Research Article

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Caspase activity in *post mortem* muscle and its relation to cattle handling practices

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Abstract

BACKGROUND: Animal handling practices are one of the factors majorly affecting animal metabolism prior to slaughter. This phenomenon increases the occurrence of meat quality defects such as dark cutting-beef, causing high economical losses in the meat industry. Under this framework, the assessment of apoptosis onset in *post mortem* muscle was proposed as a novel approach to reveal biochemical characteristics in several Spanish bovine breeds (Asturiana de los Valles, Retinta and Rubia Gallega) managed under different production systems (intensive *versus* semi-extensive) and transport/lairage conditions (mixing *versus* not mixing with unfamiliar animals). To do so, the activities of initiator caspase 9 and executioner caspases 3/7 were determined in *Longissimus thoracis et lumborum* muscle at three early *post mortem* times (2, 8, and 24 h).

RESULTS: Breed effect and transport/lairage conditions were the most relevant factors that influenced both caspase activities over *post mortem* time, showing Rubia Gallega breed a completely different behavior compared to Asturiana de los Valles and Retinta breeds. Moreover, it is postulated that apoptosis cascade is initiated via the activation of caspase 9 under hypoxic or metabolic stress followed by the activation of executioner caspases 3/7.

CONCLUSIONS: Assessment of apoptosis on *post mortem* muscle can be a novel approach to study the influence of animal handling on muscle metabolism and *post mortem* cell death and its consequences on meat quality traits.

Keywords: caspases; cell death; apoptosis; animal management; *Bos taurus*; meat quality

INTRODUCTION

Over the years, meat quality research has been focused on the study of several proteolytic enzyme groups, physical state of myofibrillar proteins, fiber structure, intramuscular fat and other quality attributes such as color or water holding capacity[1] in order to understand meat tenderness over aging time. In this sense, the relevance of apoptosis processes was not considered until Herrera-Mendez *et al.*[2] proposed cell death as one of the first events triggered during the conversion of muscle into meat. Since then, several researchers have focused on the study of metabolic responses associated with intrinsic hypoxia/ischemia that could contribute to *post mortem* proteolysis occurring during meat tenderization.[3,4] However, these studies did not investigate any relationship that can occur between *ante mortem* factors influencing animal metabolism and *post mortem* cell death. Inadequate animal management prior to slaughter could trigger a stress response through the increase of hormone secretion (catecholamines, cortisol, adrenaline, etc.), leading to the depletion of muscle glycogen reserves.[5–7] This reduction of *ante mortem* glycogen storage will modify the glycolytic metabolism in *post mortem* muscle, resulting in less lactic acid production thus impairing a decrease of ultimate pH meat to normal values (≈ 5.5). This promotes the occurrence of several meat defects such as dark cutting or DFD (dark, firm and dry) meat that causes significant economic losses in the meat industry.[8] Therefore, a careful study of *ante*

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mortem factors related to cattle (mainly breed, sex and age) and their handling procedures (mainly feeding systems, transportation, lairage and slaughter conditions) may ease the understanding of those biochemical pathways more related to meat quality traits.

Caspases constitute a family of cysteine-dependent peptidases that play an essential role in apoptosis (caspases 3, 6, 7, 8, 9, 10 and 12) and inflammatory response system (caspases 1, 4, 5, 11 and 13). Apoptotic caspases can be further classified according to their point of entry into the cell death pathway: initiator caspases (caspases 8, 9 10 and 12) and executioner caspases (caspases 3, 6 and 7). These enzymes are mainly synthesized as inactive zymogens (pro-caspases), and internal and/or external stimuli initiate a series of controlled reactions that ultimately lead to cell death. The extrinsic pathway initiates apoptosis through the activation of extracellular receptors that will finally activate initiator caspase 8. Meanwhile, the intrinsic pathway is activated by internal stimuli (metabolic and/or hypoxic stress) leading to the permeability of the outer mitochondrial membrane, favoring cytochrome-C release and the formation of the caspase 9 apoptosome complex.[9,10] This activates executioner caspases 3 and 7 that will proceed with cell dismantling.[11] Concerning activation of these enzymes in relation to stress, previous studies have found a higher caspase 1 activity in mice when animals were stressed[12] and activation of caspase 3 in Zebrafish by heat shock in response to stress.[13] In this line, some authors found a higher expression of the caspase 3 large subunit in DFD beef samples at 24 h *post mortem* times.[14]

In the present study, and for the first time, the study of apoptosis as a way to understand the influence of pre-slaughter handling practices on biochemical muscle characteristics of several bovine breeds is proposed. The research investigated the activity of executioner caspases 3/7 and initiator caspase 9 over time (2, 8, and 24 h *post mortem*) in loin samples of three Spanish beef cattle breeds managed under different feeding systems (intensive *versus* semi-extensive) and transport/lairage (mixed *versus* non-mixed with unfamiliar animals) conditions.

MATERIALS AND METHODS

Animal handling

A total of 90 ($n = 90$) male calves from three native Spanish bovine breeds were used: 29 Asturiana de los Valles (AV), 33 Retinta (RE) and 28 Rubia Gallega (RG). Calves were reared under two different production systems, intensive (I) and semi-extensive (E), following the typical procedures of each region and breed.

AV calves were suckled by their mothers under grazing conditions. They were weaned at 8 months of age and supplemented with concentrate *ad libitum* in winter period. At 10–12 months, calves were finished under one of the two systems for a period of 100 days until they were slaughtered at 13–15 months: (I) 18 yearling bulls were managed indoors and fed 8 kg/head/day of concentrate and 2 kg/head/day of barley straw, and (E) 11 yearling bulls were managed outdoors grazing and complemented with 3.5 kg/head/day of concentrate. The concentrate was composed of 84% barley meal, 10% soya meal, 3% fat, 3% minerals, vitamins and oligoelements, and pasture was mainly composed of ryegrass (*Lolium perenne*) and clover (*Trifolium repens*).

RE calves were suckled by their mothers under grazing conditions until they were weaned at 6 months. Later, calves were reared under one of the two systems until they were slaughtered at 13–14 months: (I) 13 yearling bulls were managed indoors and fed 8 kg/head/day of concentrate and 2 kg/head/day of barley straw, and (E) 20 yearling bulls were managed outdoors grazing and complemented with 4.0 kg/head/day of concentrate. The concentrate was composed of 51% corn meal, 34% barley meal, 8% soya meal, 3% fat, 4% minerals, vitamins and oligoelements, and pasture was mainly composed of ryegrass (*Lolium perenne* and *Lolium rigidum*) and clover (*Trifolium repens*).

Management of RG calves was slightly different as they are commercially slaughtered at a younger age (10 months) compared to the other studied breeds. Under the intensive system (I), 12 RG calves were weaned at 1.5 months and managed indoors with concentrate and barley straw *ad libitum*. The other 16 RG calves (E) were suckled by their mothers under grazing conditions mainly perennial ryegrass (*Lolium perenne*) and white clover (*Trifolium repens*) and supplemented with 3.5 kg/head/day of concentrate and barley straw until 7 months. Then, they were finished with concentrate *ad libitum* for 100 days. The concentrate was composed of 40% corn meal, 28% barley meal, 21% toasted soybean flour, 4% palmist extraction flour, 2% palm oil, 2% calcium carbonate, 2% fatty acid calcium salt and 1% sodium chloride.

At the time of slaughter, animals were transported in a lorry with natural ventilation and space allowance of 2.5 m^2 per animal. Within each breed and production system, half of the animals were mixed with unfamiliar individuals during transport and further lairage at abattoir installations prior to slaughter (I: eight AV, seven RE and six RG; E: five AV, twelve RE and six RG), whereas the other half were not mixed with other animals (I: ten AV, six RE and six RG, E: six AV, eight RE and ten RG). At arrival to the abattoirs, animals were located in lairage pens and were slaughtered 30 min after arrival to avoid additional stress.

Animals were slaughtered in commercial abattoirs of each region following safety and welfare conditions according to European Union regulations (Council Regulation (EC) No 853/2004 and No 1099/2009). The average carcass weights for each breed were 297.5 $\pm$ 59.1 kg for AV, 314.5 $\pm$ 23.3 kg for RE and 244.9 $\pm$ 39.0 kg for RG.

Sample collection

At 2 h *post mortem*, approximately 30 g of *Longissimus thoracis et lumborum* (LTL) muscle sample was taken at 13th rib level from the left-half carcass of each animal. Subsamples of 10 g were immediately frozen in liquid nitrogen and stored in Falcon tubes at −80 °C until further analysis. The rest of the muscle samples (20 g) were transported to the laboratory under refrigeration conditions (4 °C) and subsamples of 10 g were snap-frozen in liquid nitrogen and stored at −80 °C in Falcon tubes after 8 and 24 h *post mortem* time.

At 24 h *post mortem*, ultimate pH measurements (pHu) were taken at the sixth rib level using a penetration electrode (CRISON pH/mV-meter 506, CRISON Instruments SA, Barcelona, Spain).

Extraction of sarcoplasmic proteins

Half gram of each LTL muscle sampled at different *post mortem* times (2, 8 and 24 h) was homogenized in 2 mL of extraction buffer containing 10 mmol/L HEPES, pH 7.5, 0.1% *v/v* Brij 30%, 10% *w/v* sucrose, 1 mmol/L EDTA and 1 mmol/L PMSF using an Ika ultra-Turrax device (Yellow Line Di 25 model, IKA®-Werke GmbH & Co. KG, Staufen, Germany). The homogenate was centrifuged at 20 000 × *g* for 20 min at 4 °C (Beckman Coulter Inc.,

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Indianapolis, IN, USA), the supernatant filtered through 0.45 µm PVDF syringe filter and stored at −80°C until further analyses.

Determination of caspases 9 and 3/7 activities

Sarcoplasmic protein extracts (50 µL) were poured each in a well of a 96-well microtiter plate followed by the addition of 20 µL of a 24 mmol/L DTT solution. Microplate was pre-incubated for 30 min at 37 °C and the enzyme reaction was initiated by adding 50 µL of 0.1 mmol/L of Ac-LEHD-AMC (caspase 9) or Ac-DEVD-AMC (caspases 3/7) substrate dissolved in HEPES-CHAPS buffer (10% *w/v* sucrose, 100 mmol/L sodium chloride, 50 mmol/L HEPES pH.7.5, 0.1% *w/v* CHAPS and 1 mmol/L EDTA). Fluorescence intensity (expressed as Relative Fluorescence Units) was measured using a CLARIOstar microplate fluorometer (BMG LABTECH GmbH, Ortenberg, Germany) for 30 min at 37 °C in 2 min intervals using excitation and emission wavelengths of 360 ± 15 and 480 ± 20 nm, respectively. Three technical replicates were done for each assay.

Statistical analysis

Data were subjected to analysis of variance (ANOVA) using Linear Mixed Model procedures of SPSS statistical software (version 26.0, Chicago, IL, USA) considering the individual animal as a subject and carcass weight as a covariate in the model. Caspase activity (caspases 9 and 3/7) data were analyzed using breed, production system and transport as fixed factors and *post mortem* time as a repeated measure factor. Production system was nested within breed and transport was nested within production system and breed. For muscle pHu, the effect of *post mortem* time was excluded from the model. The parameters of the model were estimated using the restricted maximum likelihood method and the Huynh–Feldt matrix was selected for the repeated measures covariance structure following the Akaike information criterion. Additionally, least square means of dependent variables for the levels of fixed factors breed and *post mortem* time were compared using the least significant difference (LSD) test. Significance level was declared at $P \leq 0.05$.

RESULTS AND DISCUSSION

Results of statistical significances of studied factors and binary interactions are reported in Table 1. The covariate (carcass weight) included in the mixed model did not affect significantly any of the variables studied (pHu and caspase activities; $P > 0.05$) but it was maintained in the models as adjustments were improved. In the present study, all carcasses presented normal pHu values (below 5.9), but these values were significantly affected by breed ($P \leq 0.001$) while the effects of the production system and transport type were not significant. RE breed showed significantly higher values (5.64 ± 0.12) compared to the other two breeds (5.49 ± 0.06 for AV and 5.55 ± 0.14 for RG), and this could be related to its excitable temperament.[15] In the present study, other indicators apart from pHu, have been considered to evaluate the biochemical characteristics of *post mortem* muscle and final meat quality. In this sense, measurements of caspases 9 and 3/7 activities were considered as an interesting and innovative approach taking into account that animals suffering higher stressing conditions would yield higher apoptosis levels compared to animals managed under less stressful conditions. It is worth considering, however, that caspases 9 and 3/7 activities can vary depending on *post mortem* time. Therefore, these activities were evaluated at 2, 8 and 24 h *post mortem* to determine the caspase enzyme kinetics at early *post mortem* times as a key factor to better understand the biochemical characteristics of meat on the considered bovine breeds.

Table 1. Statistical significances of studied factors and binary interactions

Effects and interactions	pH[a]	Caspase 9	Caspases 3/7
Breed	***	*	ns
Production system (Breed)	ns	ns	ns
Transport (Production system (Breed))	ns	ns	**
PM Time	—	*	*
PM Time × Breed	—	***	***
PM Time × Production system (Breed)	—	ns	ns
PM Time × Transport (Production system (Breed))	—	*	**

*$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$; ns, not significant ($P > 0.05$).
[a] *Post mortem* (PM) time was not included in the mixed model.

The activity of caspase 9 was significantly affected by breed ($P \leq 0.05$) and *post mortem* time ($P \leq 0.05$). However, evolution of caspase 9 activity over time depended on breed ($P \leq 0.001$) and the transport utilized within each production system and breed ($P \leq 0.05$; Table 1). In Fig. 1, the interaction between breed and *post mortem* time on caspase 9 activity has been depicted. In RG breed, a different behavior of caspase 9 activity over time can be clearly observed, showing significantly higher activities at 2 h and lower activities at 24 h *post mortem* compared to AV and RE breeds. Intermediate and statistically no different values among breeds were observed at 8 h *post mortem*. It is assumed that caspases are activated immediately after animal exsanguination, showing the highest activity values at early *post mortem* times and then decreasing over time.[2,3,16–18] In this sense, as previously reported in the literature[19–21], this pattern was only observed in RG breed. In contrast, the low caspase 9 activity observed in RE and AV breeds at early *post mortem* times could be indicative of higher stress levels reached before slaughter. This would trigger activation of other cell defense mechanisms such as synthesis of heat shock proteins (HSPs), which has been widely described for their anti-apoptotic role.[22,23] They interact with active caspases hindering their function and, consequently, slowing down the cellular death process. This could explain the lower levels of caspase 9 activity determined early *post mortem* for RE and AV. In the scientific literature, breed effect and its relation to excitable temperament and stress susceptibility has been reviewed for several species[24] and previously reported for RE breed,[15] but there is no information available concerning the non-aggressive behavior described of AV and RG.[25,26] Low caspase 9 activity in AV breed can be explained considering that it is a double muscle breed[27] caused by myostatin gene mutation, giving rise to an increase in the number of muscle fibers. Hypertrophied animals may be more susceptible to stress for their regularly limited mobility and reduced muscle capillary density, myoglobin content and lung weight as compared to normal cattle.[28] In this line, other authors also found the same

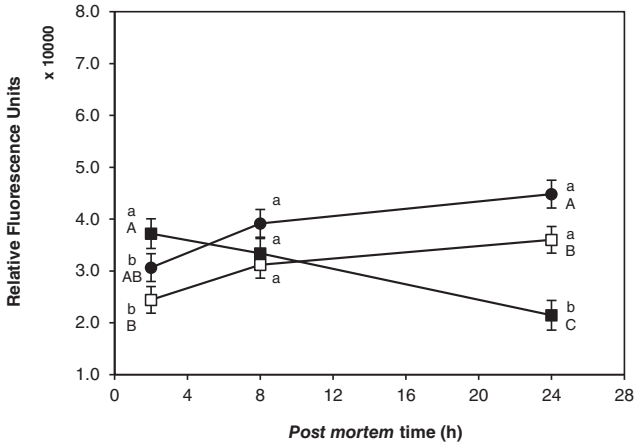

Figure 1. Interaction between breed and *post mortem* time factors for caspase 9 activity. Least square means and standard error of the means have been represented. Different capital letters indicate significant differences among breeds and different lower case letters indicate significant differences among *post mortem* times ($P \leq 0.05$). ●, Asturiana de los Valles; □, Retinta; ■, Rubia Gallega.

caspase pattern in callipyge lambs,[18] showing low caspase activity at early *post mortem* times and then increasing over time reaching the maximum at 24 h. In any case, further research would be necessary to understand the different behavior of caspase 9 activity between

breeds and their relationship with stress events.

The interaction between transport/lairage and *post mortem* time effects has been depicted in Fig. 2. While AV and RE breeds behaved with a similar increasing pattern over time and no differences between mixed and non-mixed animals (Fig. 2(a,b)), a very different pattern was observed for RG breed (Fig. 2(c)), reinforcing the idea of the preponderant role played by bovine breed in caspase activity. Initially (2 h), RG breed yielded slightly higher caspase activity values compared to the other breeds, but with no differences between mixed and non-mixed animals, after that, differences were significant between mixed and non-mixed animal groups (8 and 24 h). In RG breed, behavior of non-mixed-animals followed a similar pattern than that already shown in Fig. 1 for this breed. In contrast, mixed animals behaved differently reaching the highest caspase activity at 8 h *post mortem*. This would be explained considering the protective action exerted by HSPs that may delay apoptosis process over *post mortem* time. It is recognized that transport is one of the most stressful events that influence bovine animals through the meat production chain due to loading/unloading, duration of transport, temperature, feed/water deprivation, noise, vibration, social regrouping and unfamiliar conditions.[7,29] Mixing of unfamiliar animals prior to slaughter can considerably upset group hierarchy, thus promoting the occurrence of behavioral stress[5] and leading to muscle glycogen depletion.[30] However, as already indicated, in the present study transport and lairage conditions did not affect meat pHu while breed did.

The activity of caspases 3/7 was significantly affected by transport ($P \leq 0.01$) and *post mortem* time ($P \leq 0.05$). However, the evolution of caspase 3/7 activity over time depended on breed ($P \leq 0.001$) and on the transport utilized within each production system and breed ($P \leq 0.01$; Table 1). The binary interaction between breed and *post mortem* time effects has been depicted

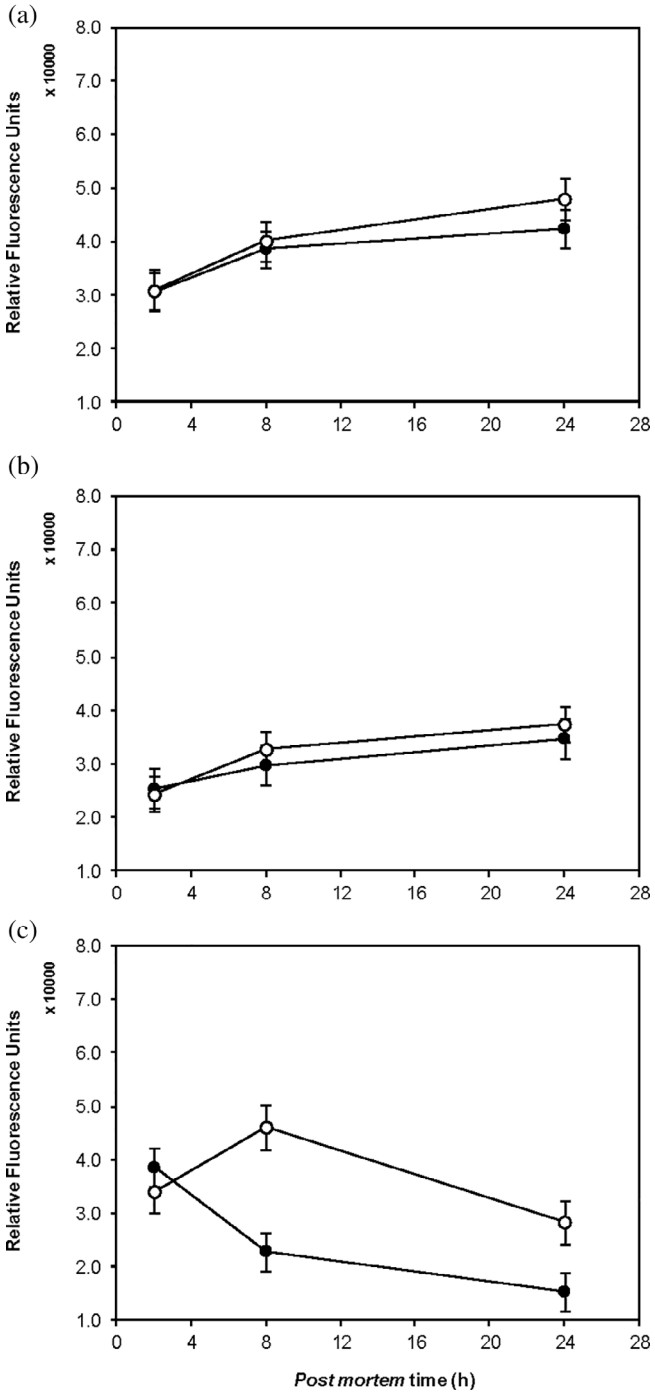

Figure 2. Evolution of caspase 9 activity over *post mortem* time as related to transport/lairage for mixed (○) and non-mixed (●) animal groups in each studied breed: (a) Asturiana de los Valles; (b) Retinta, and (c) Rubia Gallega breed. Least square means and standard error of the means have been represented.

in Fig. 3. The different behavior of caspase 3/7 activity in RG compared to the other breeds (AV and RE) can be clearly observed. Caspase 3/7 activity values of RG breed were significantly different for all *post mortem* times (2, 8 and 24 h) compared to the other two breeds that were similar to each other. At 2 and 8 h *post mortem*, caspase 3/7 activity was the highest in RG breed, while at 24 h was the lowest. It is worthwhile mentioning the differences

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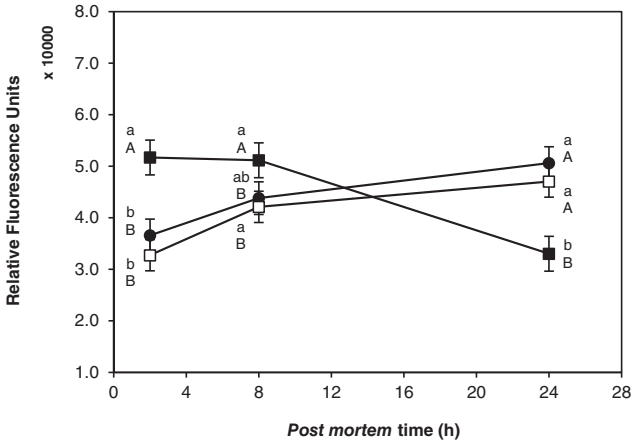

Figure 3. Interaction between breed and *post mortem* time factors for caspase 3/7 activity. Least square means and standard error of the means have been represented. Different capital letters indicate significant differences among breeds and different lower case letters indicate significant differences among *post mortem* times ($P \leq 0.05$). ●, Asturiana de los Valles; □, Retinta; ■, Rubia Gallega.

in animal age at slaughter, as RG were younger (10 months) than the rest of the animals (13–15 months). This fact could also explain, at least partially, the differences found in the *post mortem* muscle metabolism. In this line, Zhu *et al.*[31] reported that several apoptotic mechanisms, including caspase 3 activation, were more pronounced in younger rat brains as compared to more mature developmental states after induction of cerebral hypoxia-ischemia. When comparing both executioner caspase 3/7 and initiator caspase 9 activities, they showed a similar breed-dependant pattern although caspases 3/7 seemed to be slightly less sensitive, not being able to discriminate among breeds at 24 h *post mortem*. This could be explained considering that caspases 3/7 are involved in the last step of signaling caspase cascade, giving rise to a higher residual activity compared to caspase 9.

The interaction between transport/lairage and *post mortem* time for each breed is depicted in Fig. 4. As for caspase 9, AV and RE breeds behaved with a similar increasing pattern over time, with no differences between mixed and non-mixed animals (Fig. 4(a,b)), whereas a very different pattern was observed for RG breed (Fig. 4(c)). At 2 h *post mortem*, caspase 3/7 activity values for RG breed were notably higher compared to the other breeds, with no differences between mixed and non-mixed animals. However, at later *post mortem* times (8 and 24 h) differences were significant between mixed and non-mixed animal groups (Fig. 4(c)), yielding again differences between breeds as observed for caspase 9.

Several studies have described the effect of handling practices on meat characteristics[24,32,33] and some of them have reported differences on caspase 3 expression patterns between normal and DFD samples, finding higher caspase 3 regulation levels in DFD samples at 24 h *post mortem*.[14,15] However, no studies have been previously reported concerning the relationship between caspase activity and stress-related factors. From our study we hypothesized that, depending on the external stimulus affecting animals before slaughter, different caspase activation responses could be triggered. In this regard, our work suggests that the intrinsic apoptotic pathway responding to stress and physiological damage may be the preferred mechanism to initiate the proteolytic cascade through the formation of the caspase 9 apoptosome complex.

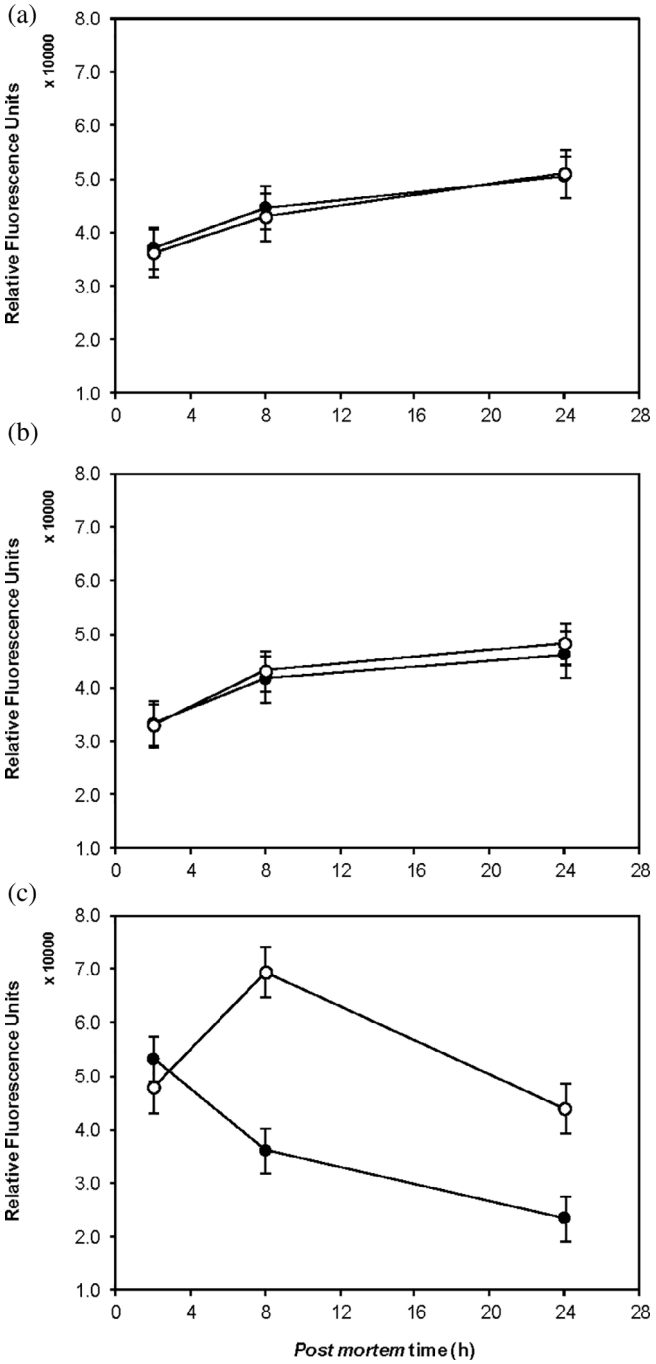

Figure 4. Evolution of caspase 3/7 activity over *post mortem* time as related to transport/lairage for mixed (○) and non-mixed (●) animal groups in each studied breed: (a) Asturiana de los Valles; (b) Retinta, and (c) Rubia Gallega breed. Least square means and standard error of the means have been represented.

CONCLUSIONS

Results obtained in the present work showed that levels of caspase activity over *post mortem* time greatly depends on breed and transport/lairage procedures, while production system

played little effect. The behavior of RG breed over the studied *post mortem* period (2–24 h) was significantly different as compared to AV and RE breeds, suggesting further research is necessary to understand the evolution of both caspase activities in each breed. Caspase activity determinations were able to discriminate among breeds at both 2 and 24 h *post mortem*, especially between those breeds behaving differently (RG *versus* AV and RE). Moreover, enhanced sensitivity of caspase 9 activity at 24 h *post mortem* with respect to caspases 3/7 would indicate its preferable use as an indicator capable to detect breed and transport differences influencing animal meat characteristics. This innovative approach could be an interesting tool to improve assessment of meat quality in the industry. However, future research is necessary to fully address the prediction of stressful situations negatively influencing muscle metabolism and meat quality through assessment of either caspase 8 or 9 activity as the main contributors of the extrinsic and intrinsic apoptotic pathway, respectively.

AUTHOR CONTRIBUTIONS

Conceptualization, Claudia Fuente-Garcia and Miguel Ángel Sentandreu; methodology, Claudia Fuente-Garcia, Mamen Oliván, Daniel Franco, Susana García-Torres, Miguel Ángel Sentandreu; investigation, Claudia Fuente-Garcia; data curation, Noelia Aldai and Luis Javier Rodríguez Barron; writing – original draft preparation, Claudia Fuente-Garcia; writing – review and editing, Claudia Fuente-Garcia, Enrique Sentandreu, Noelia Aldai and Miguel Ángel Sentandreu; supervision, Noelia Aldai and Miguel Ángel Sentandreu; project administration, Miguel Ángel Sentandreu; funding acquisition, Miguel Ángel Sentandreu All authors have read and agreed to the published version of the manuscript.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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