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Skeletal muscle calcium channel ryanodine and the development of pale, soft, and exudative meat in poultry

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ABSTRACT. The development of pale, soft, and exudative (PSE) breast fillet meat has become an economic burden for the poultry industry worldwide. PSE meat results in 1.0-1.5% loss in moisture and carcass weight, and a 2010 estimate of the Brazilian annual production put the economic loss due to PSE at over US$30 million. In the USA, PSE has caused an annual loss of up to US$200 million to the poultry industries. The underlying causes of the color abnormality in PSE meat are not fully understood. However, the likely physiological origin of PSE broiler meat is an excessive release of Ca^{2+} promoted by a genetic mutation of the ryanodine receptor (RYR), a Ca^{2+}-channel protein in the skeletal muscle sarcoplasmic reticulum. In pigs, the genetic cause of PSE meat has been identified as a point mutation in the *RYR1* gene at nucleotide 1843, which causes an amino acid substitution (Arg615 to Cys615) in the RYR. This mutation leads to an alteration in Ca^{2+} homeostasis, hypermetabolism, intense muscle contraction, and malignant hyperthermia in pigs susceptible to porcine stress syndrome. An understanding of this process represents the basis for breeding strategies aimed at eliminating the

RYR1 mutation from global pig populations, a strategy that the poultry industry intends to emulate. The aim of this study was to review the subject, with an emphasis on the most recent developments in the field.

Key words: Chicken; Ryanodine receptor; Mutation; Meat quality; Calcium; Porcine stress syndrome

INTRODUCTION

The mechanisms that control rigor mortis and a decrease in pH in animal muscles are numerous. Many efforts have been directed at understanding these processes to prevent undesirable outcomes such as pale, soft, and exudative (PSE) meat in pigs, turkeys, and chickens (Fujii et al., 1991; Chiang et al., 2004; Oda et al., 2009; Droval et al., 2012b). The recent application of molecular biology and genetic techniques has enabled detailed investigations into the molecular basis of PSE. Muscle contraction is controlled by calcium (Ca^{2+}) release from the endoplasmic reticulum via several channels, one of which is the ryanodine receptor (RYR) protein. Pigs that develop PSE meat are known to have a mutation in the *RYR* gene. This mutation results in an RYR protein that is still active, but whose altered sequence causes a greater release of calcium in muscle tissues than in those expressing the wild-type RYR protein. PSE characteristics have also been observed in poultry meat, but mutations in the genes that encode RYR in poultry have not yet been identified until now.

PSE meat

The term PSE meat is derived from its sensorial characteristics, including discoloration, sagging, and oozing at the meat surface. These physical changes result from accelerated muscle metabolism caused by the increased metabolic activities of the animals bred for rapid weight gain to speed up meat production. During the postmortem phase, anaerobic glycolysis degrades muscle glycogen, leading to the formation of lactic acid from pyruvate resulting in a lowered muscle pH. This decrease in pH is required for proper meat aging and is involved in the process of converting muscle to meat. However, in modern broiler chickens, that are selected for rapid weight gain, the glycogen reserves are rapidly consumed, leading to rapid postmortem glycolysis that lowers the pH while the carcass is still warm. Olivo et al. (2001) reported that in chicken PSE meat, a final pH of below 5.8 was reached within 15 min postmortem. The sharp drop in pH and the high temperature of the carcass cause the denaturation of myofibrillar and sarcoplasmic proteins, leading to the loss of meat exudate (Bendall and Wismer-Pedersen, 1962) and impairing the functional properties of meat, which promotes the development of PSE meat (Barbut, 1998; Brewer and McKeith, 1999; Oda et al., 2003) (Figure 1). The development of PSE meat causes problems for the poultry meat-processing industries, and PSE is estimated to create costs of over US$200 million in the USA and over US$36 million in Brazil annually (Oda et al., 2003; Shen et al., 2007). Consumers can detect the visual color abnormality of the PSE breast fillet meat at the point of the purchase and can taste flavor abnormalities due to PSE after cooking (Droval et al., 2012a).

Genetic basis of PSE meat

The occurrence of PSE meat and its association with a metabolic syndrome was studied

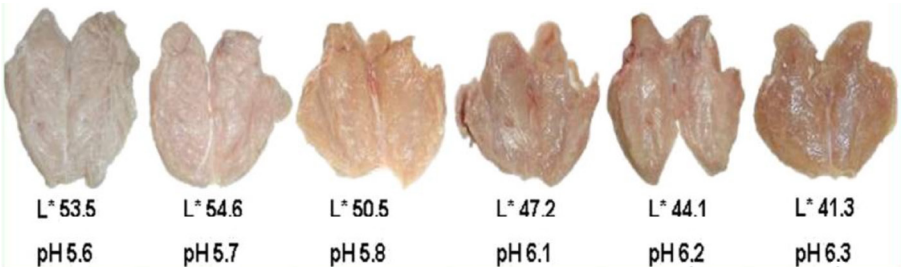

Figure 1. Chicken breast fillets. Examples of meat color that were directly affected by the final pH (Oda et al., 2003).

first in pigs (Fujii et al., 1991). A direct relationship was found between PSE meat and porcine stress syndrome (PSS), which is a condition that causes symptoms similar to those in malignant hyperthermia (MH), a known syndrome in humans. MH has been identified in patients exposed to halothane anesthesia inhalation who experience an excessive release of Ca^{2+} in their tissues during muscle contraction; this increased Ca^{2+} release causes rapid anaerobic metabolism and muscle rigidity, culminating in increased body temperature and hypermetabolism (Ball and Johnson, 1993).

The mechanism of PSS induction promoted by the excessive release of Ca^{2+} from the sarcoplasmic reticulum (SR) was proposed by MacLennan and Phillips (1992). These authors claimed that the concentration of Ca^{2+} in the SR, which stores and releases these ions, regulates muscle contraction, glycolysis, and mitochondrial function. In a normal cycle, Ca^{2+} is released during muscle contraction in a controlled manner through the SR channels and by using energy obtained from balanced aerobic and glycolytic metabolism.

Thus, Ca^{2+} release is regulated by the concentration of ATP and Mg^{2+} as well as by its own concentration in tissues. An ATPase pumps Ca^{2+} into the SR, initiating the relaxation state. Under the abnormal PSS condition, the Ca^{2+}-releasing channels are sensitive to low concentrations of Ca^{2+} and remain open, which increases the Ca^{2+} concentration within the cytoplasm. Thus, muscle contraction is maintained, resulting in muscle rigidity. Aerobic and glycolytic metabolism increases, generating lactic acid, CO_2, and heat, which may cause injury to the cell membranes and systemic PSS irregularities (Cheah et al., 1984; Shen et al., 2007). The dysfunction of the RYR1 protein is caused by a point mutation in the *RYR1* gene (Fujii et al., 1991). In pigs, this mutation consists of a nucleotide substitution at position 1843 of *RYR1*, in which a cytosine present in normal animals is replaced by a thymine in mutant animals displaying PSS. This substitution alters the amino acid at position 615 of RYR1, changing arginine present in the wild type to a cysteine in the mutant protein and resulting in dysfunction of the RYR protein. This dysfunction causes an intense muscle contraction and an acceleration of post-mortem glycolysis, which rapidly lowers the pH in muscle tissue and results in the development of PSE meat. The genetic origin of the *RYR1* mutation has also been shown to occur indirectly, as birds exposed to halothane systematically contract their legs (Owens et al., 2000; Marchi et al., 2009a).

Muscle contraction and the RYR protein

In mammals

Intracellular calcium signaling and the various steps involved in muscle contraction are very

complex events. The basic mechanism requires specialized proteins to release, bind, sequester, and restore Ca^{2+} within cells. Such proteins include RYR, DHPR (dihydropyridine) receptor, and calmodulin (CaM), among others. The structures involved in this mechanism consist of 2 membrane systems, the plasma membrane and the SR, that communicate effectively with each other through specific structures called calcium release units (Protasi et al., 2002). These structures contain proteins that have been identified as key players in this process: the RYRs, large intracellular channels (~2260 kDa) that allow Ca^{2+} release from the SR in response to the depolarization of the plasma membrane, and DHPRs, which are L-type voltage-dependent Ca^{2+} channels that are present outside of the muscle cell membranes and control the opening of the RYRs (Protasi, 2002). In addition to these 2 major proteins, recent studies revealed that CaM is also involved. CaM is a ubiquitously expressed Ca^{2+}-binding protein that binds proteins, mostly under the Ca^{2+}-dependent conditions. Studies suggest that CaM binds to RYR and DHPR in a manner that modulates muscle excitation-contraction in skeletal muscle. Nanomolar Ca^{2+} concentrations inhibit RYR activities (Hamilton et al., 2000), although later reports from Hamilton (2005) indicate that low concentrations of Ca^{2+} (low μM values) activate RYR; conversely, higher Ca^{2+} concentrations (in the μM to mM range) inhibit the activity of the RYR channel. The RYRs, named because of their strong binding affinity to the alkaloid ryanodine, have a molecular weight of approximately 2300 kDa and are formed from a homotetrameric protein comprising polypeptide subunits of 500 and 600 kDa. The protein structure has a large cytosolic N-terminal region, a modulatory central region, and a C-terminal domain. In vertebrates, there are 3 RYR isoforms, which are encoded by 3 different genes: *RYR1*, *RYR2*, and *RYR3* (Sutko and Airey, 1996; Rossi and Sorrentino, 2004). In mammals, RYR1 is expressed at high levels in skeletal muscle fibers, and RYR2 is expressed primarily in cardiac myocytes and brain; in addition, both have been detected in the central nervous system and other peripheral tissues, likely participating in signal transduction mechanisms. RYR3 appears to be expressed in various mammalian tissues, including skeletal muscle, but at levels 20-50-fold lower compared to *RYR1*, although *RYR1* is restricted to the diaphragm (Rossi and Sorrentino, 2004).

In other animals

In poultry skeletal muscle, and in amphibians and fish, two RYR eptor isoforms coexist and are co-expressed, unlike mammalian muscle cells in which only *RYR1* is expressed (Airey et al., 1990; Murayama and Ogawa, 1992). These 2 isoforms are called α and β and they correspond to the mammalian RYR1 and RYR3 proteins, respectively. A third isoform, RYR2, which is recognized by the mammalian RYR2 antibody, was found in the heart tissue of hens and appears to be homologous to the mammalian RYR2 (Ottini et al., 1996).

The isoforms αRYR and βRYR are arranged in precise locations within the skeletal and cardiac muscles, in structures known as triads and dyads, respectively, and have different activation mechanisms. These structures represent complexes that form junctions between the SR and the T tubule system; they ensure a direct interaction between DHPR and RYR, and this association is essential for activating the excitation-contraction muscle mechanism. Thus, αRYR is physically coupled with DHPR and is subject to the conformational changes in DHPR, which are induced by alterations in depolarization within the membrane and cause muscle contraction. The cardiac fibers, in contrast, are not physically associated with DHPRs and are instead activated by a Ca^{2+}-induced Ca^{2+}-release mechanism (Felder and Franzini-Armstrong, 2002). For βRYR in species that possess this Ca^{2+} channel in skeletal muscle in the same proportion as the αRYR located in a parajunctional region immediately adjacent to the SR junctional region, βRYR activation would be indirect during

the excitation-contraction event (Murayama and Kurebayashi, 2011). In summary, 2 mechanisms have been proposed to explain the release of calcium: interaction of the RYR voltage sensors with DHPR and the mechanism of Ca^{2+}-induced Ca^{2+}-release. The first mechanism is the most accepted, whereas the second remains controversial. However, it is thought that distinct processes are involved in the activation of Ca^{2+} release from the SR by different RYR isoforms. In the case of non-mammalian vertebrates in which 2 RYR isoforms are present in similar amounts, it can be assumed that the αRYR isoform is activated by an interaction with DHPR, while βRYR responds to Ca^{2+}-ion activation (Percival et al., 1994).

Figure 2 shows a schematic model of the different functions of these 2 isoforms during muscle excitation-contraction (Strasburg and Chiang, 2003). The behaviors of the α and β RYR isoforms differ depending on whether they are activated by Ca^{2+} or by ATP. In the case of βRYR, Ca^{2+} appears to destabilize the closed state, whereas ATP stabilizes the open state. The different roles of the 2 isoforms are accentuated in the absence of ATP, in which αRYR exhibits a large number of openings for a very short period. The physiological significance of this event needs to be determined, but it is speculated that these channels are more efficient in supplying the Ca^{2+} needed for the activation of adjacent channels than in increasing the concentration of Ca^{2+} in the vicinity of the myofibrils.

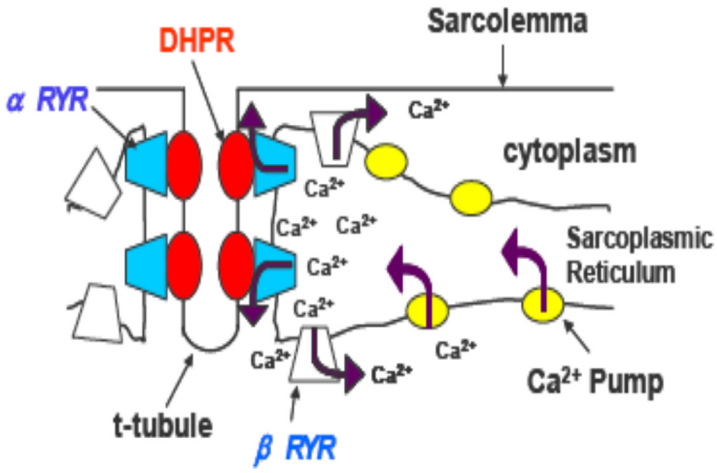

Figure 2. Schematic model of the excitation-contraction mechanism in the skeletal muscle of birds. The T-tubule depolarization causes the release of Ca^{2+} by αRYR, which is physically coupled to DHPR. The local increase in Ca^{2+} causes the opening of βRYR, located peripherally to the tubule's T/sarcoplasmic reticulum junction. The calcium ions are recaptured during the muscle relaxation by the calcium pump (Strasburg and Chiang, 2003).

Oda et al. (2009) evaluated *RYR* gene expression of the α and β isoforms in the muscle cells of PSE broiler chicken meat and non-PSE breast meat. They also analyzed the expression of these genes in a broiler line and a layer line in samples categorized as either normal or PSE by L* and $pH_{24\ h}$ values. The authors found no difference in α*RYR* expression in neither line nor meat quality. However, a decrease in β*RYR* expression was observed in the PSE meat from both lines. These results provided the first evidence that chicken PSE meat may result from excess cytosolic Ca^{2+} caused by the differential expression of the RYR proteins.

Molecular biology and poultry *RYR* mutation

As previously mentioned, RYR is extremely large (~2300 kDa), and the *RYR* gene in humans contains approximately 159,000 bp, divided into 106 exons (~15,000 bp), 2 of which give rise to the different RYR isoforms through alternative splicing of mRNA (Phillips et al., 1996).

The cytoplasmic portion of RYR is made up of 4 subunits (~500 kDa each) and is square in shape, leading to the formation of "legs", which are structures localized at the junction between the T tubules and the SR. There is a close association in this region with the outer-membrane regions, where another molecule responsible for muscle contraction, DHPR, is located (Franzini-Armstrong and Jorgensen, 1994). The N-terminal region of RYR, composed of ~4000 amino acids, interacts with DHPR, whereas the C-terminal region contains ~1000 amino acids and forms the 4 transmembrane domains (M1, M2, M3, and M4) of RYR (Brini, 2004). A schematic model of the large N-terminal region is shown in Figure 3. The junction between the T tubules and the SR is occupied by the N-terminal portion of RYR, and this N-terminal region interacts with DHPR. In addition, many human *RYR1* mutations associated with MH are located in the N-terminal region.

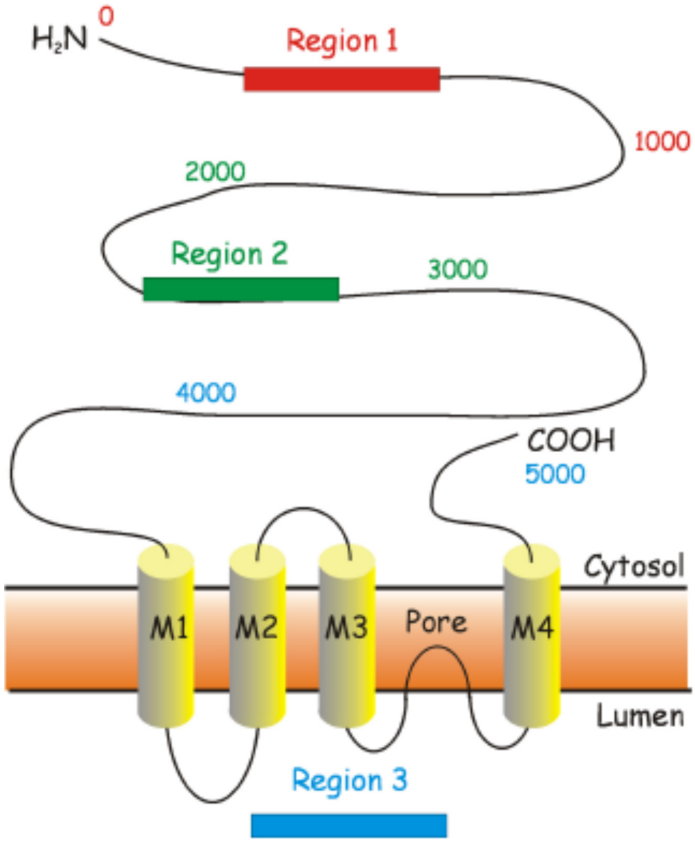

Figure 3. Schematic diagram of regions with different hotspot mutations for *RYR*. Region 1 = amino acids 0-1000; Region 2 = amino acids 2000-3000; Region 3 = amino acids 4000-5000. M1, M2, M3 and M4 = transmembrane domains. Adapted from Brini (2004).

This is also true for pigs, making this protein region a good candidate for mutational analysis in other animals. The N-terminal region between amino acids 1 and 614 is also known as hotspot 1; it contains not only mutations that impair the function of RYR in humans with MH, but also the mutation that causes PSE in pig meat. In addition to this hotspot, there are 2 other regions, located between amino acids 2162-2458 (hotspot region 2) at the N-terminus and between amino acids 4800-4900 (hotspot 3 region) in the C-terminal region, which are important protein domains that control the sensitivity of the RYR channel to regulatory compounds and that modulate Ca^{2+} release (Strasburg and Chiang, 2009). Chiang et al. (2004) screened turkey DNA and cDNA in the region encompassing amino acid residues 36-615 in the αRYR, which corresponds to the region of the human hotspot in RYR1. The authors did not identify any of the mutations in the *RYR1* gene found in pigs; however, upon examining the *RYR1* cDNA, they identified 3 different transcripts for the region corresponding to the cDNA nucleotides 1231-1947 in the human *RYR1* sequence. This included a 717-bp transcript similar to the mammalian skeletal muscle *RYR1*, the AS-81 transcript, which contains a deletion of 81 bases at the beginning of exon 13, and the AS-193 transcript, which lacks all 193 bases of exon 13. According to Chiang et al. (2004), the deletion within αRYR seems to affect the interaction between calcium channels and DHPR, which, as a result, influences the regulation of calcium release. The chicken αRYR protein and cDNA sequences can be accessed from GenBank (accessions X95266, XM_424193, XM_428139, XM_427376).

Ziober et al. (2009) reported the sequence of the cDNA encoding the chicken region hotspot 1 α*RYR* (corresponding to amino acids 386-540 relative to the turkey sequence) and analyzed the N-terminal portion of αRYR. This analysis showed that the chicken transcripts share 97 and 74% sequence identity with the turkey α*RYR* and mammalian *RYR1* sequences, respectively (Figure 4). Note that the sequences of the chicken α*RYR* transcripts described by Ziober et al. (2009) show no similarity to the *Gallus gallus* genomic sequences in the GenBank database. The reason for this discrepancy may be that the region of the α*RYR* transcript sequenced by Ziober et al. (2009) was similar to a region on chromosome 3 from *G. gallus* that contains a gap, representing a DNA fragment with a series of undetermined nucleotides.

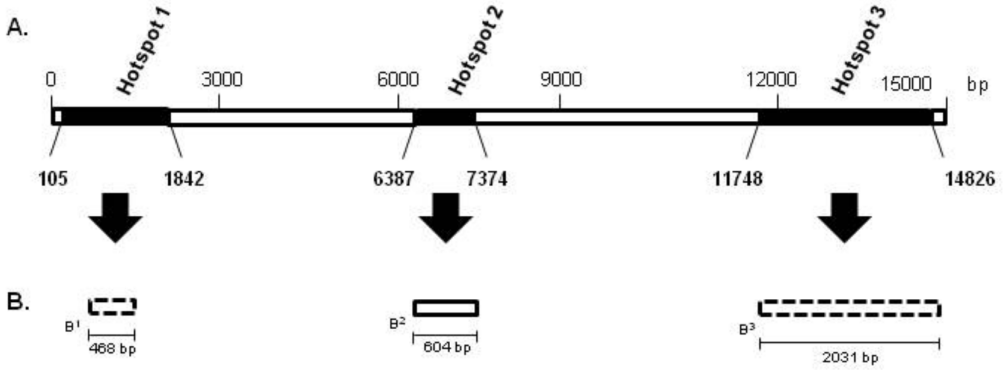

Figure 4. Schematic representation of ryanodine receptor (RYR) domains. **A.** Three regions of human RYR1 amino acid sequence (black) are referred to as hotspots because of the clustering of mutations within these domains. Amino acid residue numbers, which serve as boundaries of the hotspots. **B.** Sequences of the hotspot regions 2 and 3 of *Gallus gallus* (B^2 = 604-bp DNA from hotspot 2 of *G. gallus* are known. B^3 = 2031-bp transcript from hotspot 3 of *G. gallus* are known).

Ottini et al. (1996) sequenced an α*RYR* transcript; however, this sequence was found to correspond to the C-terminal region of the αRYR protein, which does not include the region analyzed by Ziober et al. (2009), corresponding to the N-terminal part of the α*RYR* transcript. Thereafter, Ziober et al. (2010) examined the relationship between the formation of PSE meat under halothane exposure and heat stress and the α*RYR* transcripts from chicken. The authors also sequenced a portion of the chicken mRNA that corresponded to the hotspot *RYR1* gene region and studied the effects of different treatments, such as heat stress and exposure to halothane. Ziober et al. (2010) sequenced 18 regions of chicken cDNA and reported any nucleotide deletions and substitutions. Many of the substitution mutations identified by these authors were synonymous or led to tolerable changes in protein structure, whereas the deletions resulted in transcripts with premature stop codons leading to truncated proteins that were non-functional (Table 1).

Table 1. Results of the sequence analysis of the 18 samples under halothane treatment and heat stress as well as the resulting meat quality.

Sample	Exp. to halothane	Heat stress	Meat quality	Alteration	Nucleotide	Protein	Alteration	Effect on protein
H+HS3	Yes	Yes	Normal	Substitution: T → A	#12	#4	L → H	Tolerable
H1	Yes	No	Normal	Deletion: G	#363	#9	G → A	Truncated
				Substitution: G → A	#27		(CRF)	
							(#16) TI	
H+HS5	Yes	Yes	PSE	Substitution: G → A	#31			Synonymous
				Substitution: A → G	#443	#148	T → A	Tolerable
H+HS6	Yes	Yes	PSE	Deletion: T	#56	#19	Y → T	Truncated
				Substitution: A → G	#305		(CRF)	
				Substitution: A → G	#366		(#34) TI	
C+HS3	No	Yes	Normal	Substitution: T → C	#110	#37	S → P	Tolerable
				Substitution: A → G	#290	#97	I → V	Tolerable
C3	No	No	Normal	Substitution: T → C	#110	#37	S → P	Tolerable
				Substitution: T → C	#275	#91	TI	Truncated
				Substitution: C → T	#344			
H2	Yes	No	Normal	Deletion: C	#151	#51	I → S	Truncated
				Substitution: T → C	#352		(CRF)	
							(#95) TI	
H+HS4	Yes	Yes	PSE	Deletion: C	#151	#51	I → S	Truncated
				Substitution: T → C	#352		(CRF)	
							(#95) TI	
C2	No	No	Normal	No alteration				
C1	No	No	PSE	No alteration				
C+HS1	No	Yes	PSE	No alteration				
C+HS4	No	Yes	PSE	No alteration				
C+HS5	No	Yes	PSE	No alteration				
C+HS2	No	Yes	Normal	No alteration				
H3	Yes	No	PSE	No alteration				
H4	Yes	No	PSE	No alteration				
H+HS2	Yes	Yes	PSE	No alteration				
H+HS1	Yes	Yes	Normal	No alteration				

Identification of the nucleotide alterations, proteins, and the probable effect on the protein structure is also indicated (Ziober et al., 2010). H+HS = halothane treatment and heat stress; C = control group; C+HS = heat stress without halothane treatment; CRF = change of the reading frame; TI = translation interrupted.

However, these changes in mRNA were associated with the stress generated in birds by exposing them to heat, used to induce the formation of PSE meat, and also by exposure to the anesthetic halothane, which was used to select for birds prone to developing PSE meat (Marchi et al., 2009b). It is known that stressors can interfere with α*RYR* gene expression, but it was not

possible to associate these changes directly with the occurrence of PSE meat, as altered mRNA transcripts were found in samples from both normal meat and PSE meat. As gene expression can be modulated by the environment, Droval et al. (2012b) sequenced a portion of the chicken *RYR* gene corresponding to the hotspot region 2 in human *RYR* and identified a polymorphism consisting of a replaced guanidine nucleotide at a specific position of the gene (Figure 5).

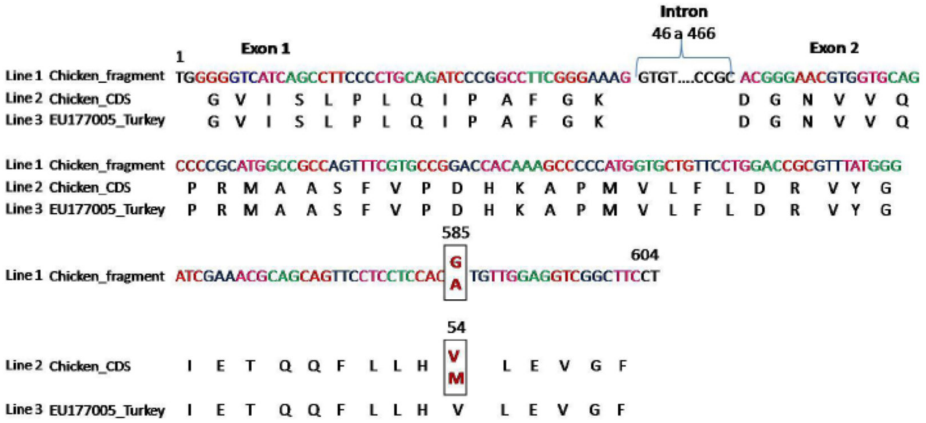

Figure 5. Alignment of the nucleotide sequence of the fragment obtained from chickens with the translation of the amino acid sequence, indicating the nucleotide (position 585) and amino acid (position 54) modified in the coding region. *Line 1* = nucleotides colored in red, blue, pink, and green correspond to the codons of the coding region and the black non-coding region. *Line 2* = amino acid sequence of αRYR in chicken. Black indicates the normal sequences and red indicates the altered amino acid sequence. *Line 3* = amino acid sequence of αRYR in turkey (2455-2513) (Droval et al., 2012b).

However, as this polymorphism occurred in both the normal meat and the PSE meat samples, it was not possible to correlate the presence of the polymorphism with PSE meat in poultry.

CONCLUSIONS

The development of PSE meat represents an economic problem for the poultry industry worldwide. Although genetic studies demonstrated that PSE pig meat is associated with a mutation in the *RYR1* gene encoding the RYR1 protein, no *RYR1* gene mutations that are involved in the development of PSE meat in chickens have yet been discovered. In turkeys, some variants in RYR transcripts were identified, but none was related to changes in *RYR* DNA or to the occurrence of PSE meat. Thus, the gene sequences involved in PSE meat formation, especially the *RYR* gene, are still being evaluated to identify mutations that would explain a similar genetic origin of poultry PSE meat as in pigs, or to identify a molecular marker for PSE meat formation. Such information might lead to the development of a molecular diagnostics tool that could be used to predict the development of abnormal broiler chicken meat.

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