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Apoptosis during the development of the hepatic steatosis in force-fed ducks and cooking yield implications

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ABSTRACT Mule ducks were force-fed for 12 d to determine whether or not signs of apoptosis could occur during the development of the hepatic steatosis induced by the huge quantities of corn ingested twice daily by the birds. Presence of apoptosis in hepatocytes was assessed through the measurements of increased activities of capsase-3 +-7, -8, and -9. From d 0 of the force-feeding period until d 8, activities of the different caspases remained at a low level. On the contrary, at d 10 and d 12, activities of all measured caspases dramatically increased, indicating that apoptosis occurred at this stage, which corresponds to the time of accumulation of large quantities of lipids in the hepatic cells.

The melting level of the liver issued from force-feeding ("foie gras") during cooking is a point of interest for processors because it could degrade the quality of this delicate dish. In this study, we used the levels of caspases activities to improve the predictability of foie gras cooking, in addition to other parameters usually used, such as its weight or lipid content. From this improvement, we suggest that part of the variability of melting during cooking of fatty livers could reside in more or less intense activity of hepatic proteases.

Key words: steatosis, apoptosis, force-feeding, cooking yield

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INTRODUCTION

France is the first and the largest producer (75%, ITAVI, 2016) of "foie gras" (fatty liver) of ducks in the world. This coveted traditional dish is issued from intense force-feeding of palmipedes during a short period (10 to 12 d). Force-feeding consists of twice daily delivering of increased quantities of corn. Due to this excessive input of energy (mainly carbohydrates from corn starch), livers rapidly develop a steatosis characterized by a huge accumulation of lipids within hepatocytes (Gabarrou et al., 1996). Many studies have focused on lipid or protein metabolism of the liver during the development of steatosis and shown that the liver is submitted to intense metabolic activities, mainly turning towards intense de novo lipid synthesis and exportation (Hermier et al., 1991; Bax et al., 2012).

According to Guicciardi et al. (2013), when liver is exposed to numerous insults, cell death occurs mainly by apoptosis and necrosis, with apoptosis being the main physiologic route to eliminate damaged cells to maintain tissue homeostasis. Apoptosis was first named around 50 yr ago to describe a morphologically distinct cell death program (Kerr et al., 1972). Apoptosis is often opposed to another major cell death program, necrosis, but mainly differs from it by the fact that apoptosis does not induce tissue inflammation. This lack of inflammation during liver steatosis development in force-fed ducks has already been reported by Bénard and Labie (1992). The development of apoptosis is a coordinated and often energy-dependent process that involves the activation of a group of cysteine proteases called caspases and a complex cascade of events (including other proteases activation, such as calpains or cathepsins) that includes the final demise of the cell (Elmore, 2007). In the present study, we aimed to follow the development of liver steatosis in force-fed ducks in order to detect signs, through caspases and other proteolytic activity modifications, of apoptosis occurrence. To our knowledge, this study is the first one that interrogates the signs of apoptosis during the development of the liver steatosis induced by force-feeding in ducks.

For stakeholders acting in the foie gras industry, the most important problem regarding product quality, apart from recent sanitary crises associated with avian flu, is the management of melting of liver during cooking. It is well known that the genetic origin of the birds, the duration and intensity of the force-feeding, the final weight of the liver, and other parameters related to breeding conditions or to the cooking process

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itself influence the cooking yield of foie gras (Theron et al., 2011, 2012, 2013). Still, a large part of its variability remains unclear. One hypothesis to explain that livers can melt more or less during cooking is that postmortem proteolysis could be to some extent more or less intense and consequently weaken in higher or lesser degree the hepatocyte structure. To minimize this, processors recommend to harvest livers from hot carcasses (just at the end of the slaughter line) and to cool them down rapidly. Should apoptosis occur in some livers, its association with increased proteolysis also could contribute to the weakening of the hepatocyte structure. This would facilitate the escape of a large quantity of accumulated lipids out of the tissue. In this study, we verify hypothesis by measuring some proteolytic activities associated with apoptosis development and its relationship with the cooking yield.

MATERIAL AND METHODS

Animals and Samples

A flock of 100 male Mule ducks was reared in the Experimental Geese and Ducks Farm (FEOC, Dordogne, France) according to common commercial procedures. At 12 wk of age (average body weight = 4.140 kg), birds were randomly assigned to 2 groups for the force-feeding period. No visible signs of a poor health were observed in birds before the beginning of the force-feeding period. The first group of ducks was force-fed for 12 d according to the traditional intensity of force-feeding (average quantity of feed distributed during the force-feeding period = 9,435 g/bird). The second group was force-fed in the same conditions but with a slightly higher quantity of feed distributed during the same period (average quantity of feed distributed during the force-feeding period = 9,835 g/bird). In the 2 groups, the composition of feed distributed during the force-feeding period was the same (38% of corn grains, 60% of corn flour, and 3% of various minerals and vitamins).

At d 0, 2, 4, 6, 8, 10, and 12 of the force-feeding period, 5 ducks of each group were randomly selected and sacrificed in a commercial slaughter-house. At the end of the slaughter line, approximately 20 min after the exsanguination following the electronarcosis, livers were harvested from carcasses and weighed. Small samples of each liver were immediately frozen in liquid nitrogen and stored at –80°C for further analysis.

When fatty livers weighed over 300 g, they were cooled for 6 h on ice and then conditioned in glass cans of 180 g before pasteurization (Vp = 70 min at 80°C). After 2 mo of storage at +4°C, cooking yields (Y) were determined by weighing cooked fatty livers after removing visible melted lipids and the percentage calculated as: Y(%) = [raw initial weight (g)—cooked weight (g)] × 100/[raw initial weight (g)].

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/EEC). Investigators were certificated by the French governmental authority for carrying out those experiments (agreement no. 31–165).

Biochemical Determinations

Total Lipid Content Near-infrared reflectance spectrometry (**NIRS**) was used to estimate total lipid content of raw entire livers, just after the harvesting, according to the method described by Marie-Etancelin et al. (2014). Measurements were performed directly on the fatty liver surface using a portable ASD Labspec Pro spectrometer (wavelength range: 350 to 2,500 nm) with a "contact probe" module.

In all frozen samples, the following measures were determined in duplicates.

Dry Matter Content After grinding in liquid nitrogen, samples were desiccated for 24 h in a drying oven (103°C) to determine the dry matter (**DM**) content (JOCE, 1971).

Zymograms of Calpains, Cathepsins, and MMP-2 As previously described by Awde et al. (2013), zymograms of calpains, cathepsins, and matrix metalloprotease-2 (MMP-2) were performed according to the methods of Raser et al. (1995); Afonso et al. (1997), and Kizaki et al. (2008), respectively. Using the procedure described by Bax et al. (2012), relative protease activity was expressed as protease activity/pool activity within each gel. To obtain a good sampling and a correct statistical analysis, a duplicate was analyzed for each sample.

A detailed protocol of the zymography of proteases from steatotic duck liver is available from Wilkesman et al. (2017).

Activities of Caspases Activities of caspases-8, -9, and (-3+-7) were measured following the instructions of the Caspase-Glo® assay technical bulletin distributed by Promega (69,660 Charbonnières-les-Bains, France). Results are expressed in released fluorescence units/mg of proteins.

Statistics

The analysis for this paper was generated using SAS software, version 9.4 of the SAS System for Windows (SAS, 2012). ANOVAs were performed with the General Linear Model (Proc GLM) completed with the Student–Newman–Keuls' post-hoc test. Because no effects of the intensity of the force-feeding, nor interactions with the number of d of force-feeding, were found to be significant, all data were pooled in a single set and further analyzed in a simple one-way ANOVA regarding the effect of the total number of d of force-feeding. Simple correlations (Proc Corr) were determined according to Pearson, while multiple linear regressions were performed forward and step by step (Proc Stepwise forward).

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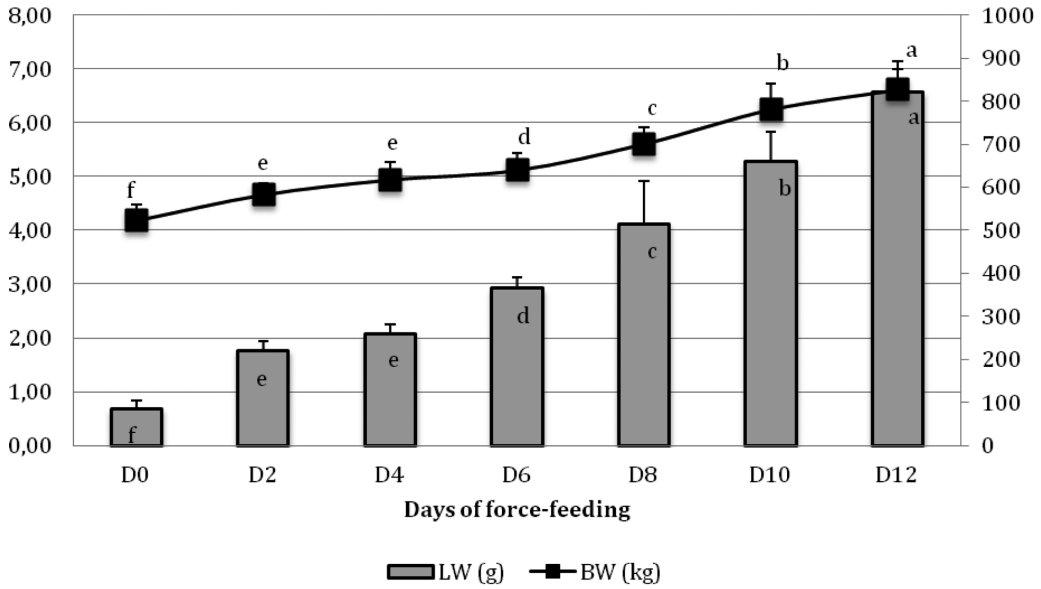

Figure 1. Evolution of body (BW in kg, left y axis) and liver (LW, in g right y axis) weights of ducks during the force-feeding period. Values are means ± SD. For each variable, means with the same superscript letter are not significantly different ($P < 0.05$).

Table 1. Variations during the force-feeding period of the weight (LW), cooking yield (Y), and percentages of lipids (L) and dry matter (DM) in the liver. Values are means ± standard deviations.

	D0	D2	D4	D6	D8	D10	D12	RMSE	p
LW (g)	85^{f} ± 20	220^{e} ± 20	358^{e} ± 22	364^{d} ± 26	515^{c} ± 100	660^{b} ± 69	822^{a} ± 70	55	<0,0001
Y (%)	-	-	-	$97,29^{a}$ ± 1,10	$93,38^{a}$ ± 5,92	$84,70^{b}$ ± 7,94	$68,38^{c}$ ± 13,30	8,31	<0,0001
L (%)	$4,14^{f}$ ± 1,39	$14,43^{e}$ ± 2,47	$32,04^{d}$ ± 3,57	$43,67^{c}$ ± 3,75	$49,48^{b}$ ±	$50,83^{b}$ ± 4,65	$56,37^{a}$ ± 3,00	3,26	<0,0001
DM (%)	$30,09^{f}$ ± 1,61	$38,51^{e}$ ± 1,54	$49,07^{d}$ ± 2,91	$57,91^{c}$ ± 2,92	$62,54^{c}$ ± 3,84	$65,46^{b}$ ± 2,23	$67,86^{a}$ ± 2,56	2,63	<0,0001

[a-f]Within a row, means with similar superscripts are not different ($P < 0.05$).

RESULTS AND DISCUSSION

The evolution of body and liver weights, followed during the force-feeding period (Figure 1), shows that during the short 12-day period, there was an increase of body weights by more than 50% and a huge increase of the liver weight from 85 to 822 g (+867%). Those increases have already been described in previous studies (Fernandez et al., 2010; Theron et al., 2012), reporting about the influence of force-feeding on growth, but it is noticeable in the present study that the final liver weights are very high. This is due to the intensity of the force-feeding and was deliberately done in order to exacerbate responses of the liver to the increased daily delivery of meals containing a high quantity of carbohydrates.

From Table 1, it becomes evident that the large increase in the liver weight all along the force-feeding period is mainly due to an increase of the DM content [+125% between d 0 (D0) and d 12 (D12)] associated with a large increase of the lipids content (+1,261% between D0 and D12) of the liver. Those values are close to those reported by Theron et al. (2013) and Bax et al. (2012) and indicated a rapid development of a steatosis (lipids accumulation) in the liver. It is induced by the large quantity of carbohydrates (mainly starch) supplied by the corn of the feed. Associated with this steatosis development, there is a slow decrease in the cooking yield that ranges from 97 to 68% after 6 and 12 d of force-feeding, respectively. According to Theron et al. (2011), the liver weight, as well as the lipid and protein contents, is known to affect negatively the cooking yield of the foie gras. This is illustrated by the strong negative correlations between cooking yield and liver weight ($r = -0.83$, $P < 0.001$) or total lipids content (-0.71, $P < 0.001$).

According to Gohda et al. (1994), levels of the activity of several hepatic enzymes are commonly used for the determination of hepatic dysfunctions. Figure 2 reports the relative activities of 3 different proteases at the beginning (D0) and at the end (D12) of the force-feeding period. It shows that both MMP-2 and calpains activities significantly increased by 120 and 205%, respectively, during that short period. Wanninger et al. (2011) and Liu et al. (2006) indicated that the level of activity of the MMP-2 enzyme increased during the development of non-alcoholic steato-hepatitis (**NASH**) to counterbalance a possible development of fibrosis associated with a dysfunction of the liver. On the contrary, Awde et al. (2013) reported that in Pekin and Muscovy ducks, the hepatic activity of the MMP-2 enzyme slightly decreased in force-fed ducks when compared to non-force-fed ones. Authors hypothesized that this probably facilitated the development of the liver

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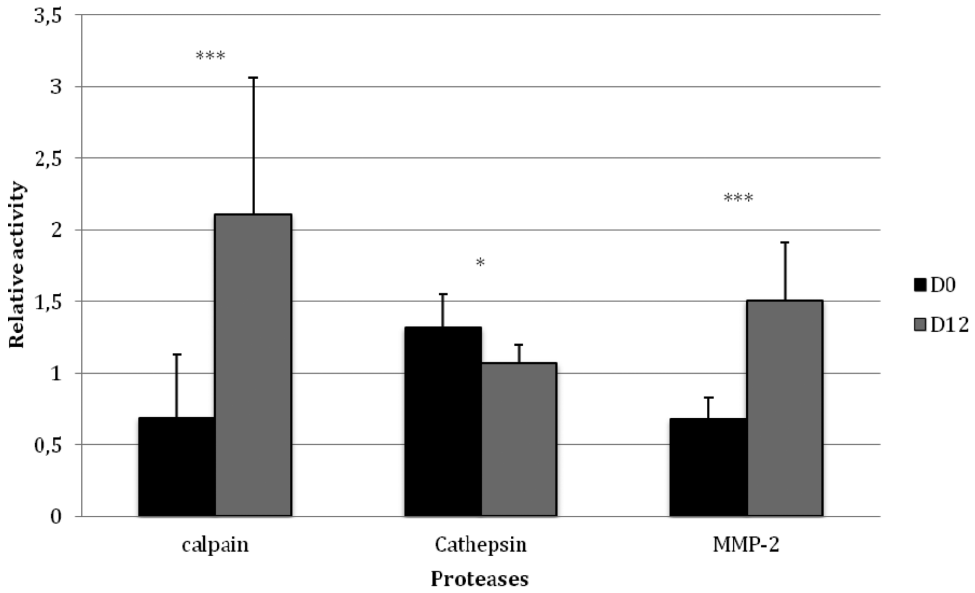

Figure 2. Comparisons of proteases relative activities between d 0 (D0) and d 12 (D12) of the force-feeding period (* = 0,01 < P < 0.05, *** = P < 0001).

during its fattening, but it is noticeable that the range of liver weights reported was much less relevant than in the present study. On the contrary, in our current study, the liver weight values reached at the end of the force-feeding period were probably extreme. Thus, it seems that the metabolism of the liver was more oriented towards the prevention of a fibrosis development, which could be the final consequence of the very high level of development of the steatosis.

According to Sakon et al. (1998) and Limaye et al. (2003), calpains are activated in non-cancerous liver tissues with various pathologies. For Momeni (2011), the activation of calpains mediates the degradation of many structural cytoskeletal and membrane proteins, as well as various proteins in the nuclear matrix, which are involved in maintaining cellular structural integrity that is essential for normal cellular function and survival, and their degradation leads to apoptosis. From the present study, it can be hypothesized that the huge solicitation of the metabolism of hepatocytes induced by the force-feeding probably turns a lot of them towards a regular cell-death pathway.

Contrary to the activities of proteases observed in this study, cathepsin's activities slightly decreased by 23% during the period of force-feeding. This decrease is associated with lipids accumulation in livers, and this has already been noticed in rats by Yang et al. (2017) and by Kadowaki et al. (2014). Those authors suggested that steatosis development as a result of toxicological products, impaired autophagy degradation of liver cells, which is mediated by lysosomal proteases such as cathepsins. In our present study, it also can be assumed that the liver steatosis, which rapidly develops during the force-feeding period, leads also to a decrease of autophagic degradation of hepatocytes and an increase in apoptosis development, as suggested above.

According to Fuchslocher Chico et al. (2017), apoptosis represents the principal suicide program in many physiological and developmental settings and can be triggered through the external or the internal pathways. Both pathways are mediated by intracellular specific proteases, called caspases, which can be divided into initiator (caspase-8 and -10 or -9 for the external and internal pathways, respectively) and effector caspases (caspase-3, -6, and -7; for a review see Shalini et al., 2015). In the present study, we measured the activities of caspase-8 and -9 for screening external and internal apoptosis induction pathways, respectively, and the activity of caspase-3 + -7 for the detection of the effectiveness of the induction of apoptosis. Figure 3 reports the respective activities of those caspases during the force-feeding period. From D0 to D8, activities of all caspases are at the lowest levels and remained more or less constant. As apoptosis is involved in normal cell turnover (Kerr et al., 1972), we can conclude that until D8 of the force-feeding period, the observed activity of caspases might illustrate the normal activity of the liver despite the rise of its metabolic activity associated with the storage of a high quantity of lipids. On the contrary, at D10, and more so at D12, we could observe a dramatic increase in the activity of all the studied caspases. This suggests that hepatocytes are now undergoing apoptosis. The high increase in caspase-3 + -7 activity indicates that hepatocytes are in a non-reversible step of the apoptosis pathway because those 2 caspases are effector ones. It signifies that some hepatocytes have entered a suicide pathway, probably because they were no longer able to support effectively the high level of metabolism imposed by the force-feeding program. However, it is also noticeable that the activity of caspase-3 + -7 becomes very variable at D10 and D12 (80 and 52%, respectively) indicating a large

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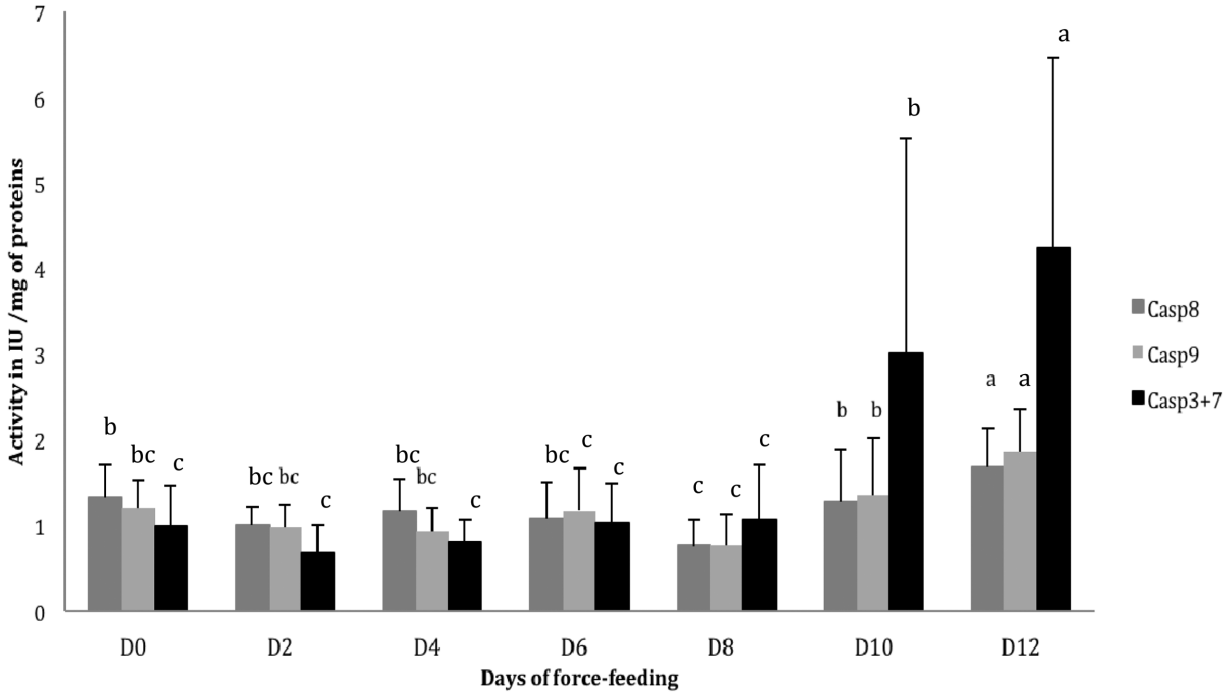

Figure 3. Evolution of the activities of the caspase-8 (Casp8), -9 (Casp9), and -3 + -7 (Casp3+7) of the liver during the force-feeding period. Values are means ± SD. Means with different letters are significantly different ($P < 0.05$).

Table 2. Pearson 's correlation coefficients ($P < 0001$) between the cooking yield (Y) and the body weight (BW), the liver weight (LW), the total lipids amount (L), and activities of caspase-3 and -7 (casp3+7), the caspase-8 (casp8) and the caspase-9 (casp9). The cooking yield was measured only after 6, 8, 10, and 12 d of force-feeding (n = 10 ducks/d).

	BW	LW	L	Casp3+7	Casp8	Casp9
Y	−0,75	−0,83	−0,71	−0,72	−0,63	−0,67

heterogeneity of adaptation among the hepatocytes. Bax et al. (2012), using a proteomic approach, also distinguished 2 distinct states of hepatocytes during the development of the liver steatosis. During the first half of the force-feeding period, the hepatic metabolism seemed to be adapted to face the high amount of hepatic glucose, while, during the second half of the period, metabolic processes aimed to maintain the cellular homeostasis despite the very high metabolic load. Regarding apoptosis development in the present study, we also distinguish those 2 different states characterized by low (from D0 to D8) or high (D10 and D12) levels of activity of caspases.

Correlation coefficients between the cooking yield and some traits of the livers are reported in Table 2. Awde et al. (2014) reported a strong negative correlation between the technological yield and body or liver weights or the total amount of lipids of the liver. For those variables, which are often used to try to estimate the percentage of melting of the foie gras during cooking, our r^2 values are higher than those reported by Theron et al. (2012). This is probably due to the range of the liver weight samples, which was very large (i.e., from 364 g to 822 g at D6 and D12, respectively). On the contrary, Theron et al. (2012) presented very similar but reduced liver weights (around 570 ± 40 g).

If apoptosis takes place in a majority of hepatocytes at the end of the force-feeding period, the proteolytic activities of the caspases are responsible for weakening cellular structures, which then increases the cooking losses and thus reduces the cooking yield. This hypothesis is well supported by the negative correlations between the activities of the caspases and cooking yields, as reported in Table 2. Consequently, we can estimate that the more developed the apoptosis, the weaker the hepatocytes and ultimately the higher the melting of the liver that will occur during cooking. In addition to simple correlations reported in Table 2, we built multiple linear regressions with the different variables available from ducks force-fed during 6 to 12 days. To predict a more precise model of cooking yield, based on r^2 and Mallows's C_p values, the combination of liver weight, the amount of lipids, and activities of the 4 studied caspases (Figure 4), was used. This combination of those 5 data considerably increases the r^2 value to 0.77 ($P < 0.001$).

CONCLUSION

Given our experimental procedure, we clearly demonstrate that the final stages of the force-feeding period of ducks can be associated with the development of apoptosis. This shows that, at least for a given number of birds, the rapid development of the hepatic steatosis, induced by ingestion of a large quantity of

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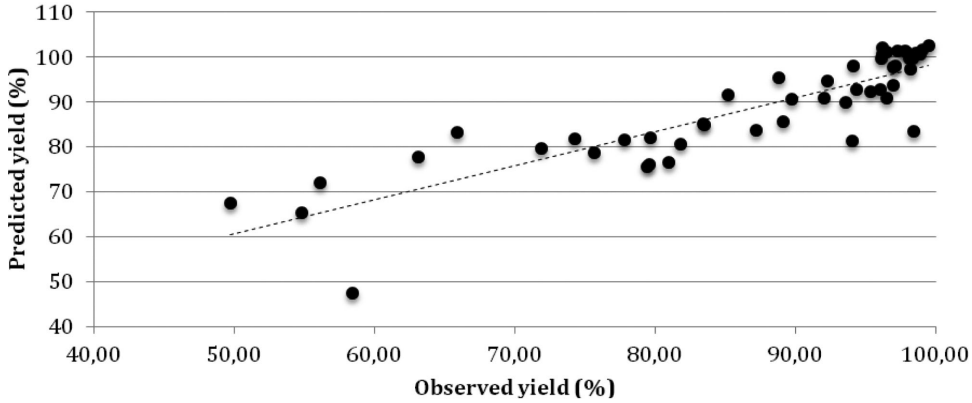

Figure 4. Plot of the observed vs. the predicted cooking yield values of fatty livers. The 5 predictive variables were the weight, and the percentage of lipids in the liver associated to the activities of capsases-3+7, -8 and -9, $r^2 = 0{,}77$, and Mallow's C(p) = 6.

carbohydrate-rich feed, irreversibly disturbs the homeostasis of hepatocytes. However, as it was observed, the final weight of livers in the experiment is higher (>750 g) than the one that is sought in commercial flocks (generally around 550 to 600 g). Furthermore, no evidence of development of apoptosis could be found before d 10, despite the increase in liver weight at this stage.

Based on the fact that the development of apoptosis is often associated with modifications of the cellular proteolysis, this paper demonstrates a better knowledge of the levels of activity of major proteases present in hepatocytes, which can be useful for predicting the cooking yield of foie gras. It also confirms that the weakening of the cellular structure of hepatocytes is a major determinant of the lipid melt in this gourmet product.

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