas pool)
20 using 50 mg purified maize starch (Roquette, France) suspended in 1 ml of sodium-
21 phosphate buffer 0.05 M (pH=6.75) and placed in a thermostatically controlled water
22 bath (40°C). Pancreatic extract (490 µl from 150 mg homogenised tissue in 2.5 ml
23 Ringer solution pH=7.4) was added to the suspension. At various times of the
24 hydrolysis process (t=30 min, 1 h, 2 h, 3 h or 4 h), an aliquot was kept and centrifuged

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

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

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

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

14 Particle size distributions in ileum contents and in the wheat (942 g/kg) pelleted
15 diets (Baltimor and Scipion cultivars) were determined (12 birds replicates for D⁺
16 Baltimor and D⁻ Scipion, and 11 for D⁺ Scipion and D⁻ Baltimor) using a Malvern
17 Mastersizer S instrument (Malvern Instruments Ltd., Worcestershire, UK), as described
18 by Hetland *et al.* (2002). Particle diameters were detected in the range from 0.02 to
19 2000 µm. Results were analysed using the Malvern 2000 Software (version 5.22).

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

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3 1 microscopy was preferred to fluorescence because the staining process to be used with
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5 2 the latter observation technique resulted in starch granule losses. These losses were
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7 3 observed by comparing successive sections treated either with the bright-field technique
8
9 4 or with the fluorescence one. Bright-field microscopy analyses were made on ileum
10
11 5 contents of D⁺ birds only (n=11 birds per diet). For bright-field microscopy, the sections
12
13 6 (16 µm) were not stained and were observed with no preliminary treatment. Bright-field
14
15 7 sections were examined with a Zeiss Axioplan 2 microscope, fitted with phase
16
17 8 interference (H/DIC II). Photomicrographs were obtained using a Kappa camera
18
19 9 DX30C and analysed with Visilog Software (version 6.3). The software was
20
21 10 programmed in order to measure the length (µm) of particles. It was also able to
22
23 11 measure the concentration of starch granules in a defined area as the mean optical
24
25 12 density (OD) of this area. Observations were made of the subaleurone area of bran
26
27 13 particles. The subaleurone area was defined as described in Figure 3, with 75 µm width
28
29 14 for the considered area. An enlarged view of the subaleurone area is also shown in
30
31 15 Figure 4. About 10 particles per animal were observed.

16 **Statistical analysis**

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

23 **RESULTS**

24 **Proventriculus and pancreas enzymatic activities**

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

Tables 3, 4 near here

9 **Gluten hydrolyses with proventriculus extracts**

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

13 **Hydrolyses of fine and coarse wheat fractions with proventriculus extracts**

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

Figure 1 near here

19 ***In vitro* digestion simulation**

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

Tables 5, 6 near here

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

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

Figure 2, Table 7
near here

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

24 **Microscopy**

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

Figures 3,4,5, Table 8
near here

DISCUSSION

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

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

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

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

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

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

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3 1 It has previously been shown that, in the human small intestine, undigested
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5 2 starch granules of barley were mainly located close to the aleurone and bran layers
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8 3 (Livesey *et al.*, 1995). In the current experiment, a large amount of undigested starch
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10 4 granules was also observed in the endosperm subaleurone area of bran particles found in
11
12 5 the ileum. Microscopic analyses of these particles showed that the amount of starch in
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14 6 the endosperm subaleurone area was higher for the hard than for the soft wheat ileum
15
16 7 samples. This suggests that the problem of access with hard wheat mainly concerned the
17
18 8 endosperm subaleurone area. Subaleurone cells from hard wheat may be more resistant
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20 9 and then, after grinding, starch granules may be more protected from enzyme
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22 10 hydrolysis. Such a difference in resistance might be related to the difference in
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24 11 composition of subaleurone cell walls between hard and soft wheats (Barron *et al.*,
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26 12 2005).

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29 13 A previous study showed that adding protease (such as pepsin) could increase *in*
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31 14 *vitro* starch digestibility of cereals (Aura *et al.*, 1999), indicating that the resistance of
32
33 15 the protein matrix surrounding starch granules may affect starch digestion. Gluten is the
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35 16 major component of the protein matrix. Gluten hydrolyses with proventriculus extracts
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37 17 showed that there were no differences between hard and soft cultivar origins of gluten,
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39 18 which suggests that gluten was not involved. It might be supposed that 10 minutes of
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41 19 hydrolysis was too short to result in observation of notable differences. However, with
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43 20 the hydrolysis of fine and coarse particles from Baltimor and Scipion wheat flours, or
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45 21 with the *in vitro* digestion simulation of whole flours, protein hydrolysis was longer (4 h
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47 22 or 6.75 h, respectively) and no significant differences were observed between hard and
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49 23 soft wheat flours. Thus, differences in starch digestibility probably did not result from
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51 24 differences in protein susceptibility to enzyme hydrolysis.

52 53 54 55 56 57 58 59 60 CONCLUSION

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

11 ACKNOWLEDGMENTS

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

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12 **Table 1.** *Chemical and physical composition of wheat samples from Baltimor and*
13 *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

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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.

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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 proventriculus¹ and pancreas² (expressed as U/mg tissue or U/g tissue) at 27 d, and kinetics of hydrolysis of pure maize starch with pancreatic extract³*

	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 nm 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 proventriculus¹ and pancreas² (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 nm 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.

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

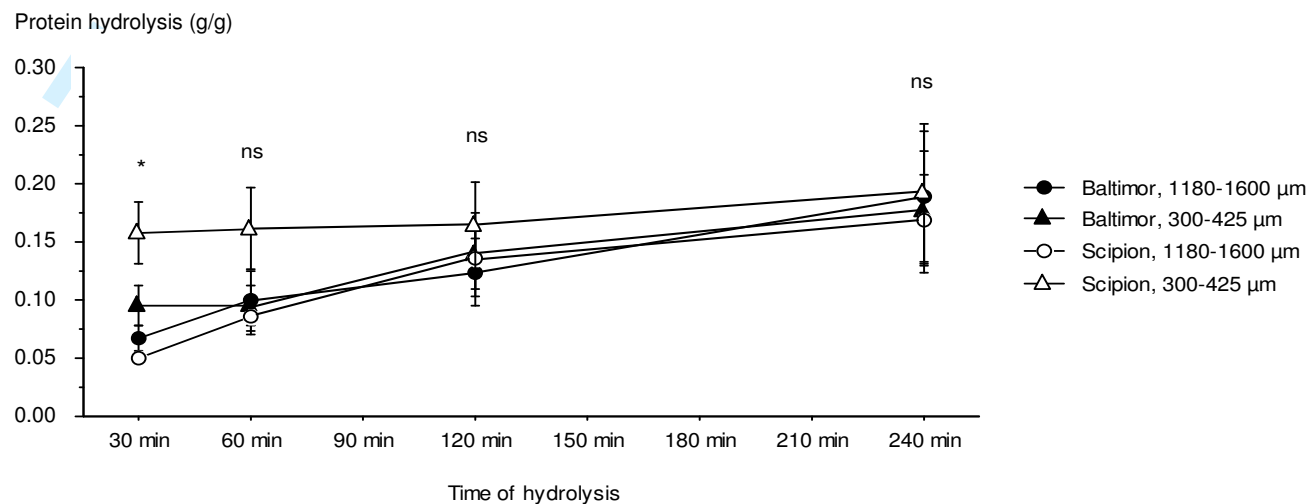
Origin of proventriculus extract pool	Wheat cultivar origin of gluten	
	Baltimore	Scipion
D ⁺ line (Baltimore fed)	0.109 ^a	0.110 ^a
D ⁺ line (Scipion fed)	0.103 ^a	0.105 ^a
D ⁻ line (Baltimore fed)	0.113 ^a	0.121 ^a
D ⁻ line (Scipion fed)	0.115 ^a	0.117 ^a
SEM ¹	0.0084	0.0083

¹ Pooled standard error for n=3 analytical replicates.

^{a, b} Means on the same line with different letter are significantly different ($P < 0.05$).

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53 **Figure 1.** Protein hydrolysis of fine (300 to 425 μm) and coarse (1180 to 1600 μm) fractions from wheat flours (Baltimor and Scipion)
 54 using a proventriculus extract from a pool of 6 chickens (D^+ line, Baltimor fed) ($\text{pH}=3.0$, $T=40^\circ\text{C}$, Proventriculus
 55 proteolytic activity added=8.3 U per mg wheat protein) (Mean \pm SEM with 2 analytical replicates).
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* particle size effect: $P=0.0159$.

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60 **Table 6.** *Protein and starch hydrolysis (g/g) after the in vitro digestion simulation*
61 *(gizzard and pancreas steps according to Tervilä-Wilo et al., 1996) on the*
62 *Baltimor and Scipion flours. Proventriculus and pancreas enzymatic extracts*
63 *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

64 ¹ Pooled standard error for n=4 analytical replicates.

65 ² P-value.

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

Line	D ⁺	D ⁺	D ⁻	D ⁻		Line	Diet	Line x Diet
Wheat	Baltimor	Scipion	Baltimor	Scipion	RSD ¹	Effect ²	Effect ²	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

70 ¹ Residual Standard Deviation.

71 ² P-value.

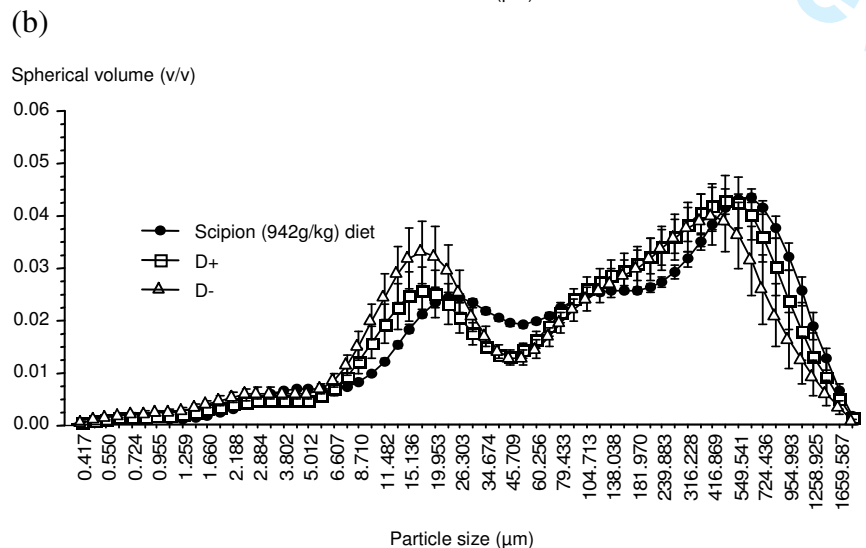
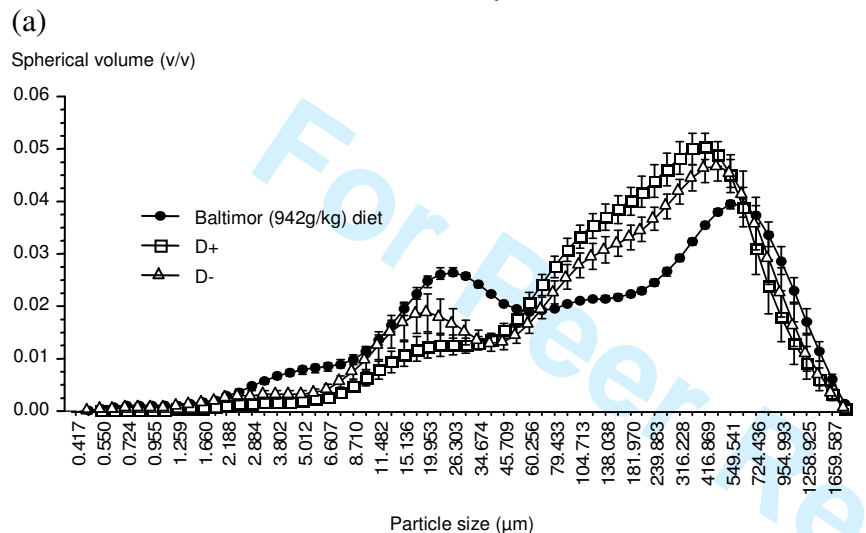
72 ³ Number of birds.

73 ⁴ Half of particles are under this size (µm).

74 ⁵ Analysis with all birds (see Figures 2(a) and 2(b)) (n=11-12).

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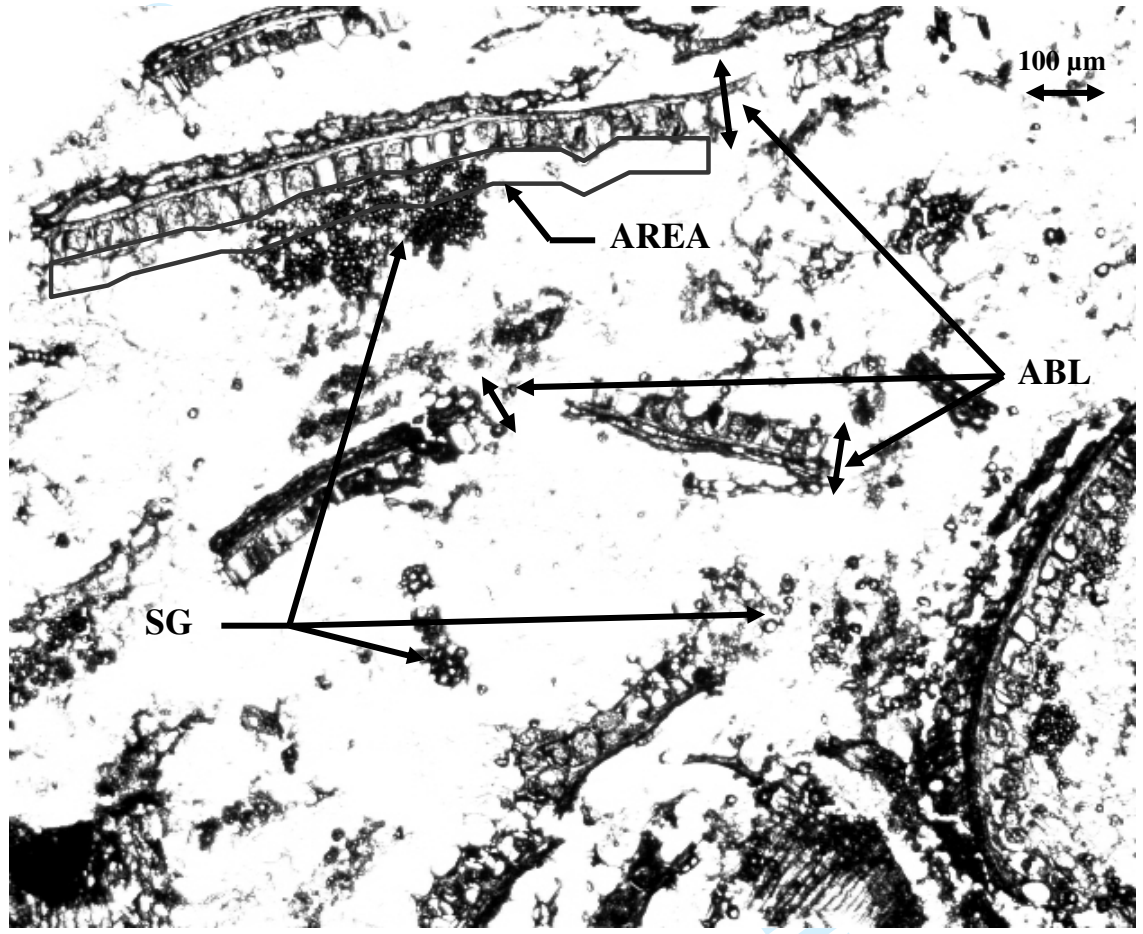
76 **Figure 2.** Particle size distribution in the wheat (942 g wheat/kg) pelleted diets (Mean±SEM, n=6) and ileum contents of D⁺ and D⁻
77 chickens fed on these diets (mean±SEM, n=11-12). (a) Baltimor wheat; (b) Scipion wheat.
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4 83 **Figure 3.** *Photomicrograph of an ileum content section. observed with bright-field*
5 84 *microscopy and analysed with Visilog Software: starch granules (SG).*
6 85 *aleurone+bran layer (ABL). example of an analysed subaleurone area*
7 86 *(75 µm width) of a bran particle (AREA).*
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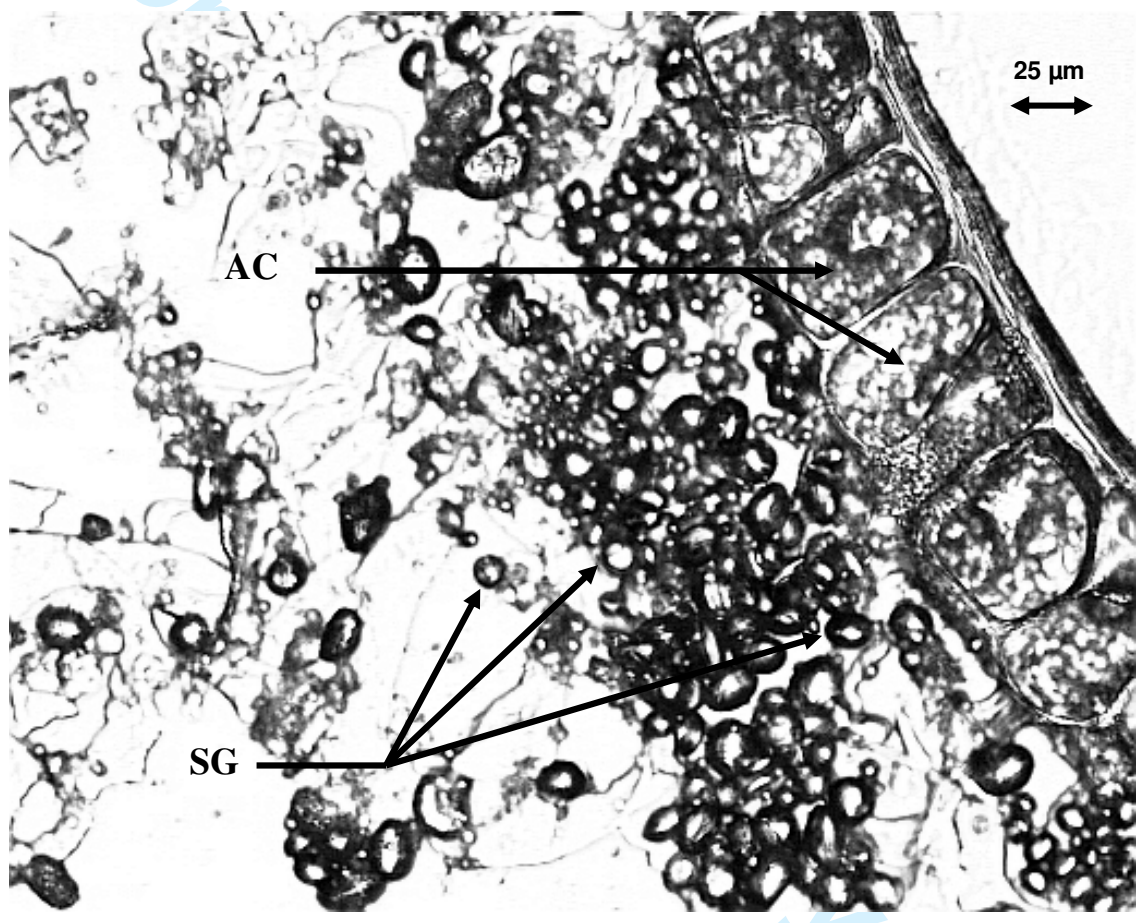
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4 108 **Figure 4.** *Enlarged view of the subaleurone area observed with bright-field*
5 109 *microscopy: aleurone cells (AC). starch granules (SG).*
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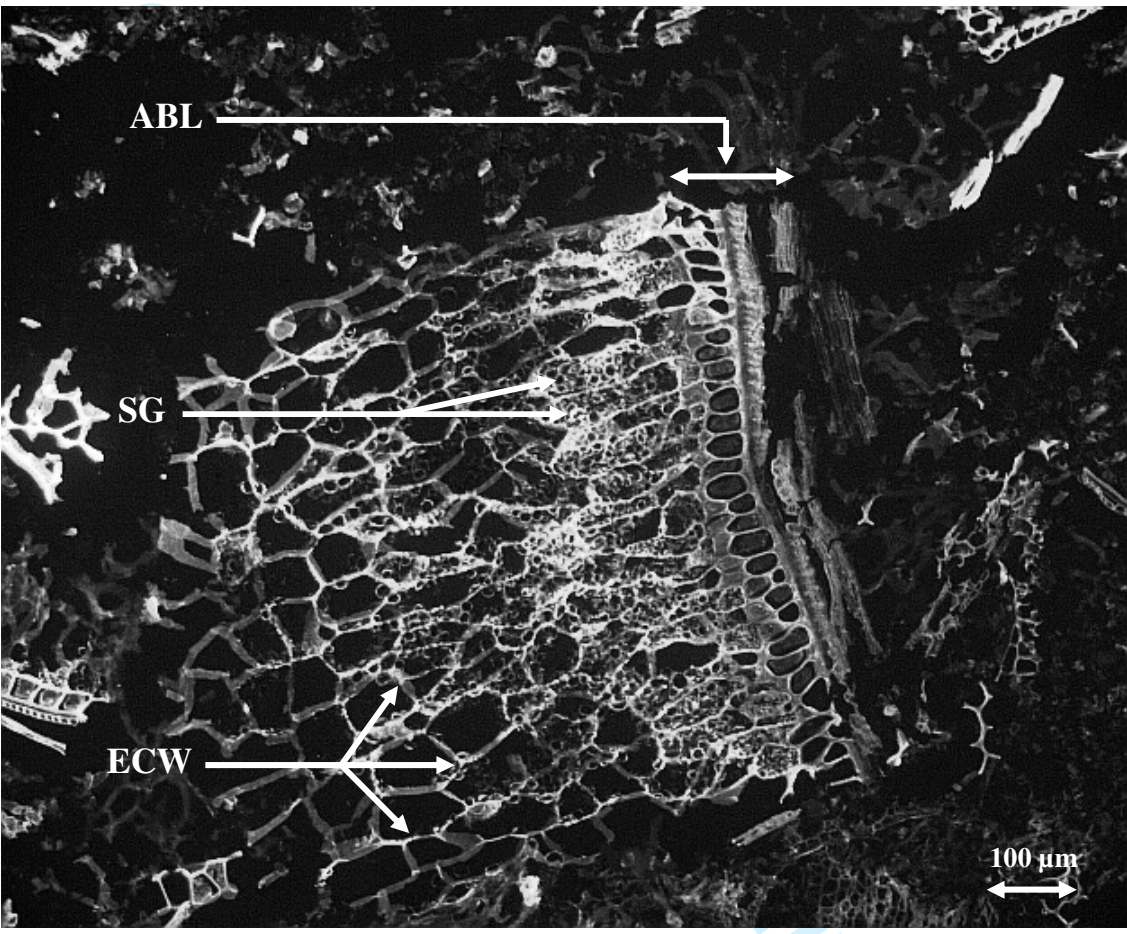


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4 132 **Figure 5.** *Photomicrograph of an ileum content section, observed with fluorescent*
5 133 *microscopy: starch granules (SG), endosperm cell walls (ECW),*
6 134 *aleurone+bran layer (ABL).*
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156 **Table 8.** *Image analyses of bran particles found in ileum contents of D⁺ birds fed*
 157 *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

158 ¹ Residual standard deviation (df=180).

159 ² *P*-value.

160 OD: Optical density. High values correspond to high starch concentration.

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