Effect of different chilling rates on the quality parameters of mule duck fatty liver

S. Awde,[*,†,‡] N. Marty-Gasset,[*,†,‡] G. Sandri,[§] A. Dalle Zotte,[§] and H. Rémignon[†,*,‡,1]

[*] *INRA, UMR1388 Génétique, Physiologie et Systèmes d'Elevage, F-31326 Castanet-Tolosan, France;* [†] *Université de Toulouse INPT ENSAT, UMR1388 Génétique, Physiologie et Systèmes d'Elevage, F-31326 Castanet-Tolosan, France;* [‡] *Université de Toulouse INPT ENVT, UMR1388 Génétique, Physiologie et Systèmes d'Elevage, F-31076 Toulouse, France; and* [§] *University of Padova, Agripolis, 35020 Legnaro (PD), Italy*

ABSTRACT The aim of this experiment was to study the effect of chilling rates on the quality features of fatty livers. Three different chilling rates were applied: ultra-fast (UF), fast (FA), and slow (SL). Technological and proteomic results were compared at time T1 when the internal temperature of livers reached 10°C and at time T2 = 24 h post mortem. Samples from the UF group reached the T1 temperature at 50 min post mortem and had the least hard livers and the lowest cooking loss percentage (25 ± 9%) at time T2 = 24 h post mortem (*P*-value of < 0.01). The FA and SL groups reached the T1 temperature after 120 and 210 min post mortem and presented higher melting (36 ± 9 and 41 ± 9%, respectively, at time T2) and harder livers compared to the UF group. In parallel, we conducted semi-quantifications of proteins by electrophoresis and proteolytic activities by mono-dimensional zymography for three families of proteases: Matrix metalloproteases (MMP), Cathepsins, and Calpains. The proteomic assays revealed less modified proteolytic activities in samples from the UF group, and less associated proteins degradations than in samples from the FA and the SL groups. Effects of the different chilling rates were mainly significant at time T2 (24 h post mortem). As a conclusion we were able to highlight an indirect positive relation between proteolysis and melting yield in ducks' fatty liver.

Key words: fatty liver, technological yield, zymography, texture, proteases

2015 Poultry Science 94:3015–3024
http://dx.doi.org/10.3382/ps/pev300

INTRODUCTION

From a sanitary point of view, animal products have to be chilled as soon as possible after slaughter. Adopting a suitable chilling method and rate is crucial and can affect the technological and organoleptic qualities of the final product. It is well known that temperature plays an important role in the determination of the texture, which is considered to be the most important quality attribute of meat products (Petäjä et al., 1985; Ali et al., 2008). Post mortem, the degradation of animal tissues proteins under the action of proteolytic enzymes contributes to this tenderization of flesh. While this phenomenon is appreciated in meat because it improves tenderness, it is undesirable in fish and leads to its softening (Cheret et al., 2007). As for other animal products, the texture of "foie gras" (fatty liver) is an important quality parameter. Based on its texture, three classes of fatty liver can be distinguished, i.e., soft, firm and hard fatty livers (Goullieux and Chanut, 2006). In fact, the texture of a fatty liver is determined by its biochemical composition and mainly by its water and fat contents. The richer the fatty liver is in water the softer it is, and the richer in fat the harder it is (Goullieux and Chanut, 2006). Softness is considered as a defect in fatty livers because it also induces the highest weight losses during chilling and/or storage because of its high water content (Cazeils, 2000). However, although hard fatty livers present the lowest weight losses during post-mortem processing, they are not desired by processors. In fact, the texture is strongly correlated to cooking yield, the major quality issue that faces the industry of fatty liver. Industrials have noticed that the harder the liver the more it melts during cooking. Melting consists of an exudation essentially of lipidic nature due to heating treatments (sterilization or pasteurization) and could be influenced by many factors such as the genetic type of force-fed palmipedes (Salichon et al., 1994): geese vs. mule, Pekin, or Muscovy ducks, the weight of the fatty liver (Blum et al., 1990) or its total lipids content (Nir and Nitsan, 1976). Rearing factors such as the duration of force-feeding or the age of birds can also influence the melting of fatty livers during cooking. Finally, technological practices such as delaying the cold storage time have also been shown to alter the

Received April 11, 2015.
Accepted August 27, 2015.
[1]Corresponding author: remignon@ensat.fr

cooking yield (Rousselot-Pailley et al., 1992). However, despite standard practices used by fatty livers processors to maintain the variability of fat loss at a lowest point, the inter-individual variability remains high. The coefficient of variation of cooking losses within a flock can exceed 50% under commercial conditions (Theron et al., 2011b). Cooking losses are then of great importance for processors because it affects their output and thus their profitability. Moreover, cooking losses cause alterations in organoleptic properties and consequently degrade the image of the final product which is sought for its particular richness in fat (Théron, 2011). Firm fatty livers are preferred by processors because they represent a good compromise between high water and fat losses resulting from soft and hard fatty livers respectively (Goullieux and Chanut, 2006). For this reason, the texture of fatty livers is manually estimated and livers are usually sorted into the 3 classes in order to reduce the melting rate variability. Nevertheless, this sorting alone is not sufficient and should be coupled with an adequate chilling method in order to obtain the highest technological yield. Surprisingly, unlike meat, the chilling rate effect on the fatty liver has been poorly studied. In meat, post mortem proteolytic activities are known to be involved in biochemical and structural changes during aging (Lametsch et al., 2002).

In this study, we investigated the effect of 3 different chilling rates (ultra-fast, fast, and slow) on proteolytic activities of three major types of proteases (calpains, cathepsins and matrix métalloprotéases-2 or MMP-2) to better understand if they could be associated with differences in the technological yield (or cooking losses) of fatty liver. We also measured the effects of storage for 24 h on both proteolytic activities and the technological yield.

MATERIAL AND METHODS

Animals

The experiments described here fully complied with the legislation on research involving animal subjects according to the European Communities Council Directive of November, 24 1986 (86/609/EC). Investigators were certificated by the French governmental authority for carrying out these experiments (accreditation n°31–11 43 501). Livers used for this experiment were issued from commercial flocks of male mule ducks (*Caïrina moschata* x *Anas platyrhynchos*) reared until the age of 13 weeks according to standard practices. At this age, ducks were force-fed by the distribution of a soak-corn mixture (42% grain–58% flour) twice daily during a period of 12 days. Ducks were then slaughtered in a commercial plant.

Experimental Methods

After slaughtering operations, fatty livers were eviscerated, weighed, and because the liver weight is known to affect the final melting rate, only fatty livers weighing from 550 to 600 g were collected (n = 72 livers). Those livers were then randomly assigned to 3 experimental groups (24 each) and submitted to a particular chilling protocol. The slow chilling (**SL)** group consisted of livers that were immediately placed in a refrigerator at 4°C (±1°C). The fast chilling livers (**FA** group) were placed in a negative air (−3 °C) blowing cooling tunnel for 15 min and subsequently placed at 4°C. The ultrafast chilling (**UF)** group were placed in the same tunnel during 3 × 15 min and subsequently placed at 4°C (±1°C). Thermic sensors (precision = 0.1°C) were randomly placed in the core of two livers per treatment to record the temperature evolution.

Fatty Liver Processing and Sampling

At the time of evisceration (**T0** = 20 min after slaughter), and before any chilling treatment, 10 g samples were collected, frozen in liquid nitrogen and stored at −80°C. The second sampling (**T1**) was carried out on half of the samples (n = 12 samples/group) when the internal liver temperature reached 10°C in each respective group. The rest of the livers were kept at 4°C until **T2** = 24 hours when the third sampling was performed (n = 12 samples/group).

At times T1 and T2, 10 g of liver samples were collected for proteomic analysis, frozen in liquid nitrogen and stored at −80°C until analysis. Additive samples of 60 g were taken from the middle part of the livers and placed into a glass can to be used for the melting test (times T1 and T2). Salt (13 g/kg) and pepper (2 g/kg) were just added to those samples and the glass cans were cooked in an autoclave at 90°C for 75 min. The cans were subsequently chilled using tap water and stored at 4°C for two months to mimic commercial mean storage period. After this storage period, livers were removed from glass cans and all visible exterior fat was trimmed by a gentle scrapping using a knife. The technological yield was then evaluated by the expression of fat losses during cooking and storage and expressed as a percentage of the initial weight:

$$\text{Technological yield (\%)} = (\text{fat trimmed liver weight} / \text{raw liver weight}) * 100.$$

And thus, melting rate (%) = 100- Technological yield.

At time T1, using a wire, two cores of 1.5 cm long were cut out from the middle part of each liver to be used for texture evaluation. Discs of a 2 cm diameter were taken from each core using a cylindrical shape cutter. The texture of discs was then evaluated after a bicycle compression test (Texture Profile Analysis) of a disc using a texturometer (compression speed 1mm/s; 60% deformation). The texture was measured by 5 parameters (hardness, cohesiveness, elasticity, gumminess and chewing) as described by Meullenet et al. (1998).

Biochemical Analysis

All biochemical analyses were performed with ground samples of liver (particle size of < 0.5 mm) and in duplicate to minimize technical variations. The dry matter content of fatty livers was determined by drying a mass (2.5 ± 0.1 g) of grounded liver in an oven at 105°C for 24 hours (Molee et al., 2005).

Total lipids content of raw samples was determined according to Folch et al. (1957). After the extraction of lipids by a chloroform/methanol solution, quantification of total lipids content of each sample was performed gravimetrically after evaporation of the chloroformic phase. Total lipid content was expressed as a percentage of the dry matter.

The protein percentage was determined by the method of Dumas (AOAC, 1989). The technique implies a complete combustion at 850°C of the protein matrix. The gases and by-products of combustion were condensed at 6°C and total nitrogen is then detected and quantified. The crude proteins content is estimated by the formula:

$$\text{Crude proteins} = \text{Total nitrogen} \times 6.25$$

Proteomic Analysis

Protein Extraction. Acylamide, BisAcrylamide and Coomassie Brilliant Blue were purchased from Bio-Rad (Marnes la Coquette, France). All other chemicals were from Sigma-Aldrich (Saint-Quentin Fallavier, France). Livers samples of 10 g were primarily crushed into powder in liquid nitrogen. Liver lysis was then achieved using Fastprep-24 (MP BioMedicals, Illkirch, France) for 20 seconds. Brielfly, 1.5 mL of extraction buffer A (50 mM Tris pH 7.5, 150 mM NaCl, 1 mM $CaCl_2$, 0.05% Brij 35, 10 μg/mL leupeptin, 1 mM PMSF) and 1.2 mL of extraction buffer B (50 mM Tris-Base pH 8.3, 20 mM EDTA, 10 mM EGTA, 0.1% ß-mercaptoethanol) were added respectively to 75 and 300 mg of liver tissue in order to extract gelatin (MMP-2 and cathepsins) and casein (calpains) degrading proteases respectively. Samples were then centrifuged at 12,000 g at 4°C for 15 min and the fat cake (extracted lipids) was removed. This operation was repeated twice to make sure to get a good separation of soluble proteins from lipids and other cell components. Finally, supernatants were collected and stored at −80°C. Total protein concentration in extracts (supernatants) was determined using Bradford's method with Bovine Serum Albumin as protein standards. For each extraction buffers, the extractability i.e., the yield of extractable proteins by a given extraction buffer (xtgelt and xtcalp for gelatin and casein degrading proteases extraction buffers, respectively) was calculated from the total quantity of proteins found in the raw liver.

MMP-2 Gelatin Zymography. Matrix metalloproteinase 2 (MMP-2) gelatinolytic activity was detected according to Kizaki et al. (2008). Briefly, samples containing 40 μg of proteins were mixed (1/4 vol/vol) with the sample buffer (150 mM Tris Base pH 6.8, 30% glycerol, 4% SDS, 40 μM $ZnCl_2$, and 0.1% Bromophenol Blue) and loaded onto a 12% polyacrylamide gels containing 0.2% gelatin A (1 mm spacers; 5 mL/minigel from Bio-Rad, Marnes la Coquette, France). Gels were then run under denaturing but non-reducing conditions (4°C, 125 V, 2 h). Proteolytic activity was finally achieved through washing gels for 30 min in TBS-T buffer (20 mM Tris, 150 mM NaCl, 0.05% Tween) and 1 h in the incubation buffer (50 mM Tris HCl pH 7.5, 6 μM $ZnCl_2$, 5 mM $CaCl_2$, 0.05% Brij, 0.02% NaN_3) with 2.5% Triton X-100. Following this, gels were incubated overnight (18 h) at 37°C in the same buffer but without Triton-X 100. Finally, zymograms were stained with a solution containing 0.1% Naphthol Blue Black for 2 h, and then washed with a destaining solution (45% ethanol and 10% acetic acid). Digestion of the gelatin due to MMP-2 activity appeared as white bands on a dark blue background.

Cathepsins Gelatin Zymography. Cathepsins activities were detected according to Afonso et al. (1997). Briefly, samples containing 15 μg of extracted proteins were loaded on 12% polyacrylamide gels containing 0.2% gelatin and run as previously described. Resulting gels were then washed twice for 30 min in a 0.1 M sodium acetate buffer solution (pH 5.2) containing 2.5% Triton X-100. Then, gels were incubated overnight with the incubation buffer (0.1 M sodium acetate + 2 mM DTT) at 37°C. Gels were then stained in a 0.5% Coomassie Brilliant Blue R-250 solution for 2 h before washing in a destaining solution (45% ethanol and 10% acetic acid). Digestion of the gelatin due to cathepsins activities appeared as white bands on a blue background.

Calpains Casein Zymography. Using the method of Raser et al. (1995), calpain activities were detected on 10% polyacrylamide gels containing 0.2% casein and previously loaded with samples containing 40 μg of proteins diluted (Vol/Vol, 1/4) in the calpain sample buffer (150 mM Tris base, 20% glycerol, 0.05% Bromophenol Blue, 0.75% ß-mercaptoethanol). Gels were run in a calpain running buffer (192 mM glycine, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 25 mM Tris base, pH 8.3) for 2 h (4°C, 100 V). Then, gels were washed twice in a calpain activation solution (20 mM Tris base, pH 7.4, 10 mM $CaCl_2$) for 30 min and incubated (overnight at 37°C) in the same buffer containing 10 mM DTT. Finally, gels were stained and destained as previously described for cathepsins. Digestion of the casein due to calpains activity appeared as white bands on a blue background.

SDS-PAGE. Proteins were separated on 10% polyacrylamide gels. Samples diluted (Vol/Vol, 4/1) in the Laemmli's sample buffer (2% SDS, 5% ß-mercaptoethanol, 10% glycerol and 62 mM Tris-HCl, pH6.8) were loaded with 60 μg proteins per lane.

SDS-PAGE analyses were performed following the method described by Laemmli (1970) using Mini-Protean II electrophoresis unit (125 V for 2h). After the run, gels were stained overnight in Coomassie Brilliant Blue G-250 (PageBlue Protein Staining Solution, FERMENTAS).

Image Analysis. Zymographic gels were scanned with an Image Scanner III using Image Master Platinum software (GE Healthcare, Uppsala, Sweden). Each liver sample was processed individually, and the value for main bands was calculated by using Image Master Platinum application. Each gel also included a pool of all samples run and was used as an internal standard. Protease activity was considered as the combination of computerized measurements of both intensity and volume of main bands for each sample. Then, relative protease activity was estimated as protease activity/pool activity within each gel.

According to the procedure described by Marino et al. (2013) the identification of proteins molecular weight was done by comparison with a known molecular weight standard (precision plus protein standard-broad range, Bio-Rad, Marnes la Coquette, France). Bands were analyzed using Image Studio Lite 3.0 free online software (www.licor.com/islite) to determine the signal intensity (optical intensity) of the defined band. The relative quantity of each band was determined as signal intensity of the defined band/signal intensity of the pool.

Band Identification by Mass Spectrometry. Bands of interest were manually excised in our laboratory and analyzed by the spectrometry platform PEFM (Plate-Forme d'Exportation du Métabolism, INRA de Theix). Proteins were extracted, and Trypsin digested was analyzed by nano-LC-MS/MS coupled to an LTQ-Orbitrap mass spectrometer according to the method described by Theron et al. (2011b). MS/MS spectra were processed by Mascot software against the *Gallus gallus* (SwissProt-TrEmbl) and *Caïrina moschata-Anas platyrhynchos* (NCBI) databases. Protein identification was validated when at least 3 peptides originating from one protein showed significant identification Mascot score (P-value of < 0.005). Only proteins with a corresponding molecular weight and with at least 10 matching peptides were retained in this study.

Statistical Analysis

For statistical analysis, the General Linear Model procedure of SAS (2011) software was used initially with a 2-way Anova to compare the effects of chilling rates and times post-mortem. When the interaction between chilling rates and times was not significant (P-value of >0.05), independent 1-way Anovas were further performed. When appropriate, means were compared according to Student-Newman and Keuls' test. Correlation values were performed according to Pearson (Proc Corr, SAS 2011).

Table 1. Pre-treatment effect of chilling groups: "UF (ultra-fast), FA (fast), and SL (slow)" on liver weight and biochemical parameters at time T0 (20 min post mortem) in mule ducks fatty liver (n = 24/group).

Chilling rate	UF	FA	SL	RMSE	$P(c)$[1]
Liver weight (g)	574.0	574.4	573.8	9.5	NS
Dry matter (%)	69.7	70.3	70.5	3.6	NS
Lipids (%)	60.5[b]	64.3[a]	60.2[b]	4.4	**
Crude proteins (%)	6.7	6.9	7.4	1.1	NS
Extractability buffer A	15.6	15.2	15.4	1.6	NS
Extractability buffer B	15.2	14.0	14.1	3.4	NS

[1] $P(c)$: effect of chilling rate. ** for P-value of <0.01, and NS for P-value of >0.05. Within a row, means assigned with different letters (a and b) are significantly different, P-value of <0.05

RESULTS AND DISCUSSION

The objective of this experiment was to study the effect of different initial chilling rates and short post mortem storage on the quality parameters of fatty liver. To ensure that any difference is not group related, we chose to compare livers at time T0 = 20 min post mortem before the application of any chilling treatment (Table 1). Because it is already known that the weight of fatty liver is one of the most important parameter affecting the melting rate, we chose to work with a very narrow range of weight (550 to 600 g) to prevent any weight-linked variability. As a whole, livers had a 70.2% dry matter content of which 62% were lipids and 7% proteins. These values are very similar to those previously reported by Salichon et al. (1994). However, while all biochemical parameters turned to have similar values for the three treatments at time T0, the percentage of lipids was found to be slightly higher (4 points) in the FA group. Nevertheless, the dry matter contents were similar in the 3 groups of samples and we conclude that the observed difference in total lipids contents can only be technically-linked.

To ensure that the different adopted methods of chilling had a real impact on the chilling rate, a couple of thermic sensors were placed in the core of 2 livers per treatment in order to monitor the temperature evolution during 6.5 h. The second sampling (T1) was performed when the internal temperature of the liver reached 10°C. This temperature corresponds to the one used in processing plants because it facilitates the hand manipulation of fatty livers. Samples from the UF group were the first to reach this temperature at T1 = 50 min, while samples from the FA and SL groups reached it after 120 and 210 min post mortem respectively (Figure 1). This clearly confirms that subjecting livers to negative air refrigeration is more efficient than the conventional chilling method i.e., placing livers in the fridge (at + 4°C). The UF group maintained a very fast temperature decrease of 0.5°C/min. However, while the FA group had a comparable initial speed during its journey in the tunnel, its chilling speed significantly decrease after being placed at 4°C, which led to an overall chilling speed of 0.2°C/min. The chilling speed of livers

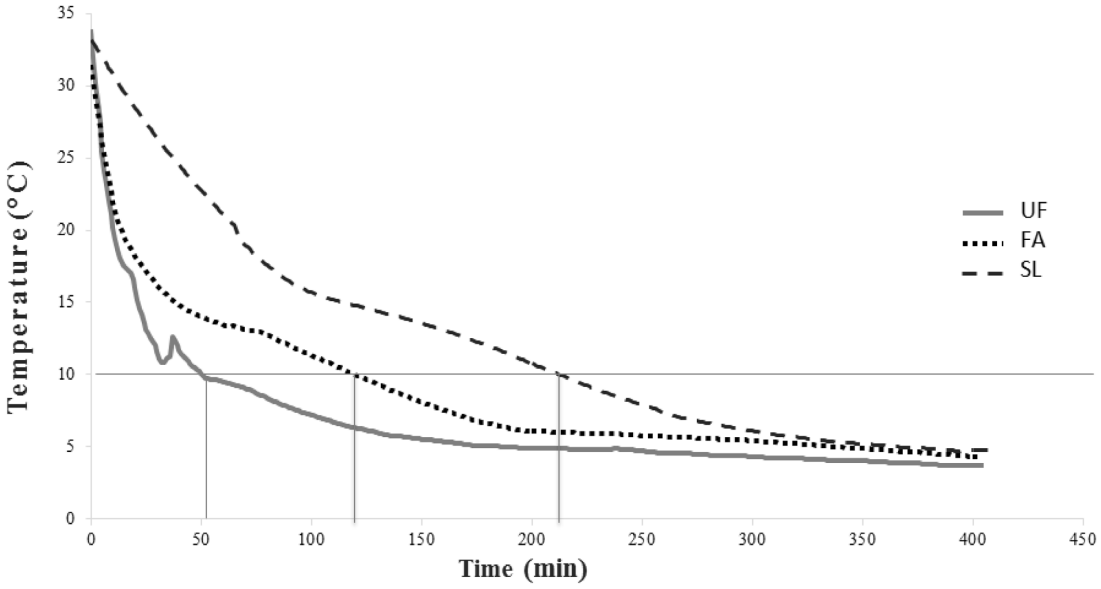

Figure 1. Internal temperature evolution of mule ducks fatty livers for the 3 different chilling methods: Ultra-Fast (UF), Fast (FA), and Slow (SL) during post mortem storage (n = 2/group).

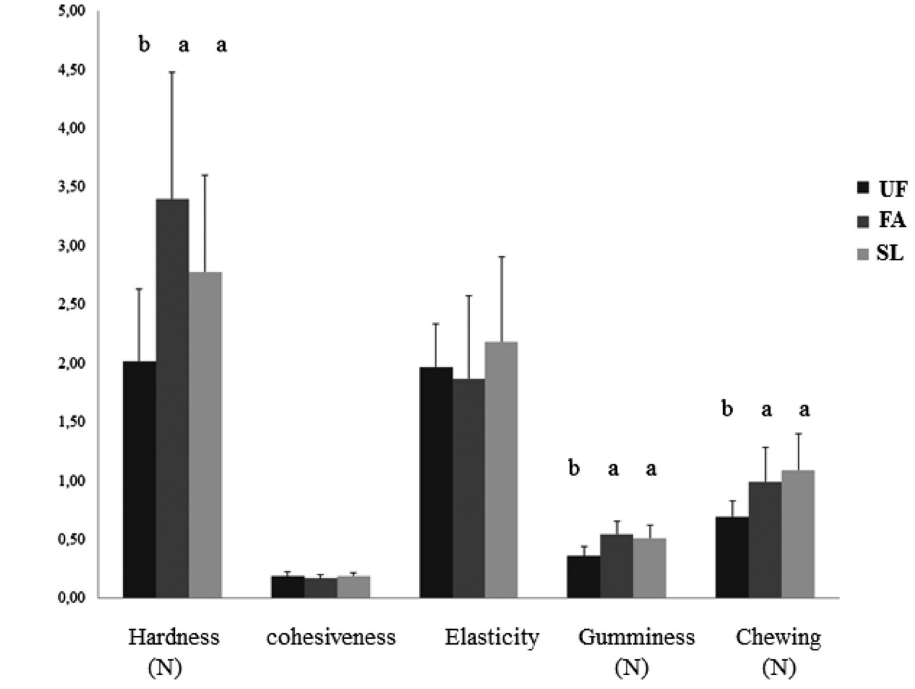

Figure 2. Comparison of texture components between the groups: UF (ultra-fast), FA (fast), and SL (slow) chilling rates at time T1 (when the internal temperature of livers reached 10°C) in mule ducks fatty livers (n = 12/group) (for each component, bars assigned with different letters are significantly different (P-value of < 0.05)).

kept from the start at +4°C was the slowest with an average value of 0.1°C/min.

The study of the effect of different chilling rates on fatty liver quality parameters was realized through the evaluation of the 5 texture components (hardness, cohesiveness, elasticity, gumminess, and chewiness) and by the measurement of the melting rate after cooking and storage. At time T1, livers from the FA and SL groups were harder, gummier and chewier than those from the UF group (Figure 2). Those differences in texture between the UF group from one side and the FA and SL groups on the other side were also reflected by a higher melting rate in these two groups compared to the first one only at time T2 (24 h post mortem, Table 2). The significance of interaction between the effect of time and that of chilling rate indicates that samples are not similarly affected by the storage for several hours at +4°C. Moreover, our results showed that the melting rate is correlated ($r^2 = 0.4279$, P-value of < 0.05) to hardness and that this correlation

Table 2. Effect of chilling treatments: "UF (ultra-fast), FA (fast), and SL (slow)" on melting rates of mule ducks fatty liver at times T1 (internal temperature = 10°C) and T2 (24 h post mortem) (n = 12/group/time).

Chilling rate	UF		FA		SL					
Time	T1	T2	T1	T2	T1	T2	RMSE	$P(c)^1$	$P(t)^2$	$P(t^*c)^3$
Melting rate (%)	22[a]	25[a]	25[a]	36[b]	24[a]	41[b]	9	**	***	*

[1] $P(c)$: effect of chilling rate.

[2] $P(t)$: effect of time.

[3] $P(t^*c)$: effect of the interaction between time and chilling rate. *,** and *** for P-value of <0.05, 0.01, and 0.001, respectively. In a row, means assigned with different letters (a and b) are significantly different (P-value of < 0.05).

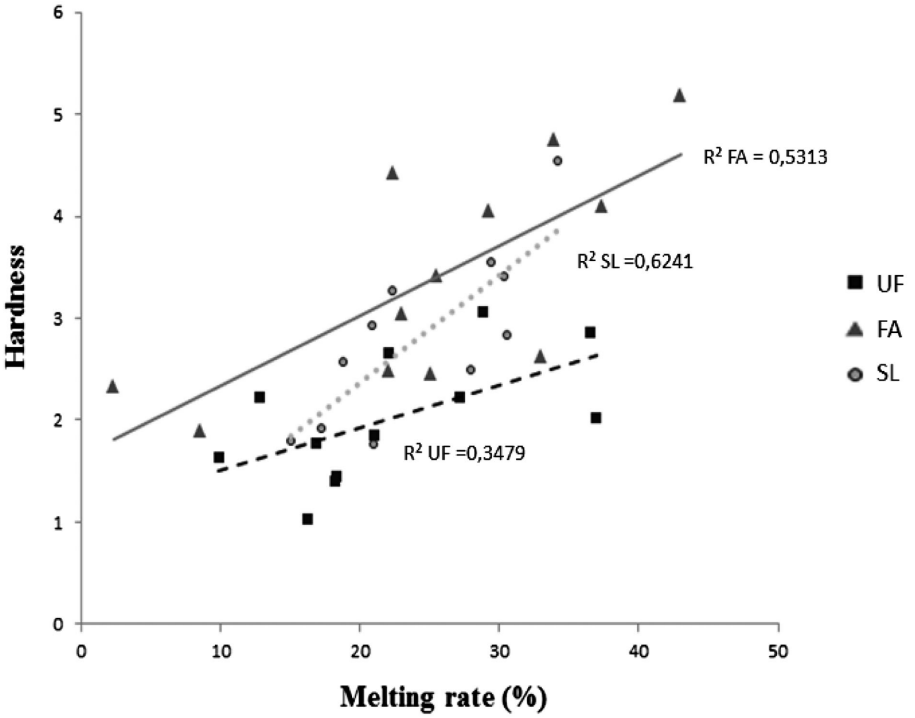

Figure 3. Correlation between melting rate and mule ducks fatty liver hardness in the 3 groups (UF: ultra-fast, FA: fast, and SL: slow chilling rate) at time T1 (temprature = 10°C) (P-value of < 0.05) (n = 12/group).

is strengthened with slower chilling rates (Figure 3). This result was not unexpected because Cazeils (2000) reported that hardness is proportional to cooking losses. Our results clearly evidence that the fastest chilling rate (UF) gave the best quality livers in comparison to the slower ones (FA and SL) of which, both texture and melting rates were similar. This result was also reported by Goullieux and Chanut (2006) who indicated that the lowest cooking losses for fatty livers were obtained with the fastest adopted chilling method. Our results are also in good accordance with field practices in processing plants where a better technological yield (i.e., lower melting rate) is obtained when the evisceration of fatty liver is performed early after death (Rousselot-Pailley et al., 1992). In fact, fatty liver processors have noticed that hot eviscerated livers (i.e., immediately after slaughter) rapidly chilled presented a higher quality than cold eviscerated ones (i.e., after cooling the entire carcass at +4°C) (Rousselot-Pailley et al., 1992). Our results, similar to those observed in the field, suggest that the mechanisms responsible for texture determinism in fatty livers are completely opposite to those reported for meat. In meat, the hardness is reputed to be proportional to the chilling speed (Olsson et al., 1994). Those differences are probably due to the composition and structural particularities of each type of tissue. For example, muscle is richer in proteins but poorer in lipids than fatty livers (proteins: 23% vs. 7% and lipids 3% vs. 60% for meat and fatty livers, respectively) (Salichon et al., 1994; Williams, 2007). Consequently, the texture of raw meat is mostly determined by proteins i.e., soluble proteins, myofibrillar proteins, and collagen (Devine et al., 1999; Reza Gheisari et al., 2009) whereas in fatty liver it is more due to the lipidic fraction i.e., lipids content and cell membrane cholesterol/phospholipids ratio (Cazeils, 2000). In fact, Theron et al. (2011a) demonstrated that cooking losses in fatty livers are caused by differences in tissue structure and lipid droplets morphology observed in high and low melting livers.

Table 3. Effect of chilling treatments: "UF (ultra-fast), FA (fast), and SL (slow)" on proteases activities of mule ducks fatty liver at times T0 (20 min), T1 (liver internal temperature = 10°C) and T2 (24 h post mortem) (n = 24/group at time T0, n = .12/group at times T1 and T2).

Chilling rate	UF			FA			SL						
Time	T0	T1	T2	T0	T1	T2	T0	T1	T2	RMSE	$P(c)^1$	$P(t)^2$	$P(t^*c)^3$
MMP-2[4]	1.1^{a,b}	1.1^{a,b}	1.3^{a,b}	1.1^{a,b}	1.4^{a}	1.1^{a,b}	1.0^{b}	1.3^{a,b}	1.1^{a,b}	0.3	*NS*	***	*NS*
Cathepsin 1	1.1^{a,b}	1.1^{b}	1.1^{a,b}	1.1^{a,b}	1.1^{a,b}	1.2^{a,b}	1.0^{b}	1.0^{b}	1.4^{a}	0.3	*NS*	*	*NS*
Cathepsin 2	1.2^{b}	1.2^{b}	1.2^{b}	1.3^{b}	1.6^{a}	1.6^{a}	1.2^{b}	1.6^{a}	1.6^{a}	0.4	**	*	*NS*
Total cathepsins	2.3^{b}	2.3^{b}	2.3^{b}	2.4^{b}	2.7^{a,b}	2.8^{a,b}	2.3^{b}	2.6^{a,b}	3.0^{a}	0.5	*	**	*NS*
Calpain-1	1.5^{a}	1.4^{a}	0.9^{b}	1.4^{a}	1.3^{a}	0.6^{b}	1.4^{a}	1.3^{a}	0.6^{b}	0.4	*NS*	***	*NS*
Calpain-2	1.3^{a}	1.4^{a}	0.9^{b}	1.3^{a}	1.0^{a,b}	0.9^{b}	1.3^{a}	1.1^{a,b}	0.8^{b}	0.4	NS	***	NS

[1] $P(c)$: effect of chilling rate.

[2] $P(t)$: effect of time.

[3] $P(t^*c)$: effect of the interaction between chilling rate and time.

[4] MMP-2: Matrix metalloproteases-2. *, ** and *** for P-value of < 0.05, 0.01and 0.001, respectively. NS for P-value of > 0.05. In a row, means assigned with different letters (a and b) are significantly different (P-value of < 0.05).

In order to further investigate the influence of different degrees of fatty liver proteolysis induced by different chilling rates, we chose to compare proteolytic activities of three families of proteases known to be involved in post mortem cellular reorganization (calpains, MMPs and cathepsins). Using one-dimensional zymography assays, we detected calpain-1 and 2, MMP-2, and two unidentified cathepsins forms (cts1 and cts2) of 37 and 24 kDa (Table 3). We also compared proteic profiles resulting from the extraction of main soluble proteins from fatty livers submitted to the 3 tested chilling rates at times T0, T1, and T2 post mortem. After a visual and a subjective comparison of the different gels, we selected and semi-quantified bands that seemed to be largely different from one time to another (molecular weights around 250, 180, 160, and 90 kDa)

Table 4. Effect of chilling treatments: "UF (ultra-fast), FA (fast), and SL (slow)" on SDS-PAGE profile of mule ducks fatty liver at times T0 (20 min post mortem), T1 (liver internal temperature = 10°C) and T2 (24 h post mortem) (n = 24/group at time T0, n = .12/group at times T1 and T2).

Chilling rate	UF			FA			SL						
Time	T0	T1	T2	T0	T1	T2	T0	T1	T2	RMSE	$P(c)^1$	$P(t)^2$	$P(t^*c)^3$
250 kDa	0.87^{a}	0.72^{a}	0.30^{b}	0.78^{a}	0.71^{a}	0.22^{b}	0.86^{a}	0.61^{a}	0.31^{b}	0.27	NS	***	NS
180 kDa	–	–	0.75	–	–	0.71	–	–	0.75	0.13	NS	–	–
160 kDa	0.70^{a}	0.66^{a}	0.62^{a}	0.69^{a}	0.67^{a}	0.65^{a}	0.69^{a}	0.56^{a}	0.67^{a}	0.16	NS	NS	NS
90 kDa	0.79^{a}	0.69^{a,b}	0.71^{a,b}	0.78^{a,b}	0.73^{a,b}	0.68^{b}	0.78^{a,b}	0.68^{b}	0.67^{b}	0.10	NS	***	NS

[1] $P(c)$: effect of chilling rate.

[2] $P(t)$: effect of time.

[3] $P(t^*c)$: effect of the interaction between chilling rate and time. *** for P-value of < 0.001 respectively. NS for P-value of > 0.05. In a row, means assigned with different letters (a and b) are significantly different (P-value of < 0.05).

Table 5. Identification of proteins, present (+) or lacking (−) in selected SDS-PAGE bands, by mass spectrometry at times T0 (20 min) and T2 (24 h post mortem) (UF: ultra-fast, FA: fast, and SL: slow chilling rate) (n = 24/group at time T0, n = .12/group at time T2).

				T0			T2		
Band	Protein	Function	Hypothetical molecular weight	UF	FA	SL	UF	FA	SL
250 kDa	Fatty acid synthase	Fatty acid synthesis	275	+	+	+	+	+	+
	Acetyl-CoA carboxylase	Fatty acid synthesis	263	+	+	+	+	+	+
180 kDa	Fatty acid synthase	Fatty acid synthase	275				+	+	+
	Acetyl-CoA carboxylase	Fatty acid synthesis	263				–	+	+
	Myosin, heavy chain 9, non-muscle	Cell shape	223				+	+	–
160 kDa	clathrin heavy chain 1	Structural	190	+	+	+	+	+	+
	Fatty acid synthase	Fatty acid synthesis	275	–	–	+	+	+	+
	Myosin, heavy chain 9, non-muscle	Cell shape	223	–	+	–	–	–	–
90 kDa	Heat shock protein 90 kDa	Response to stress	91	+	+	+	+	+	+
	Valosin containing protein	Structural / Proteolytic	90	–	–	–	+	+	+
	Alpha-actinin-4	Structural	104	–	–	–	–	+	+
	Phosphorylase	Carbohydrates metabolism	90	–	–	–	–	–	+
	Tetrahydrofolate synthase	Catalytic activity	100	–	–	–	–	–	+
	ATP-citrate lyase	Catalytic activity	119	–	–	–	–	–	+

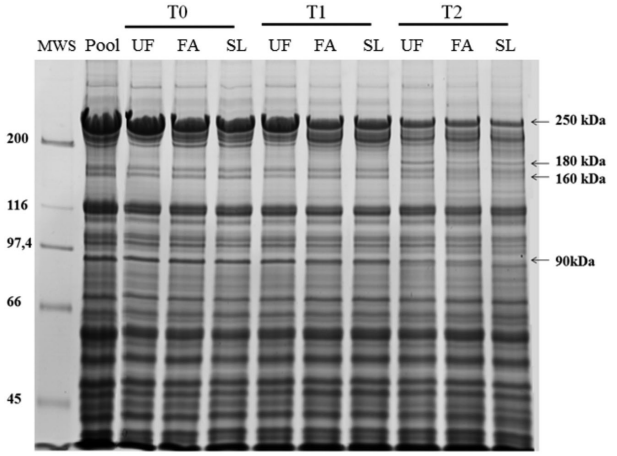

Figure 4. SDS-PAGE gel showing the obtained proteic profiles of mule ducks fatty livers using three different chilling rates (UF: ultra-fast, FA: fast, and SA: slow chilling rate) at times T0 (20 min post mortem), T1 (temperature = 10°C) and T2 (24 h post mortem). Arrows indicate further analyzed bands (n = 24/group at time T0, n = .12/group at times T1 and T2).

(Figure 4, Table 4). The semi-quantification of both proteolytic activities (from zymograms) and proteins (from SDS-Page gels) did not reveal any differences due to chilling treatments for a given time post mortem. Redmond et al. (2001) and Ali et al. (2008) also reported no differences regarding proteolytic activities and/or proteins degradation between different chilling treatments in duck, lamb and reindeer bull muscles respectively. On the contrary, other studies reported a decrease in proteolytic activities associate with low temperatures of chilling (Steen et al., 1997; Devine et al., 1999). In our study, if different chilling rates did not have a direct impact on proteases activity or proteins semi-quantification, we noted that the elapsed time post mortem had different effects for the different chilling rates. For example, while only activities of calpains were modified post mortem for the UF group, both calpains and cathepsin 2 activities were modified in samples from the FA group. Samples from the SL group exhibited the most tragic changes post mortem regarding proteolytic activities and protein bands intensities. Those results suggest that, as it is the case in muscle, the more rapidly the liver is chilled, the least proteases are active and thus the least proteins are degraded post mortem. Concerning their post mortem evolutions, proteases activities were not changed similarly. This is due to the fact that each type of proteases has functional particularities. For example, calpains are cytoplasmic enzymes and can be activated immediately if certain conditions are met. Moreover, when active, they auto-digest and this consequently causes a decrease in their activity post mortem (Cheret et al., 2007). In our study, calpains 1 and 2 activity for all chilling treatments and the one from calpain 2 for the UF group decreased from time T1 to time T2. However, the calpain 2 activity started decreasing at time T0 in all FA and SL groups. In their study, Veiseth et al. (2001) showed that the activity of the calpain-1 started dropping after 6 h post mortem and reached 60% of its initial value after 12 h post mortem. The same study showed no decrease in the m-calpain (or calpain-2) activity all along the experiment (360 h post mortem) suggesting that this isoform of calpain is not implicated in post mortem tenderization. On the contrary, in the study made by Delbarre-Ladrat et al. (2006), results showed a decrease of the calpain-2 activity in the sea bass white muscle during a 7 d post mortem storage. Meanwhile, cathepsins are lysosomal enzymes and thus need to be released from the lysosomes to be activated. This explains their delayed activation compared to calpains. This delay between calpains and cathepsins activity was also observed by Calkins and Seideman (1988). In this study, total cathepsins activity only increases between time T1 and T2 for samples from the SL group. These results are in accordance with the results obtained in muscle by Chéret et al. (2005), concerning the activity of the cathepsin D. Finally, MMP-2, like other metalloproteinases, is synthesized in a latent (zymogen) form and when suitable conditions are available, it is converted to an active form (Balcerzak et al., 2001). This might explain why in our study the MMP-2 increased between time T0 and T1. Sylvestre et al. (2002) demonstrated that MMP-2 activity remained stable from slaughter time until 21 d post mortem in lamb muscle.

Information given by SDS-Page profiles are complementary from those issued from zymographies because they can reveal subsequent alterations of the different substrates of proteases (Table 5). This is the case for the two main proteins identified in the 250 kDa band because fatty acid synthase and acetyl-CoA carboxylase were present in degraded forms presenting lower molecular weights. At the latest time, T2, the appearance of a new band at 180 kDa, which contained fragments of higher molecular weight proteins, also confirmed proteolysis developments during the time of storage. The absence of myosin heavy chain fragments in the SL group suggests that it might be more degraded and consequently moved to a lower point in the gel. The 190 kDa clathrin protein was found in the 160 kDa band. This result is not surprising, as this protein was reported to migrate as a 160 kDa band in several studies (Okamoto et al., 2000; Yamauchi et al., 2008). At time T2, and for the 3 treatments, we identified fragments from the fatty acid synthase enzyme among proteins weighing 160 kDa and this illustrates that this hyper abundant protein in fatty livers is easily degraded by proteases. At time T2 and for the lowest molecular weight, i.e., 90 kDa, a lot of different proteins were identified mainly in samples from the SL group. This also indicates that samples from the SL group witnessed a higher degree of proteolysis than the 2 other groups because of a lower chilling rate. When looking at the nature of the proteins undergoing post mortem proteolysis in the liver, we noticed that most of them are functional proteins and only few were structural ones. The actin and myosin proteins are the mostly studied proteins as a sign of post mortem

proteolysis and structural changes (Chéret et al., 2005; Reza Gheisari et al., 2009). In our study we can also see that those 2 proteins are also rapidly hydrolyzed because they were present at a lower molecular weight than their expected theoretical molecular weight. This is mainly evident in samples from the SL group. However, in this study only targeted bands of SDS-Page gels were analyzed and it will be now interesting to further investigate protein degradation with a broader approach.

In this study we showed that adopting a conventionally slow chilling rate of fatty livers increases hardness and leads to a final higher degree of melting. We also demonstrated that this higher melting rate is associated with higher proteolytic activities mainly at 24 h post mortem when proteases have been active for several hours. This post mortem proteolysis affected mainly functional proteins but also some structural ones. This enforces the idea that structural alterations are probably in the origin of higher melting rates. Finally, if the cooking of fatty livers has to be performed in the early time (0 to 3.5 h) post mortem after the evisceration, the effect of different chilling rates on the melting rate seems to be negligible. However, if a short storage time is necessary before cooking, it will be interesting for processors of fatty livers to invest in an ultra-fast chilling method in order to minimize cooking losses.

ACKNOWLEDGMENTS

Authors want to thank Isabelle Goulieux and Philippe Barret (CTCPA, Auch, France) for their help in measuring texture and cooking losses and the CIFOG (Comité Interprofessionel des Palmipèdes à Foie Gras) for their financial help.

REFERENCES

Afonso, S., L. Romagnano, and B. Babiarz. 1997. The expression and function of cystatin C and cathepsin B and cathepsin L during mouse embryo implantation and placentation. Dev. Camb. Engl. 124:3415–3425.

Ali, M. S., H. S. Yang, J. Y. Jeong, S. H. Moon, Y. H. Hwang, G. B. Park, and S. T. Joo. 2008. Effect of Chilling Temperature of Carcass on Breast Meat Quality of Duck. Poult. Sci. 87:1860–1867.

A. O. A. C. 1989. A. O. A. C. Official method 990.03. Protein (crude) in animal feed combustion method (Dumas method). JAOAC. 72:770.

Balcerzak, D., L. Querengesser, W. T. Dixon, and V. E. Baracos. 2001. Coordinate expression of matrix-degrading proteinases and their activators and inhibitors in bovine skeletal muscle. J. Anim. Sci. 79:94–107.

Blum, J. C., C. Labie, and P. Raynaud. 1990. Influence du poids et de la composition chimique du foie gras d'oie sur la fonte mesurée après stérilisation à 104°C. Sci. Aliments 10:543–554.

Calkins, C., and S. Seideman. 1988. Relationships Among Calcium-Dependent Protease, Cathepsins B and H, Meat Tenderness and the Response of Muscle to Aging - viewcontent.cgi. J. Anim. Sci. 66:1186–1193.

Cazeils, J.-L. 2000. Caractérisation de la composition lipidique des membranes plasmiques des hépatocytes de foies d'oies: relation avec le rendement technologique des foies gras. PhD Diss. INPT, Toulouse. 192p.

Chéret, R., A. Hernández-Andrés, C. Delbarre-Ladrat, M. Lamballerie, and V. Verrez-Bagnis. 2005. Proteins and proteolytic activity changes during refrigerated storage in sea bass (Dicentrarchus labrax L.) muscle after high-pressure treatment. Eur. Food Res. Technol. 222:527–535.

Cheret, R., C. Delbarreladrat, M. Lamballerieanton, and V. Verrezbagnis. 2007. Calpain and cathepsin activities in post mortem fish and meat muscles. Food Chem. 101:1474–1479.

Delbarre-Ladrat, C., R. Chéret, R. Taylor, and V. Verrez-Bagnis. 2006. Trends in Postmortem Aging in Fish: Understanding of Proteolysis and Disorganization of the Myofibrillar Structure. Crit. Rev. Food Sci. Nutr. 46:409–421.

Devine, C. E., N. M. Wahlgren, and E. Tornberg. 1999. Effect of rigor temperature on muscle shortening and tenderisation of restrained and unrestrained beef m. longissimus thoracicus et lumborum. Meat Sci. 51:61–72.

Folch, J, M Lees, and GH Sloane-Stanley 1957. A simple method for the isolation and purification of total lipides from animal tissues. Journal of Biological Chemistry 226, 497–509.

Goullieux, I., and I. Chanut. 2006. Quantification de la dureté et de la couleur du foie gras crû: influence des techniques de refroidissement. 7èmes Journées de la Recherche sur les Palmipèdes à Foie Gras. Arcachon, France. 171–175.

Kizaki, K., K. Ushizawa, T. Takahashi, O. Yamada, J. Todoroki, T. Sato, A. Ito, and K. Hashizume. 2008. Gelatinase (MMP-2 and -9) expression profiles during gestation in the bovine endometrium. Reprod. Biol. Endocrinol. 6:66.

Laemmli, U. K. 1970. Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T4. Nature 227:680–685.

Lametsch, R., P. Roepstorff, and E. Bendixen. 2002. Identification of protein degradation during post-mortem storage of pig meat. J. Agric. Food Chem. 50:5508–5512.

Marino, R., M. Albenzio, A. della Malva, A. Santillo, P. Loizzo, and A. Sevi. 2013. Proteolytic pattern of myofibrillar protein and meat tenderness as affected by breed and aging time. Meat Sci. 95:281–287.

Meullenet, J.-F., and B. g. Lyon,Carpenter, J. A., and C. e. Lyon. 1998. Relationship Between Sensory and Instrumental Texture Profile Attributes. J. Sens. Stud. 13:77–93.

Molee, W., M. Bouillier-Oudot, A. Auvergne, and R. Babilé. 2005. Changes in lipid composition of hepatocyte plasma membrane induced by overfeeding in duck. Comp. Biochem. Physiol. B Biochem. Mol. Biol. 141:437–444.

Nir, I., and Z. Nitsan. 1976. Goose fatty liver composition as related to the degree of steatosis, nutritional and technological treatments, and a simplified method for quality estimation. Ann. Zootech. 25:461–470.

Okamoto, C. T., J. G. Duman, K. Tyagarajan, K. L. McDonald, Y. Y. Jeng, J. McKinney, T. M. Forte, and J. G. Forte. 2000. Clathrin in gastric acid secretory (parietal) cells: biochemical characterization and subcellular localization. Am. J. Physiol. - Cell Physiol. 279:C833–C851.

Olsson, U., C. Hertzman, and E. Tornberg. 1994. The influence of low temperature, type of muscle and electrical stimulation on the course of rigor mortis, ageing and tenderness of beef muscles. Meat Sci. 37:115–131.

Petäjä, E., E. Kukkonen, and E. Puolanne. 1985. Effect of post-mortem temperature on beef tenderness. Meat Sci. 12:145–154.

Raser, K. J., A. Posner, and K. K. W. Wang. 1995. Casein Zymography: A Method to Study μ-Calpain, M-Calpain, and Their Inhibitory Agents. Arch. Biochem. Biophys. 319: 211–216.

Redmond, G. A., B. McGeehin, J. J. Sheridan, and F. Butler. 2001. The effect of ultra-rapid chilling and subsequent ageing on the calpain/calpastatin system and myofibrillar degradation in lamb M. longissimus thoracis et lumborum. Meat Sci. 59: 293–301.

Reza Gheisari, H., M. Aminlari, and S. Shahram Shekarforoush. 2009. A comparative study of the biochemical and functional properties of camel and cattle meat during frozen storage. Vet. Arh. 79:51–68.

Rousselot-Pailley, D., G. Guy, N. Sellier, and J. Blum. 1992. Influence des conditions d'abattage et de réfrigération sur la qualité des foies gras d'oie. INRA Prod. Anim. 5:167–172.

Salichon, M., G. Guy, D. Rousselot, and J. Blum. 1994. Composition of the 3 Types of Foie-Gras - Goose, Mule Duck and Muscovy Duck Foie-Gras. Ann. Zootech. 43:213–220.

SAS. 2011. The data analysis for this paper was generated using SAS software, Version 9.3 of the SAS System for Windows. Copyright © 2002–2010 SAS Institute Inc. SAS and all other SAS Institute Inc. product or service names are registered trademarks or trademarks of SAS Institute Inc., Cary, NC, USA.

Steen, D., E. Claeys, L. Uytterhaegen, S. De Smet, and D. Demeyer. 1997. Early post-mortem conditions and the calpain/calpastatin system in relation to tenderness of double-muscled beef. Meat Sci. 45:307–319.

Sylvestre, M. N., D. Balcerzak, C. Feidt, V. E. Baracos, and J. B. Bellut. 2002. Elevated rate of collagen solubilization and post-mortem degradation inmuscles of lambs with high growth rates: Possible relationship with activity of matrix metalloproteinases. J. Anim. Sci. 80:1871–1878.

Théron, L. 2011. Déterminisme biologique de la variabilité de la fonte lipidique à la cuisson du foie gras de canard. PhD Diss. INPT, Toulouse. 162p.

Theron, L., T. Astruc, M. Bouillier-Oudot, C. Molette, A. Vénien, F. Peyrin, Z. G. Vitezica, and X. Fernandez. 2011a. The fusion of lipid droplets is involved in fat loss during cooking of duck "foie gras." Meat Sci. 89:377–383.

Theron, L., X. Fernandez, N. Marty-Gasset, C. Pichereaux, M. Rossignol, C. Chambon, D. Viala, T. Astruc, and C. Molette. 2011b. Identification by proteomic analysis of early post-mortem markers involved in the variability in fat loss during cooking of mule duck "foie gras." J. Agric. Food Chem. 59: 12617–12628.

Veiseth, E., S. D. Shackelford, T. L. Wheeler, and M. Koohmaraie. 2001. Effect of postmortem storage on mu-calpain and m-calpain in ovine skeletal muscle. J. Anim. Sci. 79: 1502–1508.

Williams, P. 2007. Nutritional composition of red meat. Nutr. Diet. 64:S113–S119.

Yamauchi, T., T. Ishidao, T. Nomura, T. Shinagawa, Y. Tanaka, S. Yonemura, and S. Ishii. 2008. A B-Myb complex containing clathrin and filamin is required for mitotic spindle function. EMBO J. 27:1852–1862.