Post-mortem changes in chicken muscle.

Some key biochemical processes involved in the conversion of muscle to meat.

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Abstract

The post mortem changes taking place in poultry muscular tissue and the resulting meat quality, until the moment of consumption of the meat by the consumer are described.

Modern broiler chickens grow 'at the edge of what is metabolically possible'. This hypothesis is derived from the fact that muscle, and thus protein, accretion is accomplished through a dynamic equilibrium between synthesis and degradation. The cell reaches a certain maximum synthesis capacity. To grow beyond this maximum synthesis capacity the cell has to decrease it degradation. This in its turn is of great influence for meat aging. The chromatographic properties of a class of proteïnases, important for meat aging, called calpains as well as the development of a method for the quantification of the activity of these enzymes is described. Furthermore, a number of endogenous proteïnase and inhibitor activities, important for meat aging, was measured in four chicken selection lines differing in both growth rate and protein efficiency. Differences between lines were observed, the fastest growing lines showing the lowest proteolytic capacities of the calpain/calpastatin system while the slower growing animals showed higher proteolytic potentials in their breast muscles. The highly protein efficient line showed high proteïnase capacity of cathepsin H. Studies on the post mortem course of both the protein and the energy metabolism in the above mentioned selectionlines are described. The slowest growing lines showed the fastest post mortem meat tenderization while the faster growing lines showed slower tenderization, which was not yet completed within 48 hours post mortem. This suggests that increasing growth speed, which is aimed at by the poultry production industry, may at the end lead to meat aging problems and an unacceptable poultry meat quality, especially since the retail sector strives for shorter periods between slaughter and presentation to the customer, based on microbiological considerations.

The development of methods to measure the post mortem proteolytic degradation of the cytoskeletal proteins titin, nebulin, desmin and vinculin using SDS-PAGE and 'Western blotting' are described. Possible candidate fragments of the different cytoskeletal proteins were identified to serve as markers for monitoring the course of meat aging.

Stellingen

1) De algemene diskwalificatie van hydrofobe interactie chromatografie als methode om calpastatine van calpainen te scheiden is niet terecht. Zorgvuldige afstemming van de omstandigheden op de aard van het materiaal en de diersoort waaraan het onderzoek wordt verricht leidt tot een goed bruikbare methodiek. *(Koohmaraie, M., 1990. J. Anim. Sci. 68;659-665, en dit proefschrift).*

2) Meting van de myofibrillaire fragmentatie index (MFI) is slechts een zeer ruwe maat voor de afbraak van structurele spiereiwitten van het vleeskuiken en is als zodanig nauwelijks bruikbaar. *(Dit proefschrift).*

3) Bij het moderne slachtkuiken wordt de groeisnelheid van het jonge dier voornamelijk gereguleerd aan de katabole kant van het eiwitmetabolisme. *(Afgeleid uit dit proefschrift).*

4) Onderzoek naar de exacte aard van sensorische textuur eigenschappen als malsheid en sappigheid, en de nauwkeurige definitie van alle aspecten die ten grondslag liggen aan deze kwaliteitsparameters, alsmede de betrouwbare instrumentele meting hiervan, zouden in het vakgebied van de vleeswetenschappen de allerhoogste prioriteit moeten krijgen. *(Dit proefschrift).*

5) Het verdiend aanbeveling om in de humane diëtetiek meer aandacht te schenken aan het begrip voederconversie.

6) Gezien het feit dat de sociologie en fysiologie van de moderne mens (*Homo sapiens sapiens*) voortvloeien uit een ontstaansgeschiedenis die zich voor meer dan 95% als jager / verzamelaar in de vrije natuur heeft afgespeeld, zou het aan te raden zijn hiermee in meer aspecten van onze samenleving rekening te houden.

7) De afbouw van het tweede kans onderwijs en het blokkeren van de mogelijkheden tot doorstroming binnen het reguliere onderwijs vormen de belangrijkste oorzaken van het steeds groter wordende verspilling van intellectueel potentieel.

8) Eenduidige standaardisatie van de eisen die gesteld worden aan manuscripten die aangeboden worden voor publicatie in wetenschappelijke tijdschriften middels "instructions for authors" of "style guides" zal leiden tot het vrijkomen van een grote hoeveelheid onderzoektijd die besteed zou kunnen worden aan nuttiger zaken dan taalkundige- en stijlcorrectie.

9) Uit het nagenoeg ontbreken van wezenlijke verschillen bij een vergelijking van het oorspronkelijke beleidsvoornemen inzake de concentratie van het veeteeltkundig onderzoek van DLO in Lelystad, met het uiteindelijke beleidsbesluit kan geconcludeerd worden dat de medezeggenschap van de dienstcommissies van de bij de fusie betrokken instituten destijds op alle niveaus slechts symbolisch van aard was.

10) Gezien de afkeer van de gemiddelde stedeling t.a.v. geuren afwijkend van die, die voorkomen in stedelijke gebieden, zoals de geur van dierlijke uitwerpselen, verdient het aanbeveling het overheidsbeleid inzake de hinder- en milieuwetgeving zodanig bij te stellen, dat niet slechts een aantal veetelers in ernstige mate worden gehinderd in hun normale bedrijfsuitoefening, doch dat de migratie van, veelal relatief gefortuneerde, stedelingen naar de landelijke gebieden eveneens wordt beperkt.

11) Gezien het feit dat op vliegvelden, vanwege de geringe toegankelijkheid voor het algemene publiek, vaak uiterst zeldzame flora en fauna wordt aangetroffen, verdient het aanbeveling voor deze terreinen een aparte plaats in te ruimen binnen de natuurbeschermingswetgeving.

12) Het verdiend aanbeveling het oordeel over genetische manipulatie niet over te laten aan ethisch gemanipuleerde leken.

13) Urgentie is in veel gevallen omgekeerd evenredig aan importantie.

Frans Schreurs
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Wageningen, 20 januari 1999.

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September 1998

Menigeen doet erg zijn best,
voor 't geliefd gevederd vee.
Ten eerste om de eieren in hun nest,
ten tweede brengt het nu en dan een heerlijk gebraden hapje mee.
Vrij naar Wilhelm Busch

Das feinste Gemüse der Welt ist Fleisch
Oud Duits gezegde

We dance round in circles and suppose,
But the Secret sits in the middle and knows.
Robert Frost.

De reis van duizend mijlen begint met één stap.
Lao Tzu.

Voor Carla, Thom en Eric

Contents

Samenvatting*

Dit proefschrift beschrijft de veranderingen die plaatsvinden in spierweefsel in de periode na het slachten van een vleeskuiken tot het moment van consumptie van het vlees door de consument. Onderzocht zijn factoren die een rol spelen bij de omzetting van (levend) spierweefsel naar (dood) vlees. De invloed van deze factoren, betrokken bij deze omzetting, op de kwaliteit van het product, is eveneens bestudeerd.

In de loop van 1990 werd op het toenmalige Centrum voor Onderzoek en Voorlichting voor de Pluimveehouderij "Het Spelderholt" van de Dienst Landbouwkundig Onderzoek (COVP-DLO), in het kader van het onderzoekprogramma Dierlijke Productie, een onderzoekproject gestart, getiteld: "Onderzoek naar de effecten van eiwitafbraak in (pluimvee)vlees gedurende de verwerking en bewaren." Het project had de volgende doelstelling:

> Het is te verwachten dat door het verkrijgen van meer inzicht in de processen die een rol spelen bij de *post mortem* veranderingen in spiervlees, voorspellingen kunnen worden gedaan over vleeskwaliteit die optreedt ten gevolge van bepaalde verwerkingsmethoden. Als gevolg van voornoemd onderzoek zullen methoden ontwikkeld worden voor het aantonen van afbraakprocessen en de producten die ten gevolge van deze processen vrijkomen. Met deze detectiemethoden kan een signalering plaatsvinden van het tijdstip van optimale kwaliteitsontwikkeling.

In een later stadium werd hieraan een deelproject toegevoegd getiteld: "De invloed van het eiwitmetabolisme in de spier van de kip op de kwaliteit van het vlees". Het beoogde eindresultaat van dit project luidde:

> Het project zal uiteindelijk resulteren in publicatie van een rapport waarin een verklaring op cellulair en moleculair niveau zal worden gegeven voor de processen die tijdens de vleesrijping optreden.

Dit proefschrift is een weerslag van de onderzoeken die in het kader van beide bovenstaande projecten zijn uitgevoerd tussen begin september 1991 en eind februari 1998. In de loop van het onderzoek bleek dat er niet geheel voldaan kon worden aan de doelstellingen van bovenstaande projecten. De doelstelling van beide projecten was zeer, en misschien wel te, ambitieus. Toch is er gedurende die periode waardevolle kennis verzameld over de processen die een rol spelen bij de omzetting van spierweefsel van de kip in vlees. Zoveel zelfs dat op het moment van schrijven van dit proefschrift de daadwerkelijke beïnvloeding, en misschien zelfs sturing, van de vleeskwaliteit langzamerhand in zicht komt. In het kader van de steeds belangrijker wordende rol van de Integrale Keten Beheersing (IKB) en Integrale Kwaliteitszorg zijn dit belangrijke ontwikkelingen. Door sturing van de kwaliteit van het product kunnen garanties gegeven worden aan de afnemers. Er kan "op specificatie" geproduceerd worden, zowel vanuit de kenmerken van een grondstof, als naar de eisen van de afnemer toe.

In hoofdstuk 1 wordt een overzicht gegeven van de literatuur die aan de basis van beide projecten heeft gelegen. In het kort wordt achtereenvolgens ingegaan op de ontstaansgeschiedenis van het moderne vleeskuiken, de bouw en functie van spierweefsel, de achterliggende factoren bepalend voor de spiergroei en de consequenties van het voorgaande voor de vleeskwaliteit. Het merendeel echter van de literatuur, behandelt processen zoals die zich afspelen

* In afwijking van de Engelse samenvatting is voor de Nederlandstalige samenvatting gekozen voor een meer algemene beschrijving van de resultaten teneinde de inhoud van dit proefschrift voor een groter publiek toegankelijk te maken.

in spierweefsel van zoogdieren, voornamelijk runderen en in mindere mate varkens en schapen. De eerste twee diersoorten vormen wereldwijd de belangrijkste vleesproducenten waarbij opgemerkt dient te worden dat de vleeskwaliteitsproblematiek bij het rund het grootst is. Voor pluimvee geldt dat in veel mindere mate. De pluimveesector is nog steeds groeiende, en vleeskwaliteitsproblemen treden niet of nauwelijks op de voorgrond. Reden waarom binnen het onderzoek, verbonden met deze sector, nauwelijks aandacht is voor de "fysiologie van de kippenspier". Niettemin lijkt op basis van het in hoofdstuk 1 beschrevene, samen met een aantal vooronderstellingen gebaseerd op dit literatuuronderzoek, de kippenborstspier een ideaal object te zijn voor de bestudering van de processen die ten grondslag liggen aan de conversie van spierweefsel naar vlees. Met name de hypothese dat moderne vleeskuikens groeien "op het randje van wat fysiologisch haalbaar is", opent goede mogelijkheden voor de bestudering van de invloed van eiwitafbraak processen op de vleesrijping en daarmee op de kwaliteit van het product. Immers, de aanzet van spierweefsel wordt bepaald door de ligging van het dynamisch evenwicht tussen synthese en afbraak van (spier)eiwitten. Omdat er aan het synthesevermogen van de cel een fysiologisch maximum verbonden is, en vanwege het feit dat moderne vleeskuikens geselecteerd zijn op een extreem snelle spiergroei, ligt het voor de hand te veronderstellen dat verdere groeiversnelling alleen bewerkstelligd kan worden door vermindering van de eiwitafbraak. En juist die eiwitafbraak is belangrijk voor de vleesrijping.

Hoofdstuk 2 geeft een beschrijving van een groot aantal experimenten, voor een deel uitgevoerd op het "Station de Recherches sur la Viande" van het "Institut de la recherche Agronomique"(INRA-SRV) te Theix (Frankrijk) en voor de rest op het COVP-DLO later ID-DLO. Tijdens deze experimenten werden de chromatografische eigenschappen van de twee voor de post mortale eiwitafbraak belangrijke iso-enzymen van calpaïne in kippenspieren bestudeerd. Met name die eigenschappen die van belang zijn om de twee eiwitsplitsende enzymen (proteïnasen) kwantitatief van elkaar te scheiden werden uitgebreid bestudeerd. Aan de hand van de resultaten van deze studie werd een methode opgezet ter kwantificering van de enzymatische activiteit van genoemde proteïnasen.

Hoofdstuk 3 geeft vervolgens de resultaten weer van een experiment, waarbij onder andere met de in hoofdstuk 2 beschreven methoden, de enzymatische activiteit van een aantal voor de vleesrijping van belang zijnde, van nature in spierweefsel voorkomende proteïnasen en hun remmers (inhibitoren) werd gemeten in borstspierweefsel van een drietal vleeskuiken selectielijnen en een leghennen lijn. Op basis van de werkhypothese dat moderne vleeskuikens groeien "op het randje van wat fysiologisch haalbaar is", kon de volgende aanvullende hypothese geformuleerd worden:

> Op basis van het feit dat de eiwitaanzet in weefsels, en dus ook de spiergroei, de resultante is van een dynamisch evenwicht tussen synthese en afbraak van eiwitten zal bij dieren die hun maximale eiwitsynthese capaciteit hebben bereikt, extra eiwitaanzet alleen kunnen plaatsvinden door reductie van de eiwitafbraak.

Indien deze hypothese waar is, dan zou dat moeten blijken uit het feit dat extreem snel groeiende dieren dus een lagere activiteit van eiwitsplitsende (proteolytische) enzymen in hun spierweefsel vertonen.

Voor het onderzoek werd gebruik gemaakt van de volgende selectielijnen:

1. White Leghorns: een legkippen lijn met een zeer trage groei en een zeer ongunstige voederconversie m.b.t. lichaamsgroei.
2. FC-lijn: een vleeskuiken selectielijn ontwikkeld op "'t Spelderholt", met een matige groeisnelheid maar met een extreem gunstige voederconversie.

3. GL-lijn: een vleeskuiken selectielijn, ontwikkeld uit dezelfde genetische achtergrond als de FC-lijn, met een zeer snelle groei en een gemiddeld gunstige voederconversie.
4. Ross: een commerciële vleeskuiken selectielijn, als exponent van het in de praktijk gebruikelijk vleeskuiken type, met een snelle groei en een gemiddeld gunstige voederconversie.

De hypothese dat snel groeiende kuikens een lagere spiereiwit afbraak capaciteit vertonen, bleek te worden bevestigd. Daarnaast werd bij een betere, dus lagere, voederconversie (FC-lijn), een hogere activiteit van met name cathepsine H gevonden , hetgeen duidt op een efficienter (her)gebruik van eiwitten. Cathepsine H is een proteïnase met een laag pH optimum en dus waarschijnlijk pas werkzaam is in de latere stadia van de vleesrijping. Hieruit kon de conclusie getrokken worden dat de dieren die gebruikt waren bij het in hoofdstuk 3 beschreven experiment uitstekend geschikt zijn als bron van natuurlijke variatie in de proteïnasen die verantwoordelijk lijken te zijn voor de vleesrijping, en dus gebruikt kunnen worden voor de bestudering van de post mortale eiwitafbraak in vleeskuiken borstspieren. Op grond van het in dit hoofdstuk beschreven experiment kon worden verwacht dat er inderdaad verschillen zouden optreden in de vleesrijping van de verschillende hier gebruikte selectielijnen. Immers, een grotere proteolytische capaciteit van bepaalde enzymsystemen in de borstspier, zoals gevonden werd bij langzaam groeiende lijnen, zou moeten leiden tot een versnelde vleesrijping.

Vervolgens werd een onderzoek gestart naar de verschillen in de post mortale processen, betrokken bij de omzetting van spierweefsel naar vlees. Daarbij zijn twee mechanismen van belang, namelijk enerzijds het energiemetabolisme en anderzijds het eiwitmetabolisme in de spier. In hoofdstuk 4 worden de resultaten beschreven van een experiment, waarbij de verschillen in beide stofwisselingsprocessen in de tijd na het slachten werden bekeken, alsmede de invloed daarvan op de malsheid van het vlees. Daarbij werden verschillen aangetoond, waarbij met name opviel dat de Leghorns, de langzaamst groeiende selectielijn, de snelste post mortale vermalsing vertoonde. De snel groeiende Ross en GL-lijnen, vertoonden een duidelijk vertraagde vermalsing die zelfs in de meetperiode (48 uur) niet geheel voltooid was. Dit geeft een aanwijzing dat nog verdere groeiversnelling door middel van genetische selectie, zonder daarbij de vleeskwaliteit in aanmerking te nemen, op de duur moet leiden tot vleesrijpingsproblemen in de pluimveeverwerking, temeer omdat afnemers, zoals grootwinkel bedrijven, steeds stringentere versheidseisen stellen aan het product.

Daarnaast werd gevonden dat de instrumenten, die tot dan toe werden gebruikt om een indruk te krijgen van de afbraak van structurele spiereiwitten, onvoldoende gevoelig waren om deze processen daadwerkelijk in kippenspierweefsel waar te nemen. Daarom begaf het project zich vanaf hier in twee richtingen, namelijk enerzijds een vervolgexperiment om de post mortale vermalsing van spierweefsel in de vier verschillende selectielijnen zowel instrumenteel als sensorisch nader te karakteriseren, en anderzijds om methoden te ontwikkelen voor een nauwkeuriger en gevoeliger meting van de afbraak van enige voor de vleesrijping van belang zijnde myofibrillaire cytoskeletaire eiwitten, namelijk titine, nebuline, desmine en vinculine.

In hoofdstuk 5 wordt een experiment beschreven waarbij sensorische malsheidsmetingen op verschillende manieren werden vergeleken met een instrumentele afgeleide hiervan, de shear force oftewel scheurweerstand van het vlees. Daarbij werden de verschillen tussen de lijnen, die werden gevonden in shear force globaal ook teruggevonden bij de sensorische, paarsgewijze vergelijking in malsheid, waarbij telkens een stuk filet van één van de lijnen werd aangeboden samen met een stukje van een standaardmonster (Ross). De proefpersoon werd gevraagd aan te geven welk van de twee stukken het meest mals of sappig was. Het

verloop van de sensorische malsheid, en sappigheid, in de tijd kon beter bestudeerd worden met behulp van de zogenaamde lijnschaal methode, waarbij aan de proefpersonen werd gevraagd aan de malsheid en sappigheid van een product een score toe te kennen. Bij analyse van de lijnschaal resultaten, werd opgemerkt dat voor de termen malsheid c.q. taaiheid een vrijwel uni-dimensionele respons van het panel werd verkregen. Dat wil zeggen dat alle proefpersonen onder deze twee termen ongeveer hetzelfde verstaan. De analyse van de termen sappigheid c.q. droogheid viel uiteen in tenminste 3 dimensies, wat wil zeggen dat de verschillende proefpersonen tenminste 3 verschillende aspecten in deze termen aangaven. Dit geeft aanwijzingen dat de hier gebruikte, statische, sensorische methoden wellicht onvoldoende zijn als meting van malsheid, taaiheid, sappigheid en droogheid en dat de aan deze waarnemingen ten grondslag liggende fysische principes wellicht gecompliceerder zijn dan vaak wordt gedacht. Hieruit wordt geconcludeerd dat de sensorische waarneming van vleeskwaliteitskarakteristieken meer aandacht verdient in het productonderzoek dan het tot nu toe krijgt. Met name de ontwikkeling van dynamische meetmethoden, waarbij het verloop van de verschillende vormen van "mondgevoel" in de tijd, gedurende consumptie, kan worden bestudeerd. Wellicht dat dan de verschillende aspecten van de sensorische waarneming beter kunnen worden vertaald naar objectieve, instrumentele, metingen.

Hoofdstuk 6 geeft een beschrijving van experimenten die werden uitgevoerd in het kader van de ontwikkeling van methoden voor de meting van de post mortale eiwitafbraak. Daarbij werd gebruik gemaakt van SDS-PAGE (sodium-dodecyl-sulfaat polyacrylamide gel electroforese), een methode waarbij eiwitten op basis van hun grootte worden gescheiden en vervolgens zichtbaar gemaakt. Een groot aantal spiereiwitten is hiermee zichtbaar te maken maar de kleinere cytoskeletaire eiwitten komen in een zodanig laag gehalte voor in spierweefsel dat detectie met behulp van deze methode niet mogelijk is. Daarvoor is een techniek nodig die "Western blotting" wordt genoemd. Hierbij wordt gebruik gemaakt van de eigenschap van antistoffen dat zij zich specifiek binden aan de componenten waartegen zij zijn opgewekt. Vervolgens kan deze binding zichtbaar worden gemaakt en gekwantificeerd. Op deze manier werden een aantal afbraakproducten van de structurele spiereiwitten zichtbaar gemaakt die vrij goed correleerden met de post mortale veranderingen in shear force en waterbindend vermogen. De hierboven beschreven techniek heeft een aantal nadelen. Naast het feit dat deze methoden nogal gecompliceerd zijn en dus een goed getraind analist behoeven, zijn ze alleen in semi-kwantitatieve zin te gebruiken. Voor een echte kwantitatieve meting zijn andere technieken als ELISA en eventueel het gebruik van biosensoren geschikter. Aan het eind van dit hoofdstuk wordt aangegeven wat de toekomst perspectieven van deze technieken zijn.

Tot zover werden alle experimenten uitgevoerd onder standaard, niet met de normale slachtpraktijk overeenkomende, omstandigheden. Dit werd gedaan om invloeden van het slachtproces zelf zoveel mogelijk uit te sluiten. Hiermee werd een goed inzicht verkregen in het basale, niet door slachtproces of andere factoren beïnvloede post mortem metabolisme in de spier.

Hoofdstuk 7 is een weergave van een meer praktijk gericht experiment, waarbij de invloed van verschillende ante mortem verdovingsmethoden op de post mortale spierstofwisseling werd bestudeerd. In grote lijnen was de conclusie van dit experiment dat veel van de waargenomen verschillen in post mortem spiermetabolisme terug te voeren zijn op de heftige spiercontracties die optreden bij het bewusteloze dier, hetzij tijdens de electrische bedwelming, hetzij tijdens de convulsies die optreden ten gevolge van het optreden van anoxie, een toestand van zuurstoftloosheid, tijdens het gasverdoven. In sommige gevallen werd de spierstofwisseling versneld. Dit kan leiden tot vleeskwaliteitsverlies, zoals bij de kop/kop electrische

verdoving. Bij de met argongas verdoofde dieren trad eveneens een versnelling van de spierstofwisseling op, echter in mindere mate en zonder de negatieve gevolgen voor de vleeskwaliteit. De klassieke waterbad verdoving, zoals die nu in heel west-Europa en de VS wordt toegepast, gaf consequent slechtere resultaten dan de andere methoden. Met name het optreden van bloedingen in zowel de borstspier als in de dij was veel frequenter dan bij de andere methoden waarbij dient te worden opgemerkt dat ook bij kop/kop elektrische bedwelming hoge bloedingen scores werden waargenomen, echter minder dan bij de waterbad verdoving. Beide geteste gasverdovingsmethoden gaven een lage bloedingen score. Door de versnelling van het rigor mortis proces, zonder dat er negatieve gevolgen optraden voor de vleeskwaliteit, alsmede door de veel lagere bloedingenscore die werd waargenomen, werd de bedwelming door middel van inductie van een toestand van anoxie met behulp van een argon/koolzuur/stikstof mengsel als beste beoordeeld. Daarna volgden respectievelijk mechanische bedwelming m.b.v. een schietmasker en bedwelming m.b.v. een koolzuur/zuurstof/stikstof mengsel doordat deze laatste methode een vertraging van het rigor proces veroorzaakte. De "total body" elektrische bedwelming m.b.v. een waterbad volgt vanwege de vele bloedingen én de vertraging van de rigor ontwikkeling op de vierde plaats en als minst goede verdovingsmethode werd de kop/kop elektrische verdoving beoordeeld, omdat deze laatste naast veel bloedingen ook negatieve gevolgen voor de vleeskwaliteit liet zien.

Hoofdstuk 8 tenslotte is een algemene discussie waarbij de uitgangspunten van het gehele project naast de uitkomsten worden gezet waarbij deze laatste in een breder perspectief worden geplaatst. Daarnaast worden een aantal aanbevelingen voor zowel de pluimvee verwerkende industrie alsmede voor verdieping van het onderzoek gegeven.

Summary.

This thesis describes the post mortem changes taking place in poultry muscular tissue and the resulting meat quality, until the moment of consumption of the meat by the consumer. Objectives of the study were to gain insight in these processes and to predict the quality of the meat as a result of processing factors.

The experiments described in the scope of this project were carried out between the beginning of September 1991 and the end of February 1998. During this period, valuable knowledge was acquired about the processes involved in the conversion of muscle to meat in poultry.

Chapter 1 describes the literature study carried out in order to obtain a basis for this work. Successively, the history of development of the modern meat type chicken, the form and function of its muscles, factors determining muscular growth and the consequences of this for meat quality are described. Literature predominantly describes the processes occurring in mammalian tissue and mainly in beef. Processes occurring in poultry muscle are rarely studied. However, some presumptions can be made based on this literature study, indicating that chicken breast muscle is the ideal object for the study of the processes involved in muscle aging. Especially the remark, that modern broiler chickens grow 'at the edge of what is metabolically possible', is important in this scope. This hypothesis is derived from the fact that muscle, and thus protein, accretion is accomplished through a dynamic equilibrium between synthesis and degradation. As is shown, the cell reaches a certain maximum synthesis capacity. To grow beyond this maximum synthesis capacity the cell has to decrease it degradation. This in its turn would possibly be of great influence for meat aging. This indicates that poultry breast muscle is especially suited to study the effects of post mortem proteolytic degradation on meat aging and the quality of the product..

Chapter 2 describes the experiments carried out to investigate the chromatographic properties of a class of proteïnases, important for meat aging, called calpains as well as the development of a method for the quantification of the activity of these enzymes.

Chapter 3 describes the results of an experiment in which a number of endogenous proteïnase and inhibitor activities, important for meat aging, was measured in four chicken selection lines differing in both growth rate and protein efficiency. Differences between lines were observed, the fastest growing lines showing the lowest proteolytic capacities of the calpain/calpastatin system while the slower growing animals showed higher proteolytic potentials in their breast muscles. The highly protein efficient line showed high proteïnase capacity of cathepsin H.

Chapter 4 describes experiments to explore the post mortem course of both the protein and the energy metabolism. The slowest growing lines showed the fastest post mortem meat tenderization while the faster growing lines showed slower tenderization, which was not yet completed within 48 hours post mortem. This suggests that increasing growth speed, which is aimed at by the poultry production industry, may at the end lead to meat aging problems and an unacceptable poultry meat quality, especially since the retail sector strives for shorter periods between slaughter and presentation to the customer, based on microbiological considerations.

In this experiment, the measurement of myofibrillar fragmentation, originally developed for the determination of the post mortem proteolytic degradation of structural proteins in beef, was shown to be unsuitable for the study of these processes in chicken muscular tissue. From hereon, the project was aimed at two directions. On one hand, both the instrumental and sen-

sory characterization of the post mortem tenderization was pursued. On the other hand, methods were developed to measure the degradation of structural, cytoskeletal myofibrillar proteins more sensitively and accurately.

Chapter 5 describes experiments carried out to study the post mortem course of sensory tenderness and juiciness of chicken breast meat from the four selection lines in comparison with the instrumental equivalent, the shear force. Pairwise comparisons between the lines for the term tenderness were comparable with the shear force measurements. The term juiciness did not show many differences between lines. The time course of the sensory terms tenderness and toughness was comparable to the time course of the shear force measurements. Generalized Procrustes analysis showed uni-dimensionality for the terms tender and tough but at least tri-dimensionality for the terms juicy and dry.

Chapter 6 describes the development of methods to measure the post mortem proteolytic degradation of the cytoskeletal proteins titin, nebulin, desmin and vinculin. SDS-PAGE and 'Western blotting' were used to separate and visualize the individual proteins and some of their degradation products. Possible candidate fragments of the different cytoskeletal proteins were identified to serve as markers for monitoring the course of meat aging.

Up to here, all experiments were carried out under carefully controlled conditions, not comparable with normal slaughterhouse practice, in order to exclude any influences of the slaughtering process itself. Influences of this process will be superposed on the effects of the undisturbed post mortem muscle metabolism.

Chapter 7 describes an attempt to acquire insight in the influence of different stunning methods. Most of the phenomena observed were related to the heavy muscular contractions either as a result from electrical current running through the muscles, or as a result of heavy convulsions during head-only electrical and gaseous stunning methods. Some methods increased metabolic rate, sometimes resulting in decreased meat quality. Hemorrhaging, as one of the most important arguments discrediting whole-body electrical stunning, occurred also during head-only electrical stunning. The gaseous stunning methods did not result in increased incidence and severity of hemorrhages. The argon-induced anoxia stunning/killing resulted in few hemorrhages and an increase of metabolic rate without detrimental effects on meat quality.

Chapter 8, serves as a general discussion in which the objectives of the project are combined with the results. The latter are placed in a broader perspective. Some recommendations are directed towards the poultry processing industry and some suggestions are made for future research.

CHAPTER 1: INTRODUCTION

The domestic chicken (*Gallus domesticus*).

History of domestication

Archaeological evidence shows that chickens were first domesticated from the red jungle fowl (*Gallus gallus*) in south east Asia well before the eighth millennium [1]Bp and taken north to become established in China by approximately 8000 Bp. Later they were introduced to Japan via Korea in 2300 - 1700 Bp, the so-called Yayoi period. It is not sure whether the domestication of the chicken in India, around 4000 Bp, occurred as a diffusion process or as an independent process. The Iron Age (3000 - 1000 Bp) was the main period of dispersion of the domestic chicken (*Gallus domesticus*) throughout Europe, however, they were already present in some areas during the late Neolithic (4500 – 3800 Bp) and early bronze ages (3800 – 3000 Bp). Some archaeological work points to the fact that the dispersion to Europe has taken place from China through central areas of the Russian federation (West & Zhou, 1989). The fact that chickens were grown in farm-like circumstances during the iron age comes from a Roman writer Lucius Junius Moderatus of Gades (Cadiz), also called *Columella*, who lived in the reigns of the first emperors until about 70 AD. He owned farms and lived near Rome. To one Publius Silvinus he addressed his *Res Rustica*, the most comprehensive, systematic and detailed of Roman agricultural works. In part VIII of his work (Foster & Heffner, 1968) he states: "*Ac de aliis quidem forsitam ambigatur, an sint agrestibus possidenda: gallinarum vero plerumque agricolae cura solennis est. Earum genera sunt vel cohortalium, vel rusticarum vel Africanarum. Cohortalis est avis, quae vulgo per omnes fere villas conspicitur: rustica, quae non dissimilis villatacae per aucupen decipitur, eaque plurima est in insula, quam nautae in Ligustico mari sitam producto nomine alitis Gallinariam vocitaverunt*". Translated into English this means: "With regards to other animals it may perhaps be doubted whether country people should posses them but the keeping of hens by farmers is quite a general practice. They fall into three classes, the farmyard fowl, the "rustic"-hen and the African fowl. The farmyard fowl is the bird commonly to be seen on almost every farm. The "rustic"-cock which is not very different from the farm-yard bird and is caught by the wiles of the fowler, is found in the greatest number in the island in the Ligurian sea to which sailors have given the name Gallinaria (a lengthened form of the Latin word for hen)".

The domestic fowl was kept at farmyards throughout the middle ages for production of eggs, especially during a period from spring into autumn, because egg production was restricted by daylight length. Only spent hens and obsolete cocks, and sometimes larger pullets, were used for meat consumption. The holding of chickens especially for egg production and much less for meat production was common practice until far into the twentieth century. Special, dual-purpose races of domestic fowl were selected from the root types, like for instance the Barnevelder chicken. From the late forties of this century on, genetic selection programs diverged the layers from the meat type broilers. These selection programs have had a tremendous impact on the poultry industry. Layer type chickens like the White Leghorn nowadays, produce approximately 350 eggs per year, year-round, almost one egg every day.

[1] Bp = before present

Total body weights in these lines rarely exceed 1500 grams for the adult chickens. Broilers on the other hand were grown to a slaughter weight of around 2-2.5 kg at 16 weeks of age during the forties (Griffin & Goddard, 1994) Modern broiler lines grow to a slaughter weight of approximately 2.5 kg within 6 weeks time (Schreurs *et al.*, 1995).

Anatomy of domestic fowl as related to butchery

The M. Pectoralis major. The *M. Pectoralis major* or chicken 'filet', is the economically most important part. Compared to red meat animals, poultry possesses inherent advantages in body composition that are largely due to their anatomic adaptations to flight. Although domestic fowl have retained little of their ancestors' ability to fly, many of the characteristics connected to flight have been conserved, like feathers, fused and pneumatic bones, air sacs and strong flight muscles (Addis, 1986). The flight muscles constitute a major portion of the meat of domestic birds. The pectoral muscle, which depresses the wing, can generate tremendous forces. This can be illustrated by the fact that, due to electric stunning methods, the contractions of the *M. Pectoralis major* can lead to broken keel and wing bones (Ma & Addis, 1973). Situated medially to *the M. Pectoralis major* is the *M. supracoracoideus*, also called the *M. Pectoralis minor*, which elevates the wing. The *M. Pectoralis major* is generally referred to as the large breast muscle and *the M. Pectoralis minor* the small breast muscle.

The high carcass yields of domestic chickens are partly related to adaptations to flight. The breast muscle contributes up to approximately 30% of the total chicken carcass weight while the total muscle weight constitutes approximately 40%. This means that ¾ of the total muscle weight is breast muscle (Addis, 1986).

Fibertypes, blood supply, and metabolism. Chickens, and to some extent turkeys, display the largest differences in muscle color known to occur in one single animal. Breast muscle approaches the whiteness of some fish species, while leg meat is comparable to pork and sometimes beef in redness.

Avian muscle activity can be divided into tonic and phasic types although there are numerous mixed function muscles. The tonic types consist of slow contracting posture muscles and the phasic types of fast contracting muscle exhibiting locomotor functions. From table 1 it is clear that the slow, red, muscles are dependent of oxidative processes for energy supply, as is reflected in a high myoglobin content, a dense cappilarisation of the tissue and the high mitochondria content. The low capillarisation, myoglobin and mitochondria content of the white muscles indicates that these muscles are designed to work under mainly anaerobic conditions. It is therefore that these types are called fast glycolytic muscles. Their energy supply comes mainly from the degradation of glycogen to glucose and further to lactate without supply of oxygen. These muscle types contain large glycogen stores as an energy source.

Early systems for muscle classification distinguished between red and white muscle, which soon proved to be too simplified. Ashmore & Doerr (1971a,b) developed a histochemical system that classified two contraction speed types, α (fast-) and β (slow-twitch) and two color types, red and white fibers in mammalian and avian muscle. It resulted in three fundamental muscle fiber types, namely, the fast twitch white (αW, glycolytic), the fast twitch red (αR, glycolytic-oxidative) and slow twitch red (βR, oxidative) types. Table 1 shows the similarities and differences between the three major fiber types (Addis, 1986).

The flight muscles of migratory birds contain mainly αR fibers. They are capable of oxidatively degrading fuel thus generating large amounts of energy for sustained flight without the danger of fatigue. Non-migratory fowl species like pheasant, grouse and turkey, however, can exhibit spectacular bursts of flight-muscular activity but cannot sustain such performances for a long time. The domestic chicken does not fly much, if at all, but still shows powerful breast muscle activity.

Characteristic	βR	αR	αW
Color	Red	Red	White
Myoglobin content	High	High	Low
Capillary density	High	Intermediate	Low
Fiber diameter	Small	Small/intermediate	Large
Number of mitochondria	High	Intermediate	Low
Contraction speed	Slow	Fast	Fast
Contractile action	Tonic	Phasic	Phasic

Table 1 *Differences between muscle fiber types.*

Their flight muscles contain mainly αW glycolytic fibers (Wiskus *et al.*, 1973; Kiessling, 1977). The main fuels for migratory birds are fatty acids, which they accumulate in the form of subcutaneous fat reserves. The main fuel for non-migratory species is glycogen, which reserves are stored in the, predominantly white, muscle itself and in the liver.

Structure and function of muscular tissue.

Muscle tissue constitutes ca. 75% water, 18-29% protein and the 5-7% rest consists of carbohydrates, fats, minerals and other dissolved material. (Dukes, 1943). Muscle proteins can, on the basis of solubility characteristics, be divided into three groups (Lawrie 1966):

1. Sarcoplasmic proteins
   These proteins are soluble in water or low molarity saline (<50 mM) solutions. They constitute approximately 30% of the muscular proteins and consists mainly of metabolic enzymes under which the endogenous proteinases which will be dealt with below.
2. Myofibrillar proteins
   These proteins are soluble in high molarity (ca. 0.6 M KCl) saline solutions. They constitute approximately 60% of the total muscular protein. This group can be divided into 3 subgroups:
   - Contractile proteins:
     Proteins responsible for the mechanical contraction of the muscle.
   - Regulatory proteins:
     Proteins responsible for the initiation and regulation of muscular contraction.
   - Cytoskeletal proteins:
     Proteins forming the cytoskeleton and as such responsible for the integrity and rigidity of the muscle cell.

3. Connective tissue proteins

These proteins are mostly insoluble in the above mentioned solutions and form a very heterogeneous group of constituents determining the structural integrity and the attachment of the muscle to other anatomical elements, like tendons, myotendinous junctions, myo-neurological junctions etc.

Figure 1 shows a cross section of a skeletal muscle. The thick layer of connective tissue located at the outer side of the muscle is called the epimysium. The fiber bundles are surrounded by the perimysium, a much thinner layer of connective tissue while a thin layer of connective tissue surrounds the separate muscle-fibers called the endomysium.

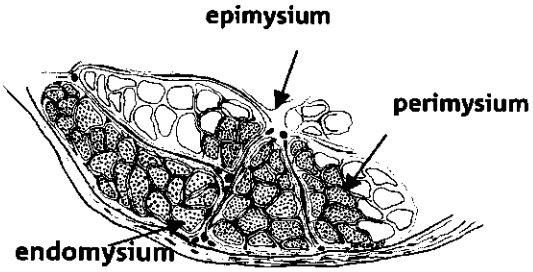

Figure 1 *Cross-section of a skeletal muscle. (drawing: Heleen Schreurs)*

A muscle fiber consists of a longitudinal, multinuclear, cylindrical cell. The multiple nuclei are derived from the fused multicellular preceding stages of muscular development. The length of these fibers varies from rather short to very long. Some fibers span the total length of the muscle, and are connected to the bones through tendons. Others begin and end within the muscle and are connected to the whole through connective tissue structures. The fiber is the smallest muscular unit capable of carrying out all muscular functions. This fiber consists of smaller subunits called myofibrils

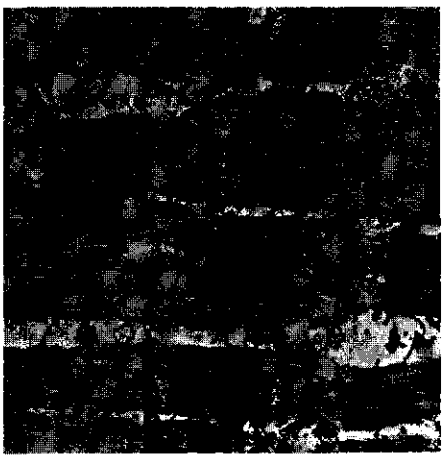

Figure 2 *Longitudinal section through striated muscle. (courtesy: A. Ouali)*

Under the light microscope, muscle tissue shows a typical alternating banding pattern of light and dark bands. Electronic microscopes are capable of giving more details at higher magnifications. Figure 2 shows an ultra micrograph of a longitudinal section of skeletal muscle. The cross-striated banding pattern is clearly visible. The dark A-bands (A = anisotropic) consist of the thick or myosin filaments with on both sides some overlap of the thin filaments. The light colored I-bands (I = isotropic) consist of thin or actin filaments on both sides of a thin but distinct Z-line. The structural unit responsible for the contraction of the muscle is the sarcomere. The sarcomere is defined as the total structure within 2 adjacent Z-lines.

Figure 3 shows a schematic representation of the structure of a sarcomere. The light I-band consists exclusively of thin filaments. These span the A-band to the H-zone. The A-band

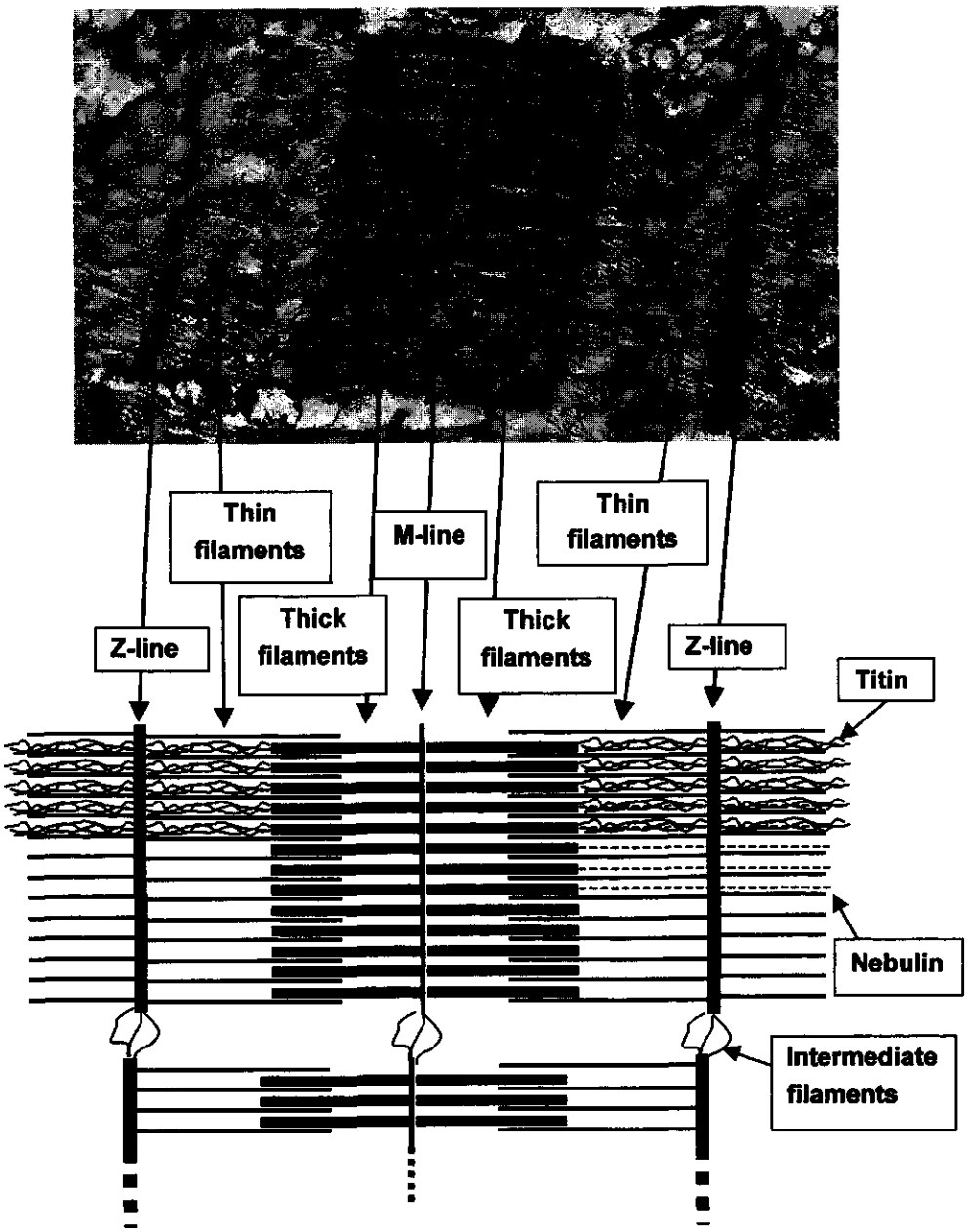

Figure 3 Schematic representation of muscle ultrastructure.

consists of thick filaments. In the middle of the A-band is a less dense region called the H-zone.

In the A-band, outside this zone, the thin filaments of the I-band and the thick filaments of the A-band overlap thus causing a dense region. The less dense H-zone consists of thick filaments only. The transverse line in the H-zone, the M-line, holds the thick filaments in lateral register, while the Z-line holds the thin filaments in lateral register. The thick filaments of the A-band consist mainly of myosin and a small amount of C-protein. Also minute amounts of H-protein and X-protein are found in the thick filaments. These proteins are bound to the surface of the thick filaments while myosin forms the core. The M-line consists of 5 lines running perpendicular to the long axis of the myosin filaments. The M-line proteins hold the myosin filaments in register both transversely and longitudinally. The proteins comprise two types called myomesin and creatinine kinase.

The I-band is composed of thin filaments and is attached to the Z-line at one end and run between the thick filaments at the other end. Main constituent of the A-band is actin, and other proteins of the thin filaments are troponin, tropomyosin, which regulate muscle contraction through mediation of Ca^{2+} ions. Also small amounts of proteins capping the actin filaments are found in the thin filaments, most important of which is β-actinin. The myofibril consists of numerous other proteins but for meat quality and the aging process, only a few are of real importance. Most of these proteins can be grouped together as the so called cytoskeletal proteins.

Table 2 shows the most important myofibrillar proteins. The cytoskeletal proteins are responsible for the stabilization of the proteins and structures involved in muscle contraction. They provide lateral and longitudinal structural integrity of the myofibril, the muscle cell and the myofibers.

The cytoskeletal proteins can be subdivided into three groups, proteins of the I-band, proteins of the A-band and proteins running through both bands. The later group comprises the so-called gap-filaments. Huxley & Hanson (1954) discovered these filaments during their studies leading to the sliding filament theory of muscle contraction. They discovered that the A-band region in myosin extracted myofibrils appeared empty without the myofibril loosing its structural integrity.

Their conclusion was that the gap between the thin filaments had to be bridged by some elastic structure, which they called the S-filaments. Locker & Leet (1975, 1976a,b) expanded these ideas and found thin and slender structures between the thick and the thin filaments in over-stretched muscles and called these the gap-filaments. Locker (1987) proposed a model in which extremely long and flexible proteins, called titin, were attached to the M-line at one side and to the Z-line at the other side. Locker & Leet (1976b) showed that these filaments are attached to the so called N_2-lines running perpendicular to the myofibrils close to the Z-line. These lines are comprised of a large protein called nebulin.

Titin was first described by Maruyama *et al.* (1976) as a very high molecular mass protein, which they called connectin. This connectin later proved to be a mixture of some large proteins (Maruyama *et al.*, 1981). Wang *et al.* (1979) isolated the largest component, which they called titin. Titin, which is a major component of muscle protein with 8% of the total myofibrillar protein content, is a very long and flexible protein capable of forming long, very thin filaments. Under the ultramicroscope the protein forms very long beaded thread like structures with the beads at distances of 4 nm, which run parallel to the thick filaments. The bead like

structures are probably folded structures that are capable of unfolding under mechanical forces thus providing extreme longitudinal flexibility (Trinnick *et al.*, 1984, Locker, 1987). Titin is probably responsible for the longitudinal integrity of the sarcomere.

Originally, Locker & Wild (1984a) found seven minor lines in the I-band region, which were running perpendicular to the length axis of the sarcomere. They called these lines N-lines. The N_1-line being localized near the Z-band. Four N_2-lines were situated around the middle region of the I-band, and two N_3-lines at the A-band end of the I-region. Wang *et al.* (1979) showed that the N_2-lines contained a large protein called nebulin which comprised 3 - 5% of the total amount of myofibrillar proteins and which was connected to titin (Robson & Huiatt, 1983). A lot of controversy is still going on about the structure and function of the N-lines and nebulin in particular but it is clearly established that nebulin serves as an attachment site for titin (Wang, 1981, Robson & Huiatt, 1983).

Protein	% of total	Location in myofibril
Myosin	43	Throughout thick filaments
Actin	22	Throughout thin filaments
Titin	8	Throughout whole sarcomere
Tropomyosin	5	Coiled around thin filaments in association with actin and troponin
Troponin	5	Coiled around thin filaments in association with actin and tropomyosin
Nebulin	3	Along thin filaments
α-Actinin	2	In Z-line
Myomesin	0.15	In M-line
X-protein	1.5	Thick filaments
H-protein	1.1	Thin filament and Z-line
Desmin	< 0.05	In Z-line (intermediate filaments)
Eu-actinin	0.2	In Z-line
Creatine kinase	0.18	In M-line
F-protein	0.18	Throughout thick filaments
I-protein	0.1	At A-I junction
Filamin	< 0.1	In Z-line
Synemin	< 0.1	In Z-line (intermediate filaments)
Vimentin	< 0.1	In Z-line (intermediate filaments)
Vinculin	< 0.1	Attachment of myofibrils to sarcolemma

Table 2 *Relative amount and location of several myofibrillar proteins*

Another group of proteins generally regarded as important to meat aging are the intermediate filaments, with in muscular tissue as the most important member being desmin. Lazarides & Hubbart (1976) first described this protein. It comprises 0.35% of the total myofibrillar

protein content (Robson & Huiatt, 1983) of skeletal muscle. In smooth muscle the desmin content can go up to 5% of the total muscle protein. Desmin is responsible for the lateral structure and integrity of the myofiber because it holds adjacent myofibrils together at Z-line level (Robson *et al.*, 1981; Robson & Huiatt, 1983). Two other intermediate filament proteins are present in skeletal muscle in much smaller proportion, vimentin and synemin (Granger & Lazarides, 1979, 1980). It is suggested that the three intermediate filament proteins form a fine proteinaceous network around the Z-disk, holding the myofibrils together in lateral register at the Z-lines.

Proteinases in chicken muscle tissue. Most endogenous proteolytic enzymes in muscular tissue are soluble in the intercellular environment and thus are called sarcoplasmic proteinases. They are involved in the protein turnover as is taking place in every cell. Activators and inhibitors that are also present in the muscle cell mainly regulate their activity. Ashgar & Batti (1987) and Goll et al. (1989) published excellent reviews on the presence and regulation of proteolytic enzymes in muscle tissue. Obinata et al. (1981) divide the proteinases present in muscular tissue into three groups, based on the pH traject in which the proteins express optimal proteolytic activity.

The alkaline proteases are generally tightly bound to the myofibrillar structure. They are very important in protein turnover of the living animal but this group can be omitted in meat research because the optimal pH is too high compared to normal meat pH to be of any importance for meat quality.

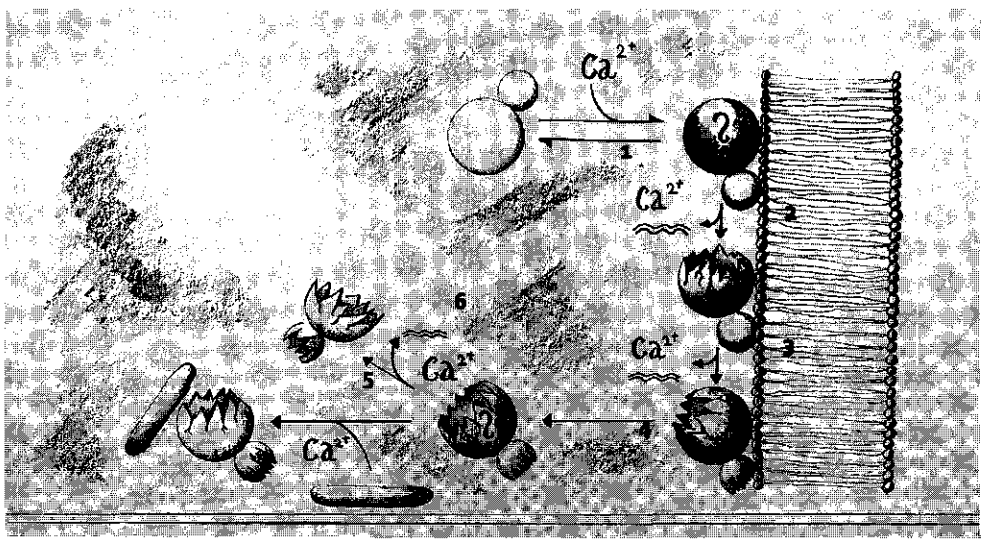

Figure 4 *Schematic representation of the mechanism of activation and autolysis of calpains. (drawn after Mellgrenn (1987) by Heleen Schreurs)*

The neutral proteinases are localised in the cytosol. The most important proteinases of this group are the neutral calcium activated proteinases, generally referred to as calpains (Murachi et al., 1981). These proteolytic enzymes need Ca2+ ions for activation. Huston & Krebs (1968) first mentioned these proteolytic enzymes. Busch et al. (1972) isolated calpain from muscular

tissue and found that it degraded Z-lines of washed myofibrils. The proteinase is capable of degrading troponin, C-protein and tropomyosin but the main substrate for calpain seem to be cytoskeletal proteins (Dayton *et al.*, 1975). Although initially only 1 type of calpain was known that needed 1-2 mM Ca^{2+} for development of 50% of the maximum activity ($½V_{max}$), another type of calpain was later discovered, needing 100-fold less for $½ V_{max}$ (Mellgren 1980; Dayton *et al.*, 1981). At present, the former is known as m-calpain while the latter is known as μ-calpain. Another regulator for calpain activity is calpastatin, an endogenous proteinaceous calpain specific inhibitor (Murachi *et al.*, 1981), capable of binding to calpain. Figure 4 shows a schematic representation of the mechanism of regulation of the calpain/calpastatin system (Mellgren 1987). Under the influence of an increased Ca^{2+} concentration the sarcoplasmic calpain associates with intracellular membrane systems (step 1). The subsequent fast partial autolysis results in activation of the proteinase (step 2). Further autolysis of the subunit responsible for membrane binding results in a diffusion of the activated proteinase to the cytosol (steps 3 and 4). Subsequent exposure of the enzymes to high Ca^{2+} concentrations for prolonged times results in an autolytic inactivation (step 5). Another possibility is the binding of a sarcoplasmic inhibitor (calpastatin) to the autolytically activated calpain thus blocking the proteolytic activity of the enzyme (step 6).

The acid proteases are all of lysozomal origin and are called cathepsins. From muscular tissue several different types have been isolated, designated cathepsin A, B, C, D, H and L (Obinata *et al.*, 1981). The types A and C are not capable of degrading native proteins and longer polypeptide chains. Types B, D, H and L are capable of degrading myofibrillar proteins. (Matsukare *et al.*, 1981) studied the influence of cathepsin L on rabbit myofibrils and found myosin heavy chain, α–actinin actin, troponin T and troponin I to be degraded. Tropomyosin and troponin C were not degraded. Schwartz & Bird (1977) studied the influence of cathepsins B and D on myofibrils. They concluded that these enzymes degraded both myosin and actin. Ouali *et al.* (1987) found cathepsin D to degrade mainly high molecular myofibrillar proteins while cathepsin B seemed to have more affinity towards smaller myofibrillar proteins. Cathepsin H seems to have less influence on myofibrillar proteins. Types B, H and L, all cysteine proteinases, have intracellular inhibitors (Bige *et al.*, 1985; Wood *et al.*, 1985; Barrett 1985; Zabari *et al.*, 1991). Most inhibitors are low molecular weight cytoplasmatic polypeptide substances. About the regulation of these enzymes systems, little is known, but it is generally assumed that compartmentalization plays a major role.

Muscle growth and protein metabolism

As mentioned above, the domestic chicken is a rapidly expanding source of animal protein in the world. Industry is constantly driving towards an increased efficiency in meat production. Efficient feed conversion is one of the major parameters exploited by meat producers. Muscular growth rate is another.

The accumulation of muscle protein is determined by the relative rates of its synthesis and degradation. Inheritance may influence growth of muscle, and inadvertently the ultimate properties of meat, by effecting an increase in average size of fibers, an increased number of fibers, or both. The existence of populations of chickens selected for egg or meat production allows for interesting comparisons.

Comparing the effects of selection for fast growth, as in broilers, over a certain period of time, it may be concluded that modern broilers show a dramatic increase in growth rate but this is accompanied by a higher fat content of the carcass (Chambers *et al.*, 1981). In general,

examination of feed efficiency differences between genetically different stocks of broilers normally reveals only small differences in the efficiency of feed utilization. However, differences in feed intake appear to be the dominant factor in growth-related changes of these genetically diverse birds (McCarthy & Siegel, 1983). Large differences in feed efficiency early post hatch, resulting in a fast initial body growth, facilitate a larger feed intake at later ages, resulting in a faster protein accretion in muscle tissue (Marks, 1991).

From some of the comparisons between layers and broilers it can be concluded that hypertrophy, and to a lesser extent hyperplasia may be the mechanisms by which genes exert effects on muscle growth. The hypothesis of a shift towards white fibers in heavily muscled animals is supported by the fact that broiler type chickens show 28% of their α fibers as αW while layers have 11% of the α fibers as αW (Aberle *et al.*; 1979). It is also noted that broilers show larger fibers than layers. The rates of protein synthesis in skeletal muscle of fully grown chickens are much lower in predominantly fast fibers than in predominantly slow fibers as reflected in the RNA concentration differences in these muscle types although synthesis per unit DNA is comparable in the two muscle types (Laurent *et al.*, 1978).

In general layers show both higher protein synthesis and degradation rates both post hatch (Muramatsu *et al.*, 1987) and in embryos of both types (Muramatsu *et al.*, 1990). The lower rates of protein degradation in broilers compared to layer strains (Hayashi *et al.*, 1985) is related to increased body weight, muscle weight and relative maturity (Saunderson & Leslie, 1988; Hocking & Saunderson, 1992). Maeda *et al.* (1984) find fractional synthesis rates to be twice that of broilers compared to layers at 4 weeks of age. However, at 8 weeks of age similar synthesis rates were found (4-5 %/day). The fractional degradation rate of broilers was much lower than that of layers at 8 weeks of age. It is suggested that faster growth of muscles is accompanied by a lower rate of protein degradation, although at ages less than two weeks, differences in protein synthesis may also contribute to differences in muscle growth (Saunderson & Leslie, 1988). *In vitro* experiments comparing broilers and layers show a higher percentage of myoblast fusion into myotubes in layer muscle cell cultures. Additionally 25 % more nuclei were detected in layer myotubes than in broiler muscle cells. The rate of incorporation of ^{3}H-leucine into total protein during pulse labeling experiments was comparable for layers and broilers, however broiler muscle cells accumulated approximately 40% more total protein per nucleus (Orcutt and Young, 1982).

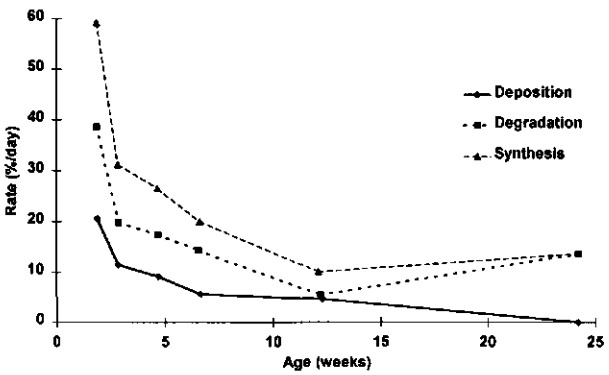

***Figure 5** Protein synthesis, degradation and resulting deposition (Drawn after Scanes, 1987).*

Muscle fiber growth obviously requires considerable deposition of structural and contractile proteins. Protein deposition or accretion is the difference between protein synthesis and degradation. Of the total amount of protein in muscular tissue of young animals, 20 to 25% is degraded and replaced per day. This turnover speed

decreases with age down to 1 to 2% in mature animals (Pearson & Young, 1989). This high rate of protein accretion in young chicks may reflect high rates of synthesis and a low rate of degradation. In fact, during postnatal growth, major decreases in the relative rate of deposition, synthesis and degradation have been described. Figure 5 shows a representation of the rates of synthesis, degradation and deposition (Scanes, 1987) at different stages in the life of domestic fowl. The synthesis and degradation of protein is not only dependent of age, but also of factors like malnutrition, muscular hypertrophy induced by exercise, muscular atrophy induced by inactivity and all kinds of pathological situations. The different reactions of muscular tissue induced by environmental factors are manifestations of one physiological process called protein turnover.

Muscular growth is characterized by different processes, like proliferation of myogenic cell lines, muscle fiber hypertrophy, and an increase in the number of myofibers. During muscular growth, mononucleous myogenic cells aggregate and fuse to a multinuclear muscle cell. Although the number of muscle fibers does not show a spectacular increase after birth, the proliferation of myogenic cells is large. The amount of DNA in muscle cells of the young rat increases more than 8-fold between the day of birth and 112 day old. This means that 88% of the DNA in muscle cells is incorporated after birth (Winick & Noble, 1966). The amount of myogenic cells increases marginally in mature animals.

Growth of muscular tissue is, as are many processes in a living higher organism, regulated hormonally (Johnson, 1989). Especially growth hormone and triiodothyronine (T_3) seem to influence growth of young chickens (Scanes, 1987). Also these two hormones show interactions. Chronic administration of growth hormone for 4 weeks to chicks decreases circulating T_3 levels. This may be the result of the stimulation of T_3 degrading enzymes (Marsh *et al.* 1984).

There is a distinct requirement for growth hormone in post-hatch growth of chickens. Not only does hypophysectomy reduce growth, but also growth hormone replacement partially overcomes the adverse effects of hypophysectomy. Growth can be stimulated in chickens by administration of exogenous chicken growth hormone (Leung *et al.*, 1986) however, mammalian growth hormone does not show any effect on growth (Scanes, 1987). The ability of this exogenous chicken growth hormone to influence growth may be related to the thyroid status of the chick. Native growth hormone was found to overcome the inhibitory effects of T_3 on growth of White Leghorn chicks (Bowen *et al.* 1987).

The most important effect of growth hormone is its influence on the lipid metabolism of the chicken. This hormone shows high lipolytic activity. After administration of GH, plasma concentrations of non-esterified fatty acids (NEFA) are elevated, indicating a liberation of fats from fat deposits (adipocytes). Moreover, hypophysectomy of young chickens is characterized by a decrease of circulating NEFA's (Scanes *et al.*, 1986). Low circulating levels of growth hormone increase the amount of lipids stored in adipose tissue (Nalbandov & Card, 1942; Gibson & Nalbandov, 1966).

From a breeding point of view, research directed to the factors promoting the proliferation of myogenous cells, and thus increasing the amount of DNA incorporated in muscle cells have a significant contribution to knowledge concerning muscular growth (Johnson, 1989). Allen (1987) reviews growth promoting factors and protein hormones, which influence the proliferation of myogenic cells. Insulin-like growth factor-I (IGF-I) and IGF-II, fibroblast growth factor (FGF) and insulin are capable of promoting proliferation of these cells. Growth hor-

mone (GH), prolactin (PRL), luteinizing hormone (LH), thyroid stimulating hormone (TSH), epidermal growth factor (EGF), platelet-derived growth factor (PDGF), and nerve growth factor (NGF) do not show these influences. Transforming growth factor β (TGF- β) inhibits the proliferation and muscle cell differentiation (Florini *et al.*, 1986; Massaque *et al.*, 1986; Olson *et al.*, 1986; Allen & Boxhorn, 1987). However, these hormones and growth factors, that have a promoting or inhibiting potential, are not necessarily involved in the regulation and control of muscular growth.

As mentioned at the beginning of this paragraph, the synthesis and degradation of muscular proteins is not only dependent of the age of the animal but can also depend on other factors like a large amount of pathological conditions, under- and over-nutrition, exercise induced muscular hypertrophy, inactivity induced muscular atrophy, and influences of endogenous or exogenous agents as well as genetic influences.

Muscular dystrophy both in human subjects as in animals is characterized by an ongoing process of degradation of muscular tissue and advancing muscular weakness. Patients with Duchenne muscular dystrophy show an increased synthesis rate of both myofibrillar and connective tissue proteins (Ioanescu et al., 1971, 1973). This suggests that the degradation and thus the activity of the proteinases is strongly increased. Maeda et al. (1987) found comparable results in genetically dystrophic chicken lines. The exact mechanisms of these processes are largely unknown. Under physiological conditions, crude cathepsin preparations did not have any effects on actin, myosin and actomyosin (Bodwell & Pearson, 1964). Iodice et al. (1966) found increased activities of cathepsins A, B and C in *M. Pectoralis* of chickens with a hereditary form of muscular dystrophy. These differences were apparent both in young, growing animals as in mature animals. The activity of cathepsin D was not increased. Kar & Pearson (1976) observed increased activities of calpains in patients with Duchenne and X-chromosome linked forms of Becker's muscular dystrophies, but not in muscular atrophies of neurological origin. Ebashi & Sugita (1979) support these results. Dayton et al. (1979) found increased calpain activities in atrophying tissue in α-tocopherol deficient rabbits. Bodensteiner & Engel (1978) showed increased amounts of Ca^{2+}-ions in muscular tissue of different myopathies thus demonstrating the important role of calpains in the increased degradation of these tissues under these circumstances.

Nutrition also influences muscular growth and protein accretion. Malnutrition causes an increase of protein degradation. The activity of cathepsins in breast and leg muscles of growing chickens is dependent of feed intake (Rosochacki *et al.*, 1986). The functional properties of red fiber myosin undergoes changes in contrast to white muscle myosin (Ashgar *et al.*, 1984). The total sarcoplasmic protein concentration in white muscle decreases during malnutrition while in red fibers no changes are observed (Ashgar *et al.*, 1986). This might be due to the decreased protein synthesis during malnutrition. However, the total protein content of the lysozomal fraction was not decreased. This points towards a relatively increased protein breakdown during nutrient deficiency. In literature, no direct relationships are reported between growth speed and calpain/calpastatin activity in leg muscle of chickens (Ballard *et al.*, 1988). However, interactions between calpain, calpastatin, Ca^{2+} and muscular proteins are known to be modified by the intracellular proximity of membranous (Suzuki *et al.*, 1988; Coolican & Hathaway, 1984; Pontremoli *et al.*, 1985) or cytoskeletal elements (Pontremoli *et al.*, 1988). It is known that phosphatidyl-inositol, a membranous component, is capable of changing the binding of calpain to membranes and the Ca^{2+} sensitivity of the isoenzymes

(Suzuki *et al.*, 1988; Coolican & Hathaway, 1984; Pontremoli *et al.*, 1985). Isovalerylcarnitine, a catabolic product of L-leucine metabolism, was identified as a potent activator of m-calpain (Pontremoli *et al.*, 1990). In chickens, a putative m-calpain activator, modulating the Ca^{2+} sensititvity, has been isolated from breast muscle (Prigge *et al.*, 1996). It is also possible that calpain is not the direct proteolytic enzyme, responsible for myofibrillar degradation during malnutrition, but merely a modifying agent, involved in enzyme activation or hormone receptor processing and translocation, thus influencing muscular growth indirectly (Goll *et al.*, 1992).

One of the most important stimulants of muscular growth is physical exercise (Hershko *et al.*, 1971). Stretch forces, applied to a muscle cause a strong stimulation of muscular growth and protein accretion (Goldspink, 1991). Rosochacki (1985) studied changes in proteolytic activity and amounts of nucleic acids in mature chicken red and white muscles in relation to tension induced muscular hypertrophy. Isometric tension induced weight gain was much larger in red than in white typed muscles. As a consequence of muscular tension, nucleic acid concentrations increased both in white and red muscles but after 1 week the amounts in white muscle decreased to the original concentration while in red muscles, the nucleic acid content still increased and stayed high during the 8 weeks the experiment lasted. The activity of proteolytic enzymes was strongly influenced by the isometric tension. Cathepsin D activity remained low in all muscles subjected to physical strain. Cysteine protease activity increased as a function of tension. It is concluded that in mature chickens, physical (isometric) tension is a strong stimulant for muscular growth but this positive effect is much larger in red than in white typed muscles.

Parkhouse (1988) treats the effects of exercise and fatigue on the degradation of myofibrillar proteins in an excellent review article. The author points to the fact that strong exercise causes serious myofibrillar degradation. Especially proteins associated with the Z-lines like α-actinin and desmin are affected. It is suggested that the decreased power that can be applied by the fatigued muscle is caused by the inability of the myofibril to transfer its contracting force through the damaged cytoskeleton. The protein synthesis rate normally increases with any form of exercise, but also the increase in lysozomal proteolytic activity during increased protein degradation is noticeable.

In recent years, the possibilities of manipulation of muscular growth using endogenous as well as exogenous reagents has gained great interest in the agricultural community. The influence of the somatotropic axis, the thyroid axis, the gonadal axis as well as the β-adrenergic system has been studied extensively (Cogburn, 1991).

Application of growth hormone (somatotropin) stimulates protein synthesis in muscular and other tissues. Pigs treated with porcine growth hormone show a much faster, leaner growth while utilizing less feed per unit of weight gain (feed conversion ratio). Cows produce more milk from less feed when bovine somatotropin is applied. Dayton and Hathaway (1989) suggest that growth hormone is unlikely to affect the protein synthesis directly. In pigs, the indirect influence of porcine growth hormone on growth is probably caused by the effect it has on the synthesis and excretion of Insulin like Growth Factor I (IGF-I). As mentioned before, administration of exogenous chicken growth hormone stimulates growth in chickens (Leung *et al.*, 1986).

In recent years, it has become quite clear that steroids can affect muscle growth under certain circumstances. However, especially in poultry, it remains unclear whether these effects

take place at the anabolic or the catabolic side of the growth equilibrium.

β-Agonists are known for their capability to inhibit the protein degradation but can also have a stimulating influence on protein synthesis. It is suggested that these influences are only directed towards muscular tissue (Buttery *et al.*, 1991). Higgins *et al.* (1988) found severely elevated calpastatin and m-calpain activity and decreased μ-calpain activities in muscular tissue of lambs treated with clenbuterol. Wang & Beermann (1988) and Kretchmar et al. (1990) confirm the decrease in proteolytic potential of the latter isozyme. This points to an inhibition of the proteolytic potential of the calpain/calpastatin system. The increased m-calpain activity could be a result of a reduced autolytic activation of the enzyme-system (De-Martino *et al.*, 1986; Mellgren, 1987). These results were confirmed by Kretchmar *et al.* (1989) who also reported decreased cathepsin B activities in clenbuterol treated lamb. The reduction of cathepsin activity under the influence of cimaterol is observed in muscle cell cultures (Béchet, 1990). Effects on the protein synthesis were not observed while the total protein degradation increased. Reeds *et al.* (1986) also report a lack of influence of clenbuterol on protein synthesis This in contrast to Harper *et al.* (1990) who report an increased protein synthesis and an unchanged protein degradation. In steers, calpastatin is found to be higher while the activity of the calpains, cathepsins and cystatins remained unchanged (Wheeler & Koohmaraie, 1992) under the influence of the β-agonist $L_{644,969}$.

Very little work on the influence of β-agonists on poultry is reported. Studies concerning the administration of cimaterol in feed to broiler chicks (Gwartney *et al.*, 1991) shows some interesting results. Muscle growth is clearly increased under the influence of the β-agonist but the effects seem to be more pronounced in red than in white typed muscular tissue. The proteolytic potential of the cathepsins was unchanged. Unfortunately, the activities of the calpain/calpastatin isozymes were not determined. At slaughter (42 and 49 days of age) the meat of the treated animals was significantly less tender than of untreated controls.

Comparison of the influence of cimaterol and clenbuterol on calpains/calpastatin and mRNA in cattle, sheep, chicken and rat in several growth trials suggest a general mechanism whereby β-agonists reduce μ-calpain activity but increase m-calpain and calpastatin activity in skeletal muscle. Parallel changes in specific mRNA's indicate that changes in gene expression or stabilization of mRNA may explain these activity changes Bardsley *et al.*, 1992).

Consequences for meat production and meat quality

Long has been known that the eating quality of meat improves by including a waiting period before consuming. The meat first needs to age. Already at the beginning of this century first reports were published on observations of changing tenderness of meat during storage (Lehman, 1907). The tenderness of beef improves in time. Since then, many studies have been carried out on the processes that take place during and after slaughter and storage. The changes observed as a result of these processes are for instance tenderization, color shifts, and reduction of water holding properties of the meat.

Most of these sensory changes are detectable both with physical and/or chemical methods. Many of the basic physicochemical processes thus resulting in a particular meat quality have been investigated but many are still unknown (Ouali, 1990, 1992).

Knowledge of these processes can be useful in predicting the quality of the meat from its original parameters, and it becomes also possible to influence the quality of the meat towards "a priory" known end parameters.

Around the moment of slaughter of an animal and during subsequent processing of the carcass, many factors can be identified that influence its muscular metabolism. *Ante mortem* factors like catching, cooping, transport and hanging onto the slaughter-line summarized into the term 'handling' and stunning as well as killing have a large impact on the metabolic status of the animal at the moment of slaughter. *Post mortem* factors like bleeding, scalding, plucking, evisceration, cooling, deboning, and packing, as well as storage time and temperature influence the course of the *post mortem* metabolism.

When an animal is killed, its intramuscular metabolism changes dramatically. Figure 6 shows a schematic representation of the processes taking place *peri-mortem* and involved in the conversion of muscle to meat. The top part of the figure shows the processes in living animals where muscle action is initiated by release of calcium-ions from a fine membranous network in the muscle cell, called sarcoplasmic reticulum (SR), caused by the arrival of a nervous pulse. During and shortly after slaughter, a large amount of nerve pulses arrive at the muscle tissue, thus releasing large amounts of calcium-ions, which in turn activate metabolic enzymes. The cytoplasmic concentration of free Ca^{2+} in living muscle cells at rest is 10^{-8} M or less. This is 10^3 to 10^5 times lower than the free Ca^{2+} concentration of the extracellular environment. The large Ca^{2+} gradient is maintained by powerful Ca^{2+} pumps located in the cell surface membrane, in the endoplasmic reticulum and in the mitochondria (Martonosi, 1984). These pumps remove the Ca^{2+} ions from the cytoplasm of the muscle cell. The cell surface membrane contains two types of Ca^{2+} pumps among which an ATP-ase activated by Mg^{2+} and Ca^{2+} ions. The SR contains an ATP-dependent Ca^{2+} pump that is distinct from the Ca^{2+}/Mg^{2+}-activated cell surface membrane ATP-ase. In skeletal muscle cells it represents 70-80% of the total protein content of the SR. Mitochondria are able to accumulate large amounts of Ca^{2+} ions by an active transport system that may be energized by electron transport or ATP-hydrolysis (Carfoli, 1982a,b; Fiskum & Lehninger, 1980). The cell surface membranes are responsible for compensation of the Ca^{2+} influx from the cell exterior. The SR mainly controls the intracellular Ca^{2+} distribution. Much of the activating Ca^{2+} required for contraction originates from the SR. Accordingly, the SR of skeletal muscle is an extensively developed network of tubules and cisternae that is arranged in a precise geometric relationship to the contractile elements. The Ca^{2+}-transport activity of fast muscle fibers is much greater than that of the slow muscle fibers, e.g. approximately in proportion to their speed of relaxation. Most important feature of both the SR as well as the mitochondria Ca^{2+} transporters, in scope of the *post mortem* metabolism, is the fact that they all need ATP for activation.

After death of an animal, cessation of blood supply occurs. With this, the supply of energy carriers and oxygen to the tissues is interrupted. Thus the energy supply is limited to the energy stores present in the muscle, which consist largely of glycogen.

Through a complicated set of pathways, this glycogen is degraded to carbondioxide and water, generating mainly adenosine triphosphate (ATP) as the major energy carrier. To fully utilize the potential (chemical) energy of the glucose molecule, the metabolites of the glucose degradation pathway have to go through three subsequent chains of biochemical reaction pathways, the glycolysis, the citrate cycle and the respiratory chain respectively. When oxygen and energy supply come to a halt, the latter two pathways are blocked. The only way then for the cell to generate at least some energy from the glycogen stores is the glycolytic pathway. This glycolysis results in the formation of rather large amounts of lactate in the cell. In living animals, this phenomenon also occurs, for instance during heavy exercise. Since blood

similar post-mortem alterations but in contrast to 'red meat' post-mortem physiology, this period is compressed in poultry (Addis, 1986).

Figure 8 shows the typical course of toughening during and after rigor development. Maximum toughening is reached within approximately 6 hours post mortem. After this time, toughness decreases gradually.

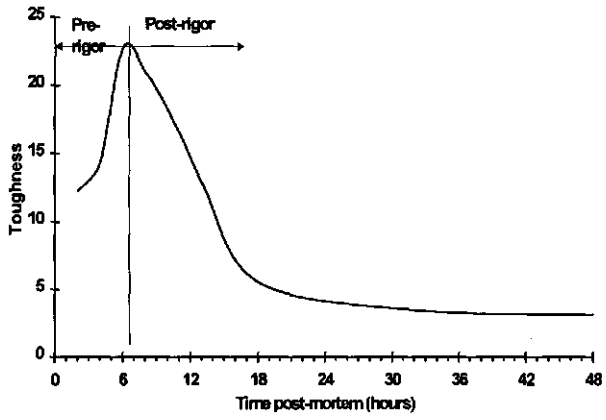

***Figure 8** Typical course of muscle toughness in chicken breast meat.*

The second part of the toughness curve cannot be explained by the events related to the energy metabolism. Another process related to the muscular protein metabolism is responsible for the tenderization during the post-rigor period. Proteolysis of key myofibrillar and associated proteins by endogenous proteolytic enzymes is the cause of meat tenderization Ouali, 1990, 1992).

As mentioned before, the proteinases responsible for the post mortem degradation of structural muscular elements are the neutral Ca2+ activated proteinases, the calpains and calpastatin, and the acid proteinases of lysozomal origin, the cathepsins.

One of the effects of proteolytic breakdown of structural proteins is myofibrillar fragmentation. Due to the degradation of proteins responsible for the lateral and longitudinal integrity, with time post mortem, the myofibrils tend to break more easily under force. Already during the late sixties and early seventies (Sayre, 1970, Olson *et al.*, 1976) studies on this subject were reported.

Microstructural changes observed during *post mortem* aging are:

- Disappearance of Z-lines
- Reduction of density of M-lines
- Loss of lateral integrity between adjacent Z-lines, M-lines and other structural elements
- Los of longitudinal integrity at Z-I junctions and at the N-lines.

(Henderson ea., 1970; Davey & Dickson, 1970;Sayre, 1970; Gann & Merkel, 1978; Walimann *et al.*, 1978; Strehler *et al.*, 1979). These changes are a result of intracellular proteolytic processes that degrade intra- and intermyofibrillar bonds. Using SDS-PAGE, breakdown of desmin and nebulin has been proven in beef (Locker & Wild, 1984b, c). Studies concerning proteolytic breakdown of titin are rather inconclusive. Lusby *et al.*, (1983) and Orcutt & Dutson (1985) report degradation of titin in beef while others (Locker & Wild, 1984b, c) have found no *post mortem* titin breakdown.

Using SDS-PAGE, proteolytic fragments of myofibrillar proteins can be found in *post mortem* muscular tissue. These fragments are rather small, 25 – 34 kD (Ouali, 1990, 1992). In

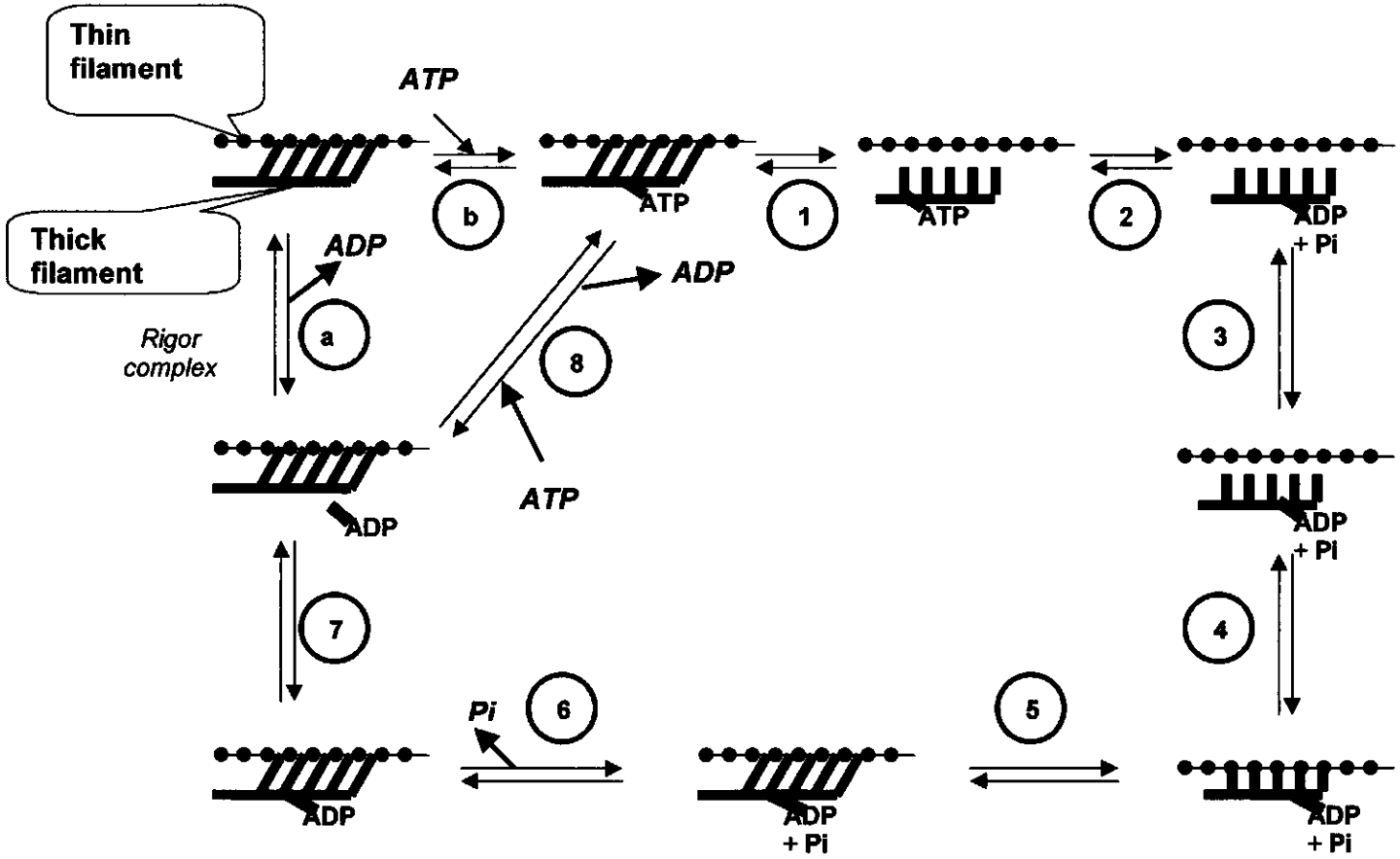

Figure 7 Schematic representation of contraction relaxation cycle and rigor mortis development.

beef, an important component of 30 kD has been described (MacBride & Parrish, 1977; Parrish *et al.*, 1981) which strongly correlates with tenderness (Penny & Dransfield, 1979; Ouali, 1984; Lepetit *et al.*, 1986). It is generally accepted that this component is a degradation product of troponin-T, however, a causal relationship behind this correlation is not clear yet.

As mentioned before, a limited amount of proteinases is involved in meat aging, their relative importance being unrevealed (Obinata *et al.*, 1981). The exact role of most of these proteinases in meat aging is unclear (Orlowski, 1990). The calpains and cathepsins B, D, H and L have been shown to play an important role in beef tenderness (Bird *et al.*, 1980; Lochner *et al.*, 1980; Marsh *et al.*, 1981; Dutson, 1983; Petäjä *et al.*, 1985).

The variation in sensitivity of the different myofibrillar proteins for degradation by different proteinases is very large. Myosin can be degraded by calpains (Dayton *et al.*, 1976b; Azanza *et al.*, 1979; Ishiura *et al.*, 1979) and cathepsins B, D (Schwartz & Bird, 1977; Dufour *et al.*, 1989) and L (Okitani *et al.*, 1980; Matsukara *et al.*, 1981, Dufour *et al.*, 1989). Actin is rather resistant to proteolytic degradation. Only Cathepsins B, D (Schwartz & Bird, 1977) and L (Okitani *et al.*, 1980; Matsukura *et al.*, 1981) are capable of degrading this protein. Troponin T and I and tropomyosin are sensitive to calpains (Penny, 1974; Penny & Ferguson-Pryce, 1979; Penny & Dransfield, 1979; Olson *et al.*, 1977; Azanza *et al.*, 1979; Dayton *et al.*, 1976; Ishiura *et al.*, 1979; Koohmaraie *et al.*, 1988) cathepsin B (Penny & Ferguson Pryce, 1979) and cathepsin L (Okitani *et al.*, 1980; Matsukura *et al.*, 1981). Troponin C seems to be unsensitive for calpains (Ishiura *et al.*, 1979) and cathepsin L (Matsukura *et al.*, 1981) although it is liberated from thin filaments (Sugita *et al.*, 1980). Calpains are capable of degrading the Z-line structure (Olson *et al.*, 1977). Cytoskeletal proteins like desmin titin and nebulin are very sensitive to proteolysis (Young *et al.*, 1980; Penny *et al.*, 1984; Koohmaraie *et al.*, 1984a, 1986, 1988b; Weber, 1984; Wismer-Pedersen & Weber, 1987; Fritz & Greaser, 1991). By far the most studies have been conducted in beef. Effects of proteolytic enzymes in poultry meat are investigated much less. Very early work of Kahn & van den Berg (1969) studied the effects of proteolytic enzymes on myofibrillar proteins of chickens of different age during storage. The authors concluded that the solubility of myofibrillar proteins increased during time *post mortem*. Fukazawa *et al.*, (1969. 1970) studied these solubility characteristics with the myofibrillar fragmentation and ultrastructural changes in pre- and post rigor muscle. They found a disappearance of the Z-lines, and the breakdown of the I-band – Z-line junction. Sayre (1970) studied the fragmentation of the Pectoralis muscle as a function of time *post mortem*. The author reports the myofibrils to break preferably at the I-Z-junction. Fragments from pre-rigor muscle are small, badly defined and strongly contracted. Meat post rigor causes the myofibrils to fragment into longer, better-defined particles. After longer storage, an increase in fragmentation occurs. In contrast to measurements in beef (Olson *et al.*, 1976) this fragmentation shows a bad correlation with tenderness. Hay *et al.*, (1973a) showed that after 48 hours *post mortem* the Z-lines in chicken breast muscle were damaged but not in leg muscles. After 168 hours *post mortem* they have disappeared completely in breast meat. In leg meat the Z-lines were damaged but the remains were still visible.

Hay *et al.*, (1973b) studied the changes of myofibrillar proteins during storage using SDS-PAGE. The authors report the development of a 30 kD component during aging both in leg as in breast meat. Samejima & Wolfe (1976) studied the myofibrillar degradation under different circumstances. They report that incubation of meat homogenates at 40 °C and pH 5.4 gives comparable results to meat aging during 7 days at 0°C. The authors report the occurrence of a

30 kD band both while incubating the homogenate at low pH as well as on carcass aging. Incubation of the meat homogenate at neutral pH did not result in the occurrence of the 30 kD component. It is concluded that the effects are most likely to descend from the influence of acid proteinases and not from neutral proteinases as is often reported for beef. Suzuki *et al.* (1985) investigated the *post-mortem* changes of connectin in chicken muscle. Connectin is characterized as a mixture of protein among which titin and nebulin. The authors found no differences in these components during aging for 3 days at 2°C.

Background and objectives of the study.

It is clear that the knowledge obtained about underlying mechanisms of meat aging in chicken is fragmented, incomplete, and often contradictory. Systematic and detailed studies of the connection of muscular growth and *post-mortem* metabolism in chicken muscle have never been conducted. Many studies report about the influence of processing parameters, especially time-temperature related, on *post-mortem* processes in chicken meat. Rarely however, these studies go beyond the onset of rigor mortis. If later events are studied, the measurements usually are related to the macro-effects of these processes, and are not concerned with molecular and intracellular mechanisms that cause these effects.

This study has the objective to study the *post-mortem* metabolism in more detail with emphasis on the post rigor proteolytic events. The differences in metabolic parameters in different broiler chicken lines, selected for growth speed (GL-line) and favorable feed conversion ratio between 3 and 6 weeks of age (FC-line) are used as natural sources of variation in the different metabolic enzyme activities. They were compared to the White Leghorn, a layer type chicken, characterized by slow growth and low feed conversion efficiency in regard to muscle growth on one hand and commercially available Ross broilers, as a standard for normal practice on the other hand. The special selection lines used in these studies were developed at the former poultry research institute "Het Spelderholt" in Beekbergen, The Netherlands and have been thoroughly characterized. Together with performance characteristics of the lines (Leenstra *et al.*, 1986, Leenstra & Pit, 1987, 1988a, b,c; Cahaner & Leenstra, 1992; Leenstra & Cahaner, 1992) groups at the institute for animal science and health (Leenstra *et al.*, 1991) together with the university of Leuven (Decuypere *et al.*, 1991, 1993a,b; Buyse *et al.*, 1992, 1994; Herremans *et al.*, 1992, 1993; Vanderpooten *et al.*, 1993) investigated the hormonal status and characteristics of fat and protein deposition in these lines in a collaborative study. Scheele *et al.* (1991, 1992) used these lines in part of their studies concerning development of metabolic diseases like ascites in the favorable feed efficiency lines.

Initially, the selection program was developed in the early eighties, to investigate the problems of increased fatness, accompanying the selection for high growth rate. This fatness increase was regarded unfavorable because objection of consumers to fat food was increasing as was the common practice of increasing sales of chicken parts as opposed to whole chicken. The excess fat would be trimmed and lost as offal with the production of valuable deboned chicken parts.

The feed conversion (FC-line) birds were selected for a low feed conversion ratio (feed consumption/body weight gain) from 21 to 42 days of age. The fast growth line was selected for individual body weight at 6 weeks after *ad libitum* feeding. Between 700 and 1000 chickens were tested in each generation of the GL-line and 576 birds of the FC-line per generation. The FC-line birds show low feed conversion ratios combined with a moderate growth speed

while GL-line broilers show extreme fast weight gain combined with moderate feed conversion ratios (Leenstra, 1988).

The thus selected lines differed clearly in their hormonal status. The growth hormone pulsatility in the FC-line is much larger than in the GL-line (Decuypere *et al.*, 1991, 1994a,b), suggesting that decreased abdominal fat and increased protein efficiency may be genetically linked to increased plasma growth hormone amplitude. The high growth hormone levels found in FC-line birds effectively down-regulate the growth hormone receptor in liver tissue compared to GL-line broilers (Vanderpooten *et al.*, 1993). GL-line broilers show higher circulating IGF-I and IGF-II levels (Decuypere 1993).The lipolytic activity of glucagon and chicken growth hormone towards abdominal adipose tissue *in vitro* is much higher in the GL-line birds than in the FC-line. This suggests that the selection criteria applied influenced the number of adipose tissue growth hormone and glucagon receptors. The number of adipose growth hormone receptors were decreased and the adipose glucagon receptors were increased in FC-line chickens compared to the GL-line birds (Buyse *et al.*, 1992). Generally, plasma triglyceride, free fatty acids and glucose levels are higher in GL-line broilers than in FC-line birds (Leenstra *et al.*, 1991).

It is clear that these selection lines are very suitable to comparative studies concerning muscle metabolism. The *in vivo* differences should reflect on the post mortem metabolic parameters. Combined with established differences between broilers and layers, these animals comprise excellent material to study the impact of muscle metabolic parameters on meat quality characteristics.

Chapter 1 of this thesis, is an expansion of the report of an extensive literature research, used as a basis for the project: "Post mortem changes in (poultry) meat during processing and storage". At the start of the project, in September 1990, no comparative studies concerning *post mortem* metabolism and subsequent meat quality of different chicken lines based on *in vivo* characteristics were available. Furthermore, there were no methods available to quantitatively determine the proteolytic potential of the calpain/calpastatin system in chickens. Therefore, the first action to take was to develop proteinase-activity assays for this proteolytic system in chicken muscles. Chapter 2 comprises most of the work, that was done, in part at the National Institute for agricultural research, meat research station (INRA-SRV) in Theix, France, and in part at the Institute for Animal Science and Health, branch Beekbergen, The Netherlands, to develop these assays based on different methods described in literature. Furthermore, adjustments to the specific muscular tissue, chicken breast muscle, had to be made in the proteinase assays concerning the measurement of the activity of different cathepsins and cystatins. These modifications are described in chapter 3. This chapter is a report of a large experiment, carried out to establish the differences in proteolytic status of the four chicken lines under investigation in this project. Chapter 3 was published as a full paper in Poultry Science (Schreurs *et al.*, 1995).

Chapter 4 reports the findings of a large experiment, that was carried out subsequently and in which the differences in *post mortem* metabolic properties of the four lines were investigated in conjunction with some *in vivo* characteristics. Part of the results of these experiments were communicated at the 41st International congress of meat science and technology (Schreurs, 1995a) and the 12th European symposium on poultry meat quality (Schreurs, 1995b).

Chapter 5 reports the findings of an experiment in which the metabolic differences of the four lines under investigation were related to meat quality, especially tenderness/toughness and juiciness/dryness of the breast meat samples.

Subsequently, as described in chapter 6, focus was directed towards course and kinetics of *post mortem* proteolytic degradation of cytoskeletal muscle proteins. A method was developed to measure the breakdown of some key myofibrillar proteins using flat-bed sodium-dodecyl-sulphate polyacrylamide-gel electrophoresis and subsequent western blotting. The changes observed in titin, nebulin, desmin and vinculin were related to changes in meat quality parameters.

Methods developed during the execution of this project were applied to a practical study, in which the impact of different *ante mortem* stunning methods on *post mortem* metabolism was investigated. Since all previous studies were conducted under standardized circumstances, the data accumulated in these experiments could serve as a basis on which the effects of the different stunning methods were superposed. Chapter 7 reports the findings of this large experiment, involving many measurements on hundreds of birds.

Finally, chapter 8 serves as an integration of the studies carried out during the execution of the project.

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Chapter 2:

Measurement of μ- and m-Calpain and Calpastatin Activity in Chicken Skeletal Muscle Tissue.

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ABSTRACT This work describes the development of a method to measure the components of the calpain/calpastatin system accurately and concurrently in chicken muscular tissue. The method describes the adaptation of both hydrophobic interaction chromatography and anion exchange chromatography to the specific tissue under study. Recovery studies are described to make sure there are no matrix effects. The within and between animal variances were determined in breast and leg muscle to investigate the discriminating capability of the method. Finally a field experiment is described in which subpopulations of divergently selected broilers were studied. The method turned out to be capable of picking up differences between individual animals as well as between subpopulations.

INTRODUCTION

The calpain/calpastatin system is one of the most abundant proteolytic systems in the animal kingdom. Almost all animals, vertebrates (Murachi, 1983) as well as non-vertebrates (Müller and Altfelder, 1991; Maeda et al., 1992; Pintér et al., 1992; Mattson and Mykles, 1993) and virtually all tissues contain one or more forms of these proteases (Huston and Krebs, 1968; Dayton et al., 1976ab; McClelland and Hathaway, 1991; Suzuki et al., 1987). Recently, calcium dependent proteinases have even been identified in plants (Reddy et al., 1994).

The enzymes play a major role in all kinds of cellular processes like protein turnover and modification or activation of intracellular enzymes (Phillips and Jakabova, 1977; Murachi et al., 1981; Goll et al., 1992ab, Saido et al., 1994) and signal proteins (Lynch and Baudry, 1984).

Calpains (EC 3.4.22.17) are calcium dependent neutral cysteine proteases. Based on this fact they have been called differently throughout the literature, like calcium activated factors (CAF), calcium activated neutral proteases (CANP) and calcium dependent proteases (CDP). However, based on the proposition of Suzuki (1991) in this report we will refer to calpains.

The calpains need calcium ions and sulphydryl-reagents for activation of their proteolytic nature and their range of optimum performance is around neutral pH values (Murachi, 1983). The first isolation of one of the isoforms of the enzyme was described by Guroff (1964). Mellgren (1980) was first to describe a form of the enzyme, isolated from canine cardiac muscle, needing only micromolar amounts of Ca^{2+} for activation of its proteolytic properties, while all forms described earlier needed milimolar Ca^{2+} concentrations for activation. The latter is several orders of magnitude higher than physiological intracellular calcium concentrations, so it was difficult to understand its role under normal circumstances. Based on the order of elution from a DEAE-cellulose column at pH 7.5 they were called calpain I and calpain II respectively (Murachi, 1983). According to Suzuki (1991) these isoenzymes will be referred to as μ-calpain and m-calpain respectively.

The calpains both consist of a light (28 kD) and a heavy (80 kD) subunit forming a heterodimer protein of approximately 110 kD. The light chains of both isoforms are identical (Kawasaki et al., 1986; Kikuchi et al., 1984) in contrast to the heavy chains (Suzuki,1990).

The protease itself is sensitive to proteolytic (autolytic) degradation. This process causes a shift towards a higher calcium sensitivity of the autolyzed form of either isoenzyme (Dayton, 1982; Goll *et al.*, 1990). The autolyzed form of μ-calpain consists of a 76 and a 18 kD subunit, while autolyzed m-calpain consists of 78 and 18 kD subunits.

Calpains attracted the attention of meat scientists when it became clear that they were probably involved in post mortem degradative processes of myofibrillar protein (Busch *et al.*, 1972; Olson *et al.*, 1977; Dayton *et al.*, 1981ab; Parrish *et al.*, 1981; Szpacenko *et al.*, 1981; Etherington, 1984; Koohmaraie, 1988,1992). Since then a vast amount of data have been published on the relation of calpains with meat quality, which have been reviewed by Ouali (1992) and Koohmaraie (1992). Most of this research however was conducted on beef, and to a lesser extent on lamb and pork. However, avian muscular tissue was very rarely subject to investigation.

One of the early publications on post mortem proteolysis in chicken muscle (Bandack-Yuri and Rose, 1961), describe two forms of proteolytic activity from chicken breast muscle, one of which has its optimum activity at pH 7, while the other had its optimum at pH 4. The authors conclude from their study that the activity present in the tissue cannot account for the

changes taking place post mortem and therefore proteolysis is probably of minor importance for meat aging. What the authors probably did not realize at that time was that many proteolytic systems have a built in control system. Most of the tissues contain several endogenous protease-inhibitors. For the calpain system this inhibitor is called calpastatin (Murachi, 1983). This component sometimes is capable of completely blocking the calpain activity present in a muscle cell homogenate (Suzuki *et al.*, 1987). It is therefore impossible to measure the activity of this class of proteases in crude tissue extracts directly (Gopalakrishna and Barsky, 1985; Bardsley *et al.*, 1992). In order to measure the activity of the different components of this proteolytic system several methods have been developed to separate and quantify the isoenzymes and the inhibitor.

As mentioned before, most published methods were developed for analysis of mammalian tissues. Very rarely, chicken muscles have been subject of studies in this field. Those, that had, often were inconclusive of the composition of the calpain/calpastatin system in this species. The first isolation of chicken calpain described only one isoform consisting of a protein of only one 80 kD subunit (Ishiura *et al.*, 1978) and with intermediate calcium requirements. It was found that the isolated calpain had no specific substrate specificity , however, it was only capable to hydrolyze larger proteins into relatively large cleavage products. From the myofibrillar proteins, troponin subunits and to a lesser extent tropomyosin, were cleaved into smaller digestion products (Ishiura *et al.*, 1979). The same research group isolated two forms of calpain from human muscle (Suzuki *et al.*, 1979) using identical methods as in their work on chicken calpain. Later, it was concluded that in chicken muscle, μ-calpain was produced from m-calpain by limited autolysis (Suzuki *et al.*, 1981). This was proven untrue for porcine skeletal muscle calpains shortly afterwards by Dayton (1982).

Kawashima *et al.* (1984) published a paper describing the comparison of rabbit and chicken muscle calpains. They found m-calpain in chicken muscle but μ- and m-calpain in rabbit muscle. However, the first was composed of two subunits, a heavy chain of 81 kD and a 28 kD light chain. The authors describe a calcium sensitivity between that of rabbit μ- and m-calpain, but in the μ-molar range. A lot of attention has been drawn towards the further characterization of this chicken m-calpain (Crawford *et al.*, 1987; Nagainis *et al.*, 1988; Ballard *et al.*, 1988) and for a long time it was believed that this was the only isoform present in chicken muscular tissue. Based on several considerations, Wolfe *et al.* (1989) concluded that chicken muscle tissue must contain both μ- and m-calpain. In their subsequent studies they even isolated three isoenzymes. They called the third isoform high-m-calpain based on the fact that the latter needed high millimolar concentrations of Ca^{2+}-ions for activation. However, it is not very likely that this variant is physiologically active. From these reports it can be concluded that the isolation of μ-calpain from chicken muscle is much more difficult than from mammalian tissue. This is probably caused by the fact that calpastatin and μ–calpain from chicken muscle, in contrast to their mammalian counterparts, are carrying close to equal charges under the circumstances described in the early papers (Wolfe *et al.*, 1989).

In the past different methods for the routine measurement of calpain and calpastatin activity in muscular tissue have been described, most of the work being dedicated towards mammalian tissue. In general they all rely on some form of chromatography for the separation of calpastatin from the calpain isoenzymes and the separation of the calpain-isoforms from eachother. When not carefully adjusted to the material under study they can lead to a severe underestimation of the actual enzyme activity.

Suzuki and Goll (1974) were among the first to describe a method for the quantitative measurement of calpain activity. They used rabbit *Longissimus dorsi* and leg muscles and fractionated the crude tissue extracts by salting out with ammonium sulfate like in their first

publication related to muscle research (Busch *et al.*, 1972). As an indicator for proteolytic activity, they used the release of soluble materials from washed myofibril preparations. However, since both the calpastatin as well as the μ-calpain iso-enzyme were unknown at that time, no purification step was included to separate the different components of the system. It may therefore be concluded that it is doubtful whether the assay was capable of quantitating the calpain activity accurately.

The first chromatographic methods for isolation of calpains from crude tissue extracts were carried out by means of ion exchange chromatography using DEAE-cellulose (Dayton *et al.*, 1976ab; Ishiura *et al.*, 1978, 1979; Azanza *et al.*, 1979; Hathaway *et al.*, 1982). As mentioned before, only m-calpain was detected using this technique.

Using DEAE-sephadex, in addition to application of iso-electric precipitation of the enzymes from the primary extract prior to chromatography, Mellgren (1980) managed to distinguish two different iso-enzymes of calpain by ion exchange chromatography using a very shallow salt-gradient. The activity of the newly discovered iso-enzyme however, was very small compared to the major peak. The use of precipitation techniques to exclude the calpastatin from a subsequent chromatography step tend to dissociate the subunits of both calpains leading to changes in proteolytic activity (Nagainis *et al.*, 1988; Wolfe *et al.*, 1989). The observation of the monomerous chicken calpain by Ishiura *et al.* (1978) is probably also a result of this fact.

Karlsson *et al.* (1985) developed a one column technique to separate the calpain isoforms and calpastatin using hydrophobic interaction chromatography on phenyl-sepharose using a protocol excluding calcium ions. However, the method was developed for rabbit brain extracts and is very time consuming (minimally 15 hours per run). Koohmaraie (1990) compared the use of hydrophobic interaction chromatography on phenyl sepharose with ion exchange chromatography on DEAE-cellulose for the separation of calpains and calpastatin from bovine *Longissimus dorsi* muscle extracts. The author concluded that the latter technique was superior in terms of recovery of proteolytic activity over the first. However, since no attempt was undertaken to carefully adjust the separation parameters, there is some space for improvement of the hydrophobic interaction chromatography based assay.

The time consuming nature of these low pressure chromatographic techniques does not allow a large amount of samples to be processed in a time appropriate for a quantitative assay. The only way to overcome this limitation is the parallel use of multiple chromatography setups suitable for gradient elution and fraction collection. This will demand a large investment in chromatographic equipment. In general, this is true for all techniques involving one or more low pressure chromatography steps using gradient elution.

One way to avoid these problems, is application of chromatographic methods based on the use of small packed columns which operate under hydrostatic pressure and utilize stepwise elution rather than a gradient elution. Several different techniques have been described in literature. Use of ion exchange chromatography with stepwise elution is not appropriate because the peaks of μ-calpain and calpastatin from chicken muscular tissue are inseparable under the circumstances used for this technique (Wolfe *et al.*, 1989). Gopalakrishna and Barsky (1985) have described a method for hydrophobic interaction chromatography using small columns. Their method is based on the fact that hydrophobic regions are introduced in the calpain molecule when Ca^{2+}-ions are bound. Introduction of calcium ions however leads to rapid autolysis and subsequent loss of proteolytic activity (DeMartino *et al.*, 1986; Crawford *et al.*, 1987; Cong *et al.*, 1989). Autoinactivation is avoided in the described assay by addition of leupeptin, a substance known to inhibit the autolysis of calpains. Here a complication is introduced because calpain activity cannot be measured in the presence of leupeptin. This means

that, after the hydrophobic interaction chromatography step, the leupeptin has to be removed. Excluding the leupeptin from the eluate in which the activity has to be determined, as described in the routine isolation procedure (Gopalakrishna and Barsky, 1985), leads to exposure of the calpains to calcium-ions and subsequent possible loss of activity. In the same paper a calcium independent isolation procedure is described. It constitutes an interesting starting point for the development of a method for calpain activity measurement using stepwise elution from a hydrophobic interaction chromatography column. The authors however disqualify the method because of low recoveries for m-calpain. This may be caused by weak binding of this isoenzyme to phenyl sepharose and partial elution in the run-through fraction where it is not detectable due to the presence of calpastatin.

Taking into account that the methods were tested with bovine brain and rat tissue calpains and calpastatin, and keeping in mind the much different chromatographic behavior of the chicken muscle proteinases (Wolfe *et al.*, 1989), carefull adjustment of separation parameters may lead to a useful separation method of the latter proteolytic system.

Application of modern chromatographic techniques applying high flow rates and high pressures is another way to overcome the limitations associated with classical low pressure liquid chromatography. An attempt was made by Ouali and Talmant (1990) to use a TSK-DEAE-5PW high performance liquid chromatography (HPLC) column for ion exchange chromatography. They used a protocol similar to the classic DEAE based ion exchange chromatography protocols adapted to HPLC dimensions to determine calpains and calpastatin in different muscles of beef, pork and lamb. They succeeded in the clear separation of μ- and m-calpain and calpastatin in all fast twitch muscle extracts of the three examined species. Separation of μ-calpain from calpastatin in slow twitch muscle from lamb and beef and intermediate twitch muscle from beef however was poor, thus demonstrating the necessity of careful adjustment of the separation methods to the material under study. The authors even had to exclude the data on μ-calpain from their report.

Iversen *et al.* (1993) developed a high performance method for the separation of the calpains and calpastatin from porcine *Longissimus dorsi* muscle based on ion exchange fast protein liquid chromatography (FPLC) using Q-sepharose fast flow. Although this method is an intermediate pressure liquid chromatography method, the authors state that only three assays per day could be completed. Aging of poultry meat is not often studied in meat research. hen it is investigated, mostly effects of proteolytic breakdown of structural proteins are studied (Samejima and Wolfe, 1976; Paxhia and Parrish, 1988; Chou et al., 1994), but rarely the enzyme systems involved. Since chicken meat ages extremely fast compared to mammalian species (Etherington et al.,1987), it can be of great value to acquire knowledge about the kinetics of the enzyme systems involved, like the calpains. It is therefore necessary to develop reliable assays to be able to quantitate the proteolytic potential of chicken muscular tissue. Object of this study was to set up these calpain activity assays based on some published methods and to check whether they were useful and accurate enough to estimate the activity of the enzyme system in chicken muscle extracts. Also a field experiment was carried out to see whether the developed method is able to pick up differences between genetically and fenotypically different broiler lines.

MATERIALS AND METHODS

In all cases, water used was of mili-Q® quality. Regular laboratory chemicals and casein (nach Hammarsten) were supplied by Merck unless otherwise stated. Pepstatin A was from Sigma. Animals used for production of muscle extracts were broilers of undefined brand ex-

cept in the last two experiments. Details will be given below. Column chromatography was carried out with Pharmacia programmable fast performance liquid chromatography equipment attached to a personal computer.

Preparation of crude muscle extracts

Animals were stunned mechanically, immediately killed by neck-cut, and bled for 3 minutes. After death of the animals, the breast muscles, and in some cases also leg muscles, were carefully excised and either transported on ice to the laboratory for extraction or immediately frozen in liquid nitrogen and subsequently stored at -85 °C. Time between slaughter of the animals and extraction of fresh tissue never exceeded 15 minutes. Extractions were carried out by homogenizing muscle samples in 3 volumes of ice cold extraction buffer (50 mM Tris.acetate, pH 8.5 containing 10 mM 2-mercaptoethanol (MCE), 5 mM EDTA and 150 nM pepstatin A). Samples from the freezer were homogenized in an initially frozen state to avoid any influence of prior thawing. Homogenization was carried out in a Warring Blendor with three subsequent bursts of 30 s at maximum speed with 1 minute cooling intervals on ice. The homogenates were stirred at 0 oC for 30 minutes after which they were centrifuged at ca. 30.000 x g for 30 minutes at 4 °C. The supernatants were filtered through glass wool and used for further analysis.

Preparation of μ- and m-Calpain and Calpastatin Isolates

Crude muscle extract was loaded onto a Ø5 x 20 cm Q-sepharose fast flow column[4]. After 1 liter of TEM-buffer (20 mM Tris.Cl pH 7.5 containing 0.4mM EDTA, 1 mM sodium azide and 10 mM MCE) proteins were eluted with a linear gradient of 0 to 0.6 M NaCl in 2 liters of TEM buffer. Fractions of 16 ml were collected.

The fractions containing m-calpain activity were pooled and concentrated to 50 ml on an Amicon YM 30 membrane, dialyzed overnight against 1 liter of TEM buffer and stored at 0 °C until use. Aliquots of the m-calpain concentrates were checked for presence of calpastatin and μ-calpain. All preparations used were free of μ-calpain or calpastatin activity. The concentrates were stable at 0 °C for at least 2 months (results not shown).

The fractions collected prior to the m-calpain peak were pooled and solid NaCl was added up to a 0.5 M concentration. This pool was loaded onto a Ø2.6 x 10 cm phenyl sepharose high performance column. After 250 ml TEM buffer containing 0.5 M NaCl proteins were eluted with TEM buffer containing 50% ethyleneglycol. The eluting peak containing the μ-calpain was concentrated to 50 ml on an Amicon YM 30 membrane, dialyzed overnight against 1 liter of TEM buffer and stored at 0 °C until use. The isolates were checked for presence of calpastatin and m-calpain. The preparations used were always free of contaminating calpastatin and m-calpain. The isolates were stable at 0 °C for at least 2 months (results not shown).

Calpastatin was isolated from the crude muscle extracts by heating under vigorous stirring in a boiling water bath until a temperature of 80 °C was reached. Subsequently it was cooled, again under vigorous stirring, in an ice bath to 0 °C. After centrifugation for 15 minutes at 30.000 x g and 4 °C, the clear calpastatin extract was concentrated to 50 ml on an Amicon YM 30 membrane, dialyzed overnight against 1 liter of TEM buffer and stored at 0 °C until use. These crude isolates were checked for the presence of residual proteolytic activity. None of the preparations showed any proteolytic activity. These crude isolates were stable for at least 1 month (results not shown).

Calpain activity assay

The routine calpain activity assays were carried out according to Beynon and Bond (1989), with the following modifications. Casein was used as a substrate in a concentration of 5 mg/ml assay buffer (50 mM Tris.CL, pH 7.5 containing 10 mM β-mercaptoethanol and 0.02% sodium azide), and 0.4 ml of sample was mixed with 0.4 ml of substrate solution. Subse-

quently, 0.2 ml of a 50 mM $CaCl_2$ solution was added and the reactionmixture was incubated at 30 °C for 30 min. Identical reactionmixtures with the $CaCl_2$ substituted by EDTA served as blanks.

One unit of calpain activity is defined as the amount of enzyme, capable of causing an increase in optical density at 280 nm of 0.001 in 30 minutes. Values exceeding 1000 Units of calpain activity were dilluted and reassayed.

Calpastatin activity assay

The routine calpastatin activity assay was carried out according to Geessink (1993). In brief, samples were heated in a boiling water bath for 3 min. After centrifugation, dillution series of the clear supernatants were mixed with defined amounts of m-calpain and assayed for calpain activity as described above. The calpastatin activity was calculated from the slope of the linear part of the inhibition curve.

One unit of calpastatin activity is defined here as the amount of inhibitor capable of completely blocking one unit of m-calpain activity under the described circumstances.

Preliminary experiments

Ion exchange chromatography. Approximately 250 ml of crude muscle extract, prepared as described above, was dialyzed overnight against 5 l of TEM buffer. After centrifugation at 30.000 x g for 1 h at 0 °C, 30 ml samples were applied to either a Ø1.6 x 40 cm DEAE-sephacel column (flowrate: 0.5 ml/min.) or a mono-Q HR 10/10 column[6] (flowrate: 4 ml/min). After 5 column volumes of TEM buffer proteins were eluted with a continuous gradient of NaCl from 0 to 0.6 M in either 2, 5, or 10 column volumes. Fractions of 10 ml were collected in the DEAE-sephacel experiments while fractions of 1 ml were collected in the Mono-Q runs. The calpain/calpastatin activity was assayed in all fractions. Fractions containing calpastatin activity were pooled, made pH 6.0 with acetic acid, and to 0.5 M with solid NaCl, and applied to an Econopac column packed with 5 ml Phenyl sepharose CL-4B equilibrated .with TEM buffer containing 0.5 M NaCl. After 15 ml of the same buffer, proteins were eluted with 10 ml of TEM buffer containing 50% ethylene glycol. The effluents of the column were collected batch-wise and assayed for proteolytic and inhibitory activity.

Hydrophobic interaction chromatography. Approximately 250 ml of crude muscle extract was made to 0.5 M with solid NaCl and subsequently dialyzed overnight against 5 l of TEM buffer at pH 7.0 containing 0.5 M NaCl. The samples were centrifuged at 30.000 x g for 1 h at 0 °C and subsequently 30 ml aliquots were applied to a Ø1.6 x 15 cm phenyl sepharose CL-4B column at a flow rate of 1 ml/min. After 5 column volumes of TEM buffer containing 0.5 M NaCl, the column was eluted with a continuous linearly decreasing gradient from 0.5 to 0 M NaCl in 2, 5, or 10 column volumes. Subsequently, the column was eluted with 2 column volumes of TEM buffer containing 50% ethyleneglycol. Fractions of 5 ml were collected and calpain and calpastatin activities were measured. Subsequently, fractions containing proteolytic activity were pooled, diluted with water to a conductivity equal to the conductivity of the TEM buffer, and applied to a mono-Q HR5/5 column equilibrated with TEM buffer. Proteins were eluted with a 10 column volume gradient of 0 to 0.6 M NaCl in TEM buffer. Fractions of 1 ml were collected and calpain activity was determined.

Hydrophobic interaction chromatography

All experiments were carried out tenfold, in disposable econopack columns, with a gel-bed volume of 5 ml. Every column was fitted with a top frit to avoid running dry. All chromatographic matrices were supplied by Pharmacia biotech. Columns of phenyl sepharose CL-4B, phenyl sepharose fast flow (high substitution level), phenyl sepharose fast flow (low substitution level), butyl sepharose fast flow and octyl sepharose fast flow were used unless otherwise stated.

Experiment 1: pH dependence of calpain binding to the column matrix. Approximately 100 units of partly purified μ-calpain or 350 units of partly purified m-calpain or calpastatin were made to a pH of 5.5, 6.0, 6.5, 7.0, 7.5 and 8.0 with concentrated HCL or NaOH respectively and solid NaCl was added to make 0.5 M in a total volume of 5.0 ml. These samples were applied to all columns. The columns were washed with 10 ml of TEM buffer with the same pH as the applied samples. The total effluent of these two steps were pooled for activity measurements (Fraction 1). Subsequently the columns were eluted with 15 ml TEM buffer (Fraction 2) and then with 15 ml TEM buffer containing 50 % ethyleneglycol (Fraction 3). The proteolytic or inhibitory activity of the eluates was measured with the activity assays as described above.

Experiment 2: NaCl concentration dependence of calpain binding to the matrix. Amounts equal to experiment 1 of purified μ- or m-calpain or calpastatin were made up to 0, 0.1, 0.2, 0.3, 0.4, and 0.5 M NaCl respectively and to pH 6.0 and subsequently subjected to hydrophobic interaction chromatography as described under experiment 1 with the exception that the columns were preequilibrated with the same salt concentration in TEM buffer as the samples were applied in. The proteolytic or inhibitory activity of the eluates was measured with the activity assays as described above.

Experiment 3: dependence of elution from ethyleneglycol concentration. Partly purified μ- (approximately 100 units) or m-calpain (approximately 350 units) were made up to 0.4 M NaCl and pH 6.0 in TEM buffer and subsequently subjected to hydrophobic interaction chromatography as described under experiment 1 with the exception that the columns were preequilibrated with 0.4 M NaCl in TEM buffer of pH 6.0. The columns were subsequently eluted with TEM buffer containing 0, 10, 20, 30, 40, 50 60 and 70% ethyleneglycol. The proteolytic activity of the eluates was measured with the calpain activity assays as described above.

Experiment 4: spiking recovery of calpain activity in isolates. To preparations of partly purified μ- (approximately 100 units) and m-calpain (approximately 350 units), defined amounts (in terms of proteolytic activity) of the same proteins were added and these were subjected to hydrophobic interaction chromatography as described in experiments 1 to 3. Samples of pH 6.0 were made up to 0.4 M NaCl before they were applied. Phenyl sepharose CL-4B, phenyl sepharose fast flow (high substitution) and phenyl sepharose fast flow (low substitution) columns were pre-equilibrated with TEM buffer at pH 6.0 containing 0.4 M NaCl. These columns were washed with TEM buffer at pH 6.0 containing 0.4 M NaCl. Subsequently the columns were eluted with TEM buffer containing 50% ethyleneglycol. The proteolytic activity of the eluates was measured with the calpain activity assay as described above.

Experiment 5: spiking recovery of calpain activity in muscle extracts (matrix effects). To crude tissue extracts, equal amounts as in experiment 4, of the calpains were added and these were subjected to hydrophobic interaction chromatography as described under experiment 4. Samples of pH 6.0 were made up to 0.4 M NaCl before they were applied. The columns were washed with the same pH and salt concentration in TEM buffer. Subsequently the columns were eluted with TEM buffer containing 50% ethyleneglycol. The latter eluates were subjected to ion exchange chromatography on a mono-Q HR 5/5 column after which the fractions collected were used for the measurement of calpain activity by means of the activity assay as described above.

Ion exchange chromatography

Fifty milliliters of crude muscle extract produced as described above was made to 0.4 M NaCl and pH 6.0 and applied to a 10 ml phenyl sepharose CL-4B column, equilibrated with

TEM buffer at pH 6.0 and containing 0.4 M NaCl. The column was washed with 30 ml of the same buffer and subsequently eluted with 20 ml TEM buffer containing 50% ethyleneglycol.

The calpain containing eluate from the hydrophobic interaction column was diluted with a 10 ml solution of 0.4 mM EDTA and 10 mM mercaptoethanol and subsequently applied to a mono-Q HR 10/10 column equilibrated with TEM buffer. After washing the column with 5 column volumes of TEM buffer the columns were eluted with different linear gradients from 0 - 0.6 NaCl. Fractions of 2 ml were collected and assayed for proteolytic and inhibitory activity as described above.

Measurement of calpains and calpastatin in breast and leg muscles

Ten Ross broilers at an age of 40 to 48 days were used in this experiment. The animals were electrically stunned and subsequently killed by neck cut and bled for 3 minutes. Immediately after killing, breast muscles were coarsely minced, divided into 5 equal portions and the samples were wrapped in aluminum foil and frozen in liquid nitrogen. Leg muscles were immediately deboned and excessive fat and connective tissue were removed. These muscles were also coarsely minced, divided into five equal portions, wrapped in aluminum foil and frozen in liquid nitrogen. All samples were stored in -80 °C until further analysis.

Ten grams of the frozen muscle samples were homogenized twice for 1 min., with 1 min. intermediate cooling periods on ice, in 40 ml of ice cold extraction buffer in an Ultra turrax homogenizer with three subsequent bursts of 30 s at maximum speed with 1 minute cooling intervals on ice. The samples from the freezer were homogenized in an initially frozen state to avoid any influence of prior thawing. The homogenates were incubated at 0 °C for 1 hour after which they were centrifuged at 30.000 x g for 30 minutes at 4 °C. The supernatants were filtered through glass wool to remove contaminating lipids and used for further analysis.

In these extracts calpastatin activity was determined according to the calpastatin activity assay as described above.

To 25 ml of the extracts, solid NaCl was added to make up to .4 M and the pH was adjusted to pH 6.0 with HCl. The samples were applied to 10 ml phenyl-sepharose CL-4B columns previously equilibrated in TEM buffer containing 0.4 M NaCl at pH 6.0. The columns were washed with 30 ml of the same buffer after which they were eluted with 20 ml TEM buffer containing 50 % ethylene glycol. The eluates were diluted with 10 ml dilluent (0.4 mM EDTA and 10 mM β-mercaptoethanol) and 25 ml of this mixture were applied to a Mono-Q® HR10/10 column that was equilibrated with TEM buffer. The column was developed with a linear 40 ml gradient of 0.0 to 0.6 M NaCl in TEM buffer. Effluents between 40 and 48 ml and between 52 and 62 ml were collected and calpain activity was determined according to the routine calpain assay described above. This proteolytic activities were determined in serially diluted samples according to the routine calpain activity assay as described above. Total calcium-dependent proteolytic activities in the original samples were calculated from the slope of the linear part of the dillution curves.

Variances within and between chickens were calculated using the SPSS General Linear Models module utilizing the simple factorial ANOVA procedure.

Field experiment

Chickens used in this experiment were a generous gift from Dr. Bernard LeClerq from the National Institute for Agricultural Research (INRA-Tours), Poultry research station in Nouzilly, France. From two lines, divergently selected for leanness, five male and five female animals were killed by decapitation and after bleeding for approximately 3 minutes, the breast muscles (*Pectoralis major*) were removed, wrapped in aluminum foil and immediately frozen in liquid nitrogen. The samples were transported to the laboratory in liquid nitrogen and sub-

sequently stored at -80 °C until analysis. Analysis for calpain and calpastatin activity was carried out as described above.

RESULTS AND DISCUSSION

As stated before, many methods for measuring the calpain/calpastatin activity have been described in literature. Most of them are developed for the measurement in beef (Etherington et al., 1987; Koohmaraie et al., 1991; Ouali and Talmant, 1990; Koohmaraie, 1990), lamb (Whipple and Koohmaraie, 1992; Etherington et al., 1987; Ouali and Talmant, 1990; Wheeler and Koohmaraie, 1991), pig (Etherington et al., 1987), rat (Fagan et al., 1983; Gopalakrishna and Barsky, 1985) and rabbit (Suzuki and Goll, 1974; Vidalenc et al., 1983; Karlsson et al., 1985; Ou and Forsberg, 1991). Only a few methods were described to measure the enzymes in chicken muscle. Etherington et al. (1987) compared beef, calf, lamb, pig *M. Pectoralis profundus* with chicken *M. Pectoralis superficialis* calpain activities. They used hydrophobic interaction chromatography to separate the calpains from the calpastatin after which they separated the calpains on a Mono-Q anion exchange column. As the authors stated in their paper, in all species similar chromatograms were obtained, although the actual elution conditions for μ- and m-calpain were different for each species. This suggests that the isoenzymes differ in separation characteristics among species. Therefore, it is probably not possible to transfer a method developed for tissue of one species to that of another species without loss of sensitivity and/or specificity. Moreover, because of the different distribution of the calpains and calpastatin among the different muscle types, a method, developed with a specific muscle in mind might not be appropriate for utilization with another muscle in the same species (Ouali and Talmant, 1990).

Koohmaraie (1990) compared ion exchange chromatography on DEAE-sephacel and hydrophobic interaction chromatography on phenyl-sepharose (type unknown) for the separation of calpains and calpastatin from beef *M. Longissimus dorsi.* On the basis of his results the author disqualified hydrophobic interaction chromatography for separation of the proteolytic and inhibitory activities. For comparative reasons, the basic chromatographic properties of chicken muscle calpains and calpastatin in both ion exchange chromatography and hydrophobic interaction chromatography were determined in preliminary experiments.

Preliminary experiments

During ion exchange chromatography columns were eluted with 2, 5 and 10 column volumes of elution buffer. The 2 and 5 column volume elutions did not give sufficient resolution between the μ-calpain and the calpastatin peak to be able to detect any calcium dependent proteolytic activity in the area were μ-calpain was expected to elute. Figure 1a shows a typical elution profile of an extract from breast muscle on a DEAE-sephacel column which was eluted with 10 column volumes of elution buffer. The μ- and m-calpain peaks are very well separated but calpastatin and μ-calpain activity are very closely together. Calculating the total calpain activity from the pooled fractions and correcting for dilution, we find a μ-calpain activity of approximately 220 units per gram of wet tissue and an m-calpain activity of around 1500 units per gram wet tissue. The combined data for calpastatin show around 850 units of inhibitor per gram of wet tissue. Figure 1b shows a typical elution profile of the same extract on a mono-Q column which was also eluted with 10 column volumes of elution buffer. The μ-calpain activity peak is very small compared to the calpastatin activity and compared to the elution profile of the DEAE- separation. Calculating the totally recovered μ-calpain activity from the elution profile, we find around 100 units per gram wet tissue and for m-calpain

Figure 1a: DEAE-sephacel

Crude tissue extract

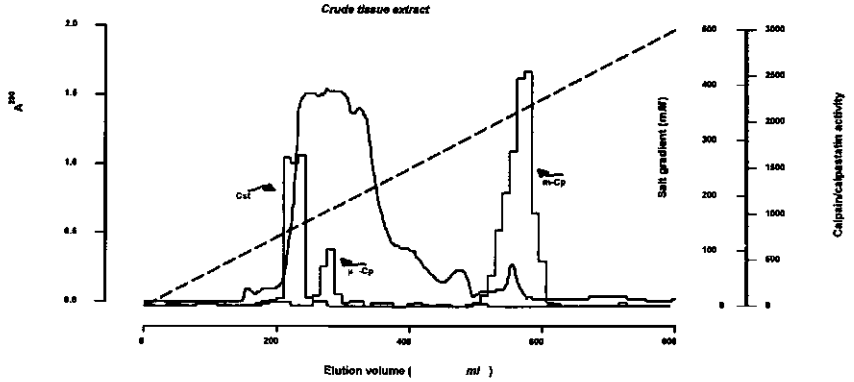


Figure 1b: Mono-Q

Crude tissue extracts

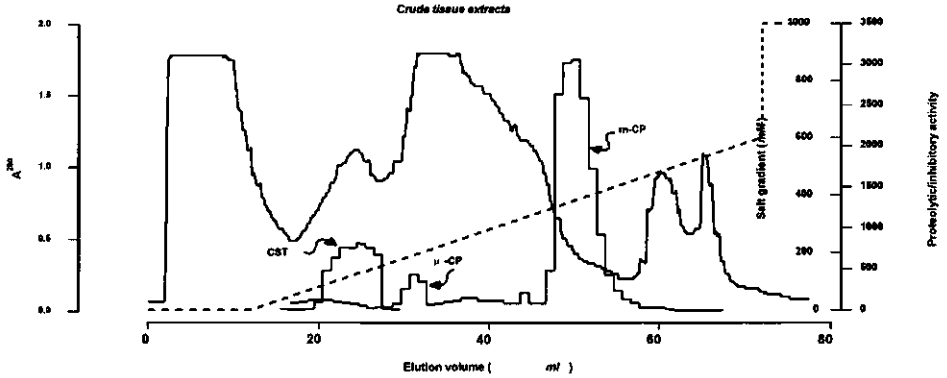
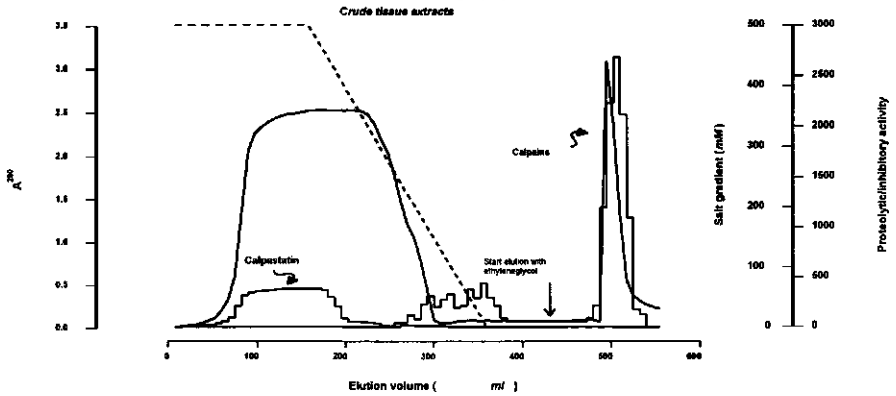


Fig 1c: Hydrophobic interaction chromatography

Crude tissue extracts



approximately 2200 units per gram wet tissue were detected. Calpastatin inhibitory activity was approximately 700 units per gram wet muscle weight.

Literature data on calpains in chicken muscle show a wide variety. Etherington et al (1990) measured μ-calpain activities in breast muscle of ca. 20 and m-calpain values of approximately 80 units per gram wet muscle weight. Johari *et al.* (1993,1994) found total calpain activities in breast muscle of approximately 1000 units per gram wet muscle weight and inhibitor concentrations of around 300 units per gram wet tissue weight. Ballard *et al.* (1988) found ca 1000 units per gram wet tissue weight of total calpain activity and ca. 360 units of calpastatin inhibitory activity per gram wet muscle weight in leg muscle tissue. Etherington *et al.* (1990) used a colorimetric detection method measuring TCA soluble casein fragments and hence another definition of the unit activity and so these data are not directly comparable but the fact that μ-calpain constitutes about 20% of the total calpain activity and m-calpain accounted for the residual 80% is a comparative figure. Although it is difficult to compare the absolute activities found in different studies using different methodologies for quantitation, the relationships between the different components within a single study can be compared. In the present study, μ-calpain constituted 13 and 5 percent of the total calpain activity found for the DEAE- and Mono-Q separation respectively. These low figures can be related to a possible large overlap of μ-calpain and calpastatin peaks in these preliminary experiments. Johari *et al.* (1993,1994) found the calpastatin activity to constitute around 30% of the total detected calpain activity in breast meat while Ballard *et al.* (1988) found around 36% of inhibitory activity compared to the total calpain activity. In the present preliminary experiments we found calpastatin to be comparable with approximately 50% and 30% of the total calpain activity for DEAE- and Mono-Q ion exchange chromatography respectively.

To check whether and to what extent the calpastatin peak was overlapping the μ-calpain activity, the peaks containing the inhibitory activity were pooled and subjected to hydrophobic interaction chromatography. The last eluates from the hydrophobic interaction columns did not contain any residual calpastatin activity so it could be concluded that the μ-calpain and the calpastatin were completely separated by hydrophobic interaction chromatography. In the DEAE-sephacel separation the calpastatin peak turned out to contain approximately 35% (28 - 47%, n=3) of the μ-calpain originally present, while in the mono-Q separation the calpastatin peak showed approximately 62% (49 - 73%, n=3) residual μ-calpain activity. Separation of μ-calpain and calpastatin could be improved by elution with an even more shallow salt gradient over the region where these components are eluted, but run-times would become unacceptably long for routine measurements. It was therefore concluded that simple ion exchange chromatography shows insufficient resolution to be of practical use for a routine assay of the activities of the calpain/calpastatin system in chicken muscular tissue. This is supported by the fact that Johari *et al.* (1993, 1994) as well as Ballard *et al.* (1988) report data on either total calpain or m-calpain and calpastatin but either make no distinction between the calpain isoenzymes or ommit data on μ-calpain respectively. Etherington *et al.* (1987, 1990) report data on μ- and m-calpain but use a two step separation using hydrophobic interaction chromatography and ion exchange chromatography optimized for measurements in different muscles of different species. The fact that Koohmaraie (1990) and Iversen *et al.* (1993) were able to separate bovine and porcine *Longissimus dorsi* respectively on a DEAE- and a Q-sepharose column underlines the fact that chromatographic properties of the chicken calpain/calpastatin system are rather different from its mammalian counterpart (Wolfe *et al.*, 1989). Moreover, Ouali and Talmand (1990) showed that even with high resolution techniques like HPLC, it was not possible to separate calpastatin and μ–calpain in muscle tissues of a contractile type different from *Lon-*

gissimus dorsi where the ratio of the inhibitor and μ-calpain was higher, and the inhibitor more or less overlapped the μ-calpain peaks.

The elution properties found for the DEAE matrix correspond rather well with the elution properties found by Birkhold and Sams (1994) although the proteinase and inhibitor peaks found in the present study eluted later than they did in the study of Wolfe et al. (1989). However, the DEAE-cellulose used in the latter study was of another type and the columns were eluted with KCl instead of NaCl. Moreover, the general picture of elution with overlapping peaks of calpastatin and μ-calpain is equal. The elution properties of the μ-calpain found for the mono-Q matrix do not correspond very well with the findings of Iversen *et al.* (1993) for the porcine enzyme system. This study reports elution of calpastatin at approximately 120 mM and μ-calpain between 180 and 300 mM NaCl. Finally, m-calpain elutes at 380 to 600 mM of salt. In their study, the authors show calpastatin and μ-calpain to separate very well. This again underlines the chromatographic differences between chicken and mammalian μ-calpain.

To investigate the chromatographic behavior of the isoenzymes in hydrophobic interaction chromatography crude muscle extracts were applied to a phenylsepharose CL-4B (1.6 x 15 cm) column. Figure 1c shows a typical elution profile. The calpastatin was found in the unretarded fraction. Development with a decreasing salt gradient did not result in elution of any calpain activity. In some cases however, some very undefined desorbtion of calpain activity could be observed. Application of a step gradient of 50 % ethyleneglycol in TEM buffer resulted in elution of a sharp peak containing calpain activity. These observations are very much in compliance with the observations made by Wolfe *et al.* (1989). They often found elution characteristics of chicken μ–calpain on phenyl sepharose to be much in contrast to mammalian μ-calpain (Gopalakrishna and Barsky, 1985; Karlsson *et al.*, 1985). This suggest that at pH 7.0, chicken μ-calpain both is considerably more hydrophobic and has a lower charge than its mammalian counterpart.

The results were confirmed by rechromatography of an aliquot of the pooled activity peaks, both proteolytic and inhibitory, on a Mono-Q (0.5 x 5 cm). The m-calpain peak eluted around 400 m*M* sodium chloride, the μ-calpain around 140 m*M* sodium chloride and the calpastatin in a rather broad range between 80 and 200 m*M* sodium chloride.

In general, it can be concluded from these results, that anionexchange chromatography methods are not suitable for the complete separation of μ-calpain and calpastatin in chicken muscular tissue.

In the case of the chicken calpain/calpastatin system, probably the most sensible solution to overcome these problems would be the combination of the pre-separation of calpastatin from both calpain isoenzymes by hydrophobic interaction chromatography and the subsequent separation of both isoenzymes by ion exchange chromatography. As a matter of fact, this method has been described by Etherington et al. (1987), but Koohmaraie (1990), on the basis of some experiments with bovine M. Longissimus dorsi, criticized the method because it gave much lower recoveries than the low pressure ion exchange technique. For reasons outlined above and with the different properties of chicken μ-calpain compared to the mammalian proteinase in mind, the following steps in the study were carried out to carefully adapt the combined hydrophobic interaction chromatography/ion exchange chromatography method to this tissue.

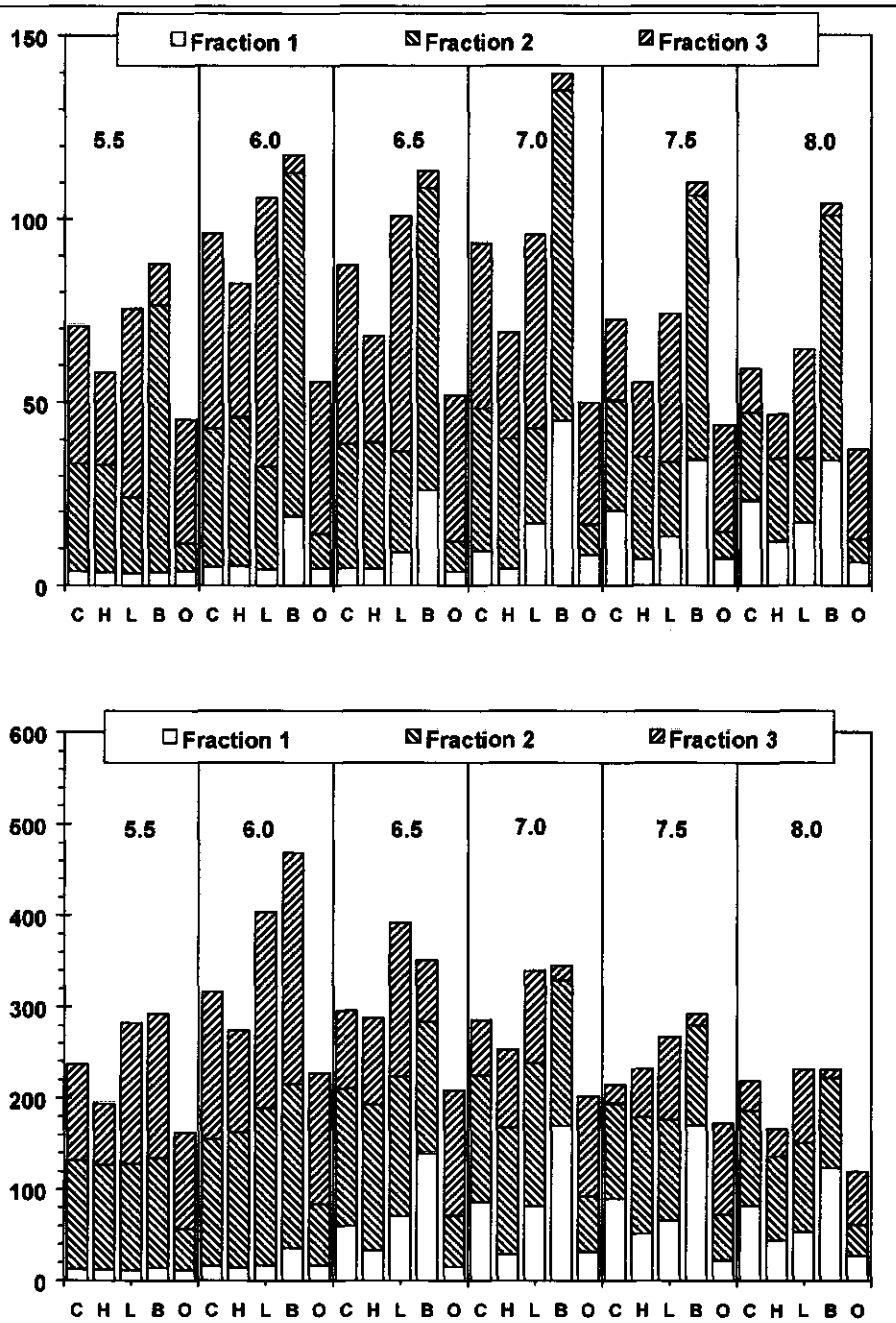


Figure 2 Hydrophobic interaction chromatography of calpains: effect of pH.
Top: μ-calpain; Bottom: m-calpain; C: Phenyl-sepharose CL4B; H: Phenyl-sepharose Fast Flow (High substitution) ; L: Phenyl-sepharose Fast Flow (Low substitution); B: Butyl-sepharose Fast Flow; O: Octyl-sepharose Fast Flow.

Hydrophobic interaction chromatography

Experiment 1: pH dependence of calpain binding to the column matrix. This experiment was carried out to investigate the influence of the pH on the separation of the calpains from calpastatin on different hydrophobic interaction chromatography matrices. Figure 2(top) shows the results of the separation of μ-calpain under the circumstances as described under materials and methods. Fraction 1 contains the unretarded proteins while fractions 2 and 3 contain the proteins that are eluted by either the saltless or the ethyleneglycol-containing eluents. The proteins found in fraction 2 are less tightly bound to the columns than the proteins found in fraction 3. It is clear that at low pH's, the majority of the calpain activity, over 90%, is retarded. At pH 6.0, the butyl matrix does not bind approximately 16% of the applied μ-calpain. At pH 7.0 most matrices with the exception of phenyl-sepharose fast flow, high substitution, release considerable amounts, over 10% of the applied proteolytic activity, in the unretarded fractions. These percentages are even larger at higher pH's.

Figure 2(bottom) shows the results for the separation of m-calpain on the same matrices under the same conditions. At pH 5.5 and 6.0, the unretarded fractions also show very little break through of unretarded proteolytic activity. From pH 6.5 upwards, the amounts of unretarded m-calpain quickly become much larger.

Both figures show a decreasing recovery of calpain activity in the eluates 2 and 3. For m-calpain, the amount of eluted activity from the matrices is over 95% for binding at pH 5.5 and well over 90% for binding at pH 6.0. For μ–calpain the fractions 2 and 3 contain over 95% of the applied activity except the octyl-sepharose matrix. This matrix binds this isoenzyme so tight at pH 5.5 and 6.0 that even with 50% ethyleneglycol, not all activity is recovered from the column. This probably results from a denaturative effect of the strong hydrophobic binding of the iso-enzyme at this pH. For m-calpain, the elution of around 93% of the applied calpain activity at pH 5.5 and 6.0 is probably due to the same effect. The elution of the same iso-enzyme around 92% from the butyl matrix is probably due to a larger breakthrough of unretarded proteinase.

In all cases calpastatin eluted from all matrices unretarded. None of the tested hydrophobic matrices exhibited any binding of the inhibitor under the described circumstances.

From this experiment it can be concluded that binding of both proteinases to the hydrophobic matrices is most suitable at pH 6.0 and that the butyl-sepharose matrix is probably unsuited to be used in the separation of calpains from calpastatin due to worse binding of the proteinases at lower pH values.

Experiment 2: NaCl concentration dependence of calpain binding to the matrix. Figures 3(top) and 3(bottom) show the effects of NaCl concentration on the binding to different hydrophobic matrices for μ- and m-calpain respectively. Already at 0.1 M NaCl the μ-calpain starts to bind to the different matrices. This is in accordance with the findings of Gopalakrishna and Barsky (1989). The only matrix that does not bind this isoenzyme very well at low salt concentrations is the butyl sepharose matrix, which was already identified in experiment 1 as inferior to the other matrices for our purpose. At 0.3 M salt concentration the unretarded fractions still do contain very little (<10% of applied) proteinase except with the butyl matrix (ca. 35%). At 0.4 M NaCl all matrices, with the exception of butyl sepharose, showed binding of over 95% of the applied μ-calpain.

For m-calpain, the unretarded fractions contained rather large amounts of proteinase up to NaCl concentrations of 0.3 M. This indicates a much looser binding of the m-calpain to the hydrophobic matrix than the μ–calpain. At 0.4 M salt however, the binding of the proteinase

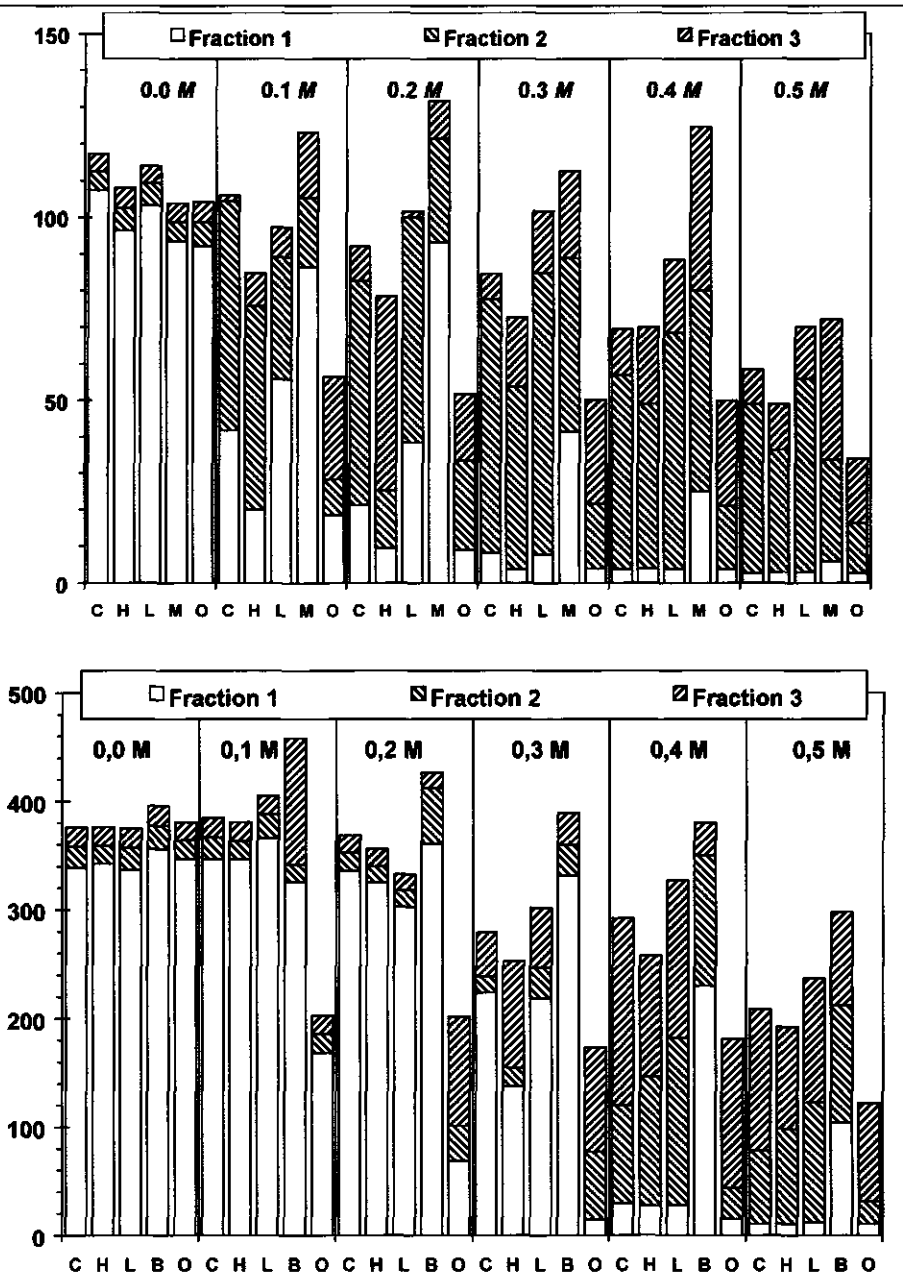


Figure 3 Hydrophobic interaction chromatography of calpains: effect of salt concentration..
Top: μ-calpain; Bottom: m-calpain; C: Phenyl-sepharose CL4B; H: Phenyl-sepharose Fast Flow (High substitution) ; L: Phenyl-sepharose Fast Flow (Low substitution); B: Butyl-sepharose Fast Flow; O: Octyl-sepharose Fast Flow.

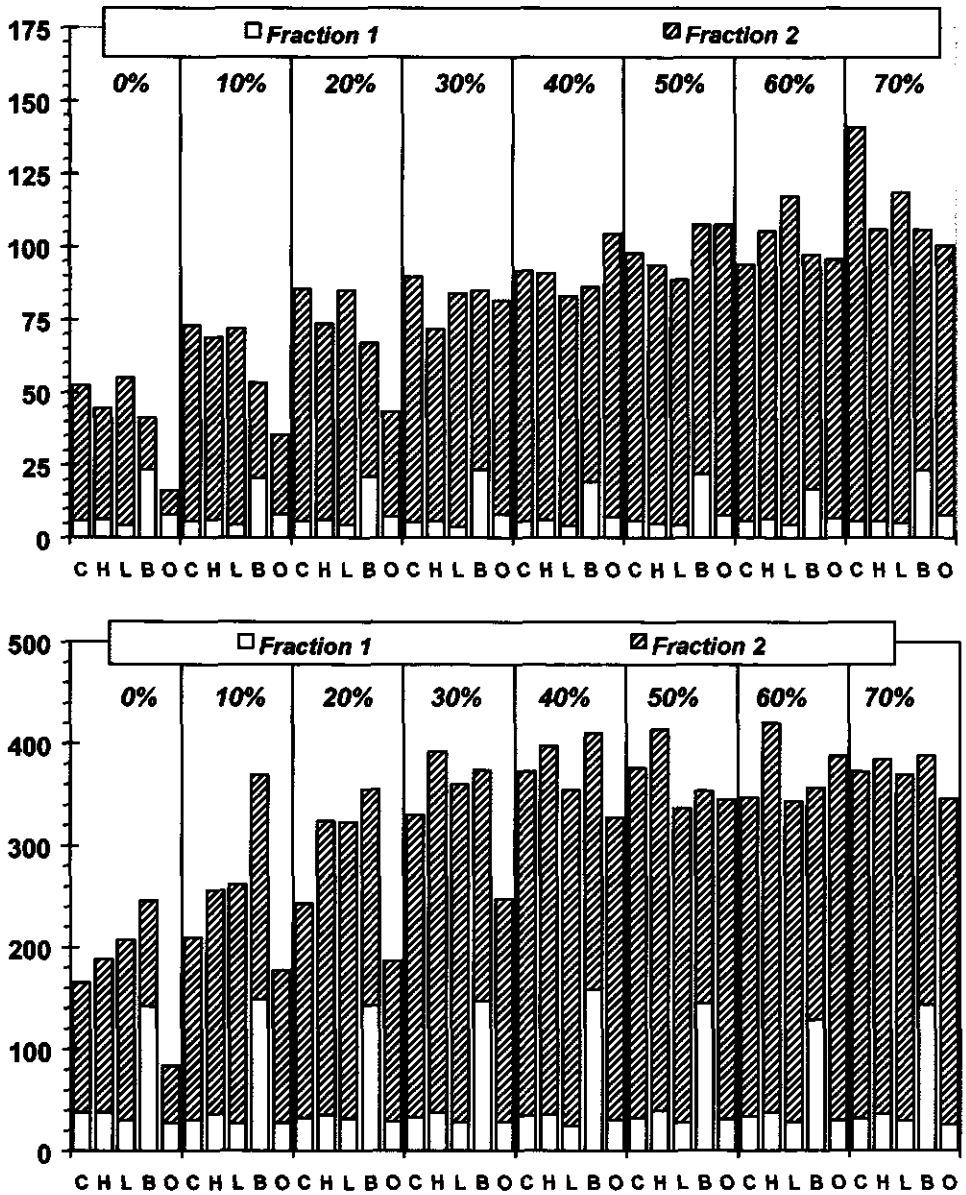


Figure 4 Hydrophobic interaction chromatography of calpains: effect of elution with ethylene glycol. Top: μ-calpain; Bottom: m-calpain; C: Phenyl-sepharose CL4B; H: Phenyl-sepharose Fast Flow (High substitution) ; L: Phenyl-sepharose Fast Flow (Low substitution); B: Butyl-sepharose Fast Flow; O: Octyl-sepharose Fast Flow.

to the hydrophobic matrix is over 90 % except for the butyl matrix. The total activity recovery from the octyl matrix is low, compared to the phenyl matrices, which is probably due to denaturative effects of to strong hydrophobic interactions, as described under experiment 1.

In all cases calpastatin eluted from all matrices unretarded. None of the tested hydrophobic matrices showed any binding of the inhibitor under the described circumstances.

From this experiment it can be concluded that for the binding of both proteinases, a minimum concentration of 0.4 M NaCl is needed. At 0.3 M NaCl, considerable amounts of m-calpain are lost in the unretarded fractions. Use of 0.5 M NaCl would even increase recoveries slightly, but since the next step in the procedure is planned to be the separation of both proteinase isoenzymes by means of ion-exchange chromatography, it is necessary to reduce the salt concentration in the eluate as much as possible. This can be achieved by keeping the salt concentration in the application buffer as low as possible to reduce column wash-out.

Experiment 3: dependence of elution from ethyleneglycol concentration. Figures 4(top) and 4(bottom) show the effect of different ethyleneglycol concentrations on the elution of calpains from the different hydrophobic matrices. Since calpastatin did not bind to the matrices in experiments 1 and 2 it was no longer tested in the following experiments.

As was found in the preliminary experiments, elution from the matrices with a low-salt buffer containing no organic modifier resulted in very limited recoveries, especially for μ-calpain. M-calpain is recovered from the columns with acceptable recoveries when eluted in minimally 30% ethyleneglycol while μ-calpain elutes from the columns with good recoveries when buffers with minimally 50% ethyleneglycol are used. As mentioned before, the butyl matrix is not suited for hydrophobic interaction chromatography of calpains under the circumstances described in this study.

From this experiment it can be concluded that when 50% ethyleneglycol is used as an organic modifier for the elution of calpains from hydrophobic matrices, recoveries of well over 90% of the originally applied activities are achieved for both μ- and m-calpain.

Experiment 4: spiking recovery of calpain activity in isolates. Experiment 1 through 3 showed the phenyl sepharose matrices to perform superior to the two other hydrophobic matrices. However, since all three have different densities in hydrophobic groups attached to the solid phase, and different coupling chemistries applied (Pharmacia, personal communication), they show different binding characteristics for the calpains. Figures 5(top) and 5(bottom) show the results of recovery studies conducted with partly purified calpains on these matrices. It is clear that under the binding and elution circumstances as determined in experiments 1 through 3, the recoveries are quite well. Even when small quantities of calpain (100 U) are added to the bulk, recoveries are 73% on the CL-4B matrix, 78% on the low substitution fast flow matrix and 101% on the high substitution fast flow matrix for μ-calpain and 96% on the CL-4B matrix, 110% on the low sub matrix and 102% on the high sub matrix for m-calpain are found. When larger amounts of proteinase are applied, comparable results are found.

These results show clearly that provided that the separation circumstances are carefully adjusted, acceptable recoveries can be achieved with hydrophobic interaction chromatography as a means of separating calpains. However, since these results are achieved on partly purified calpains, they have to be confirmed for real tissue extracts. For this purpose, experiment 5 was carried out.

Experiment 5: spiking recovery of calpain activity in muscle extracts (matrix effects). Figure 6(top) shows the results of the addition of defined amounts of μ-calpain to crude tissue extracts. For phenyl-sepharose CL-4B recoveries average to 97% (range 70 -

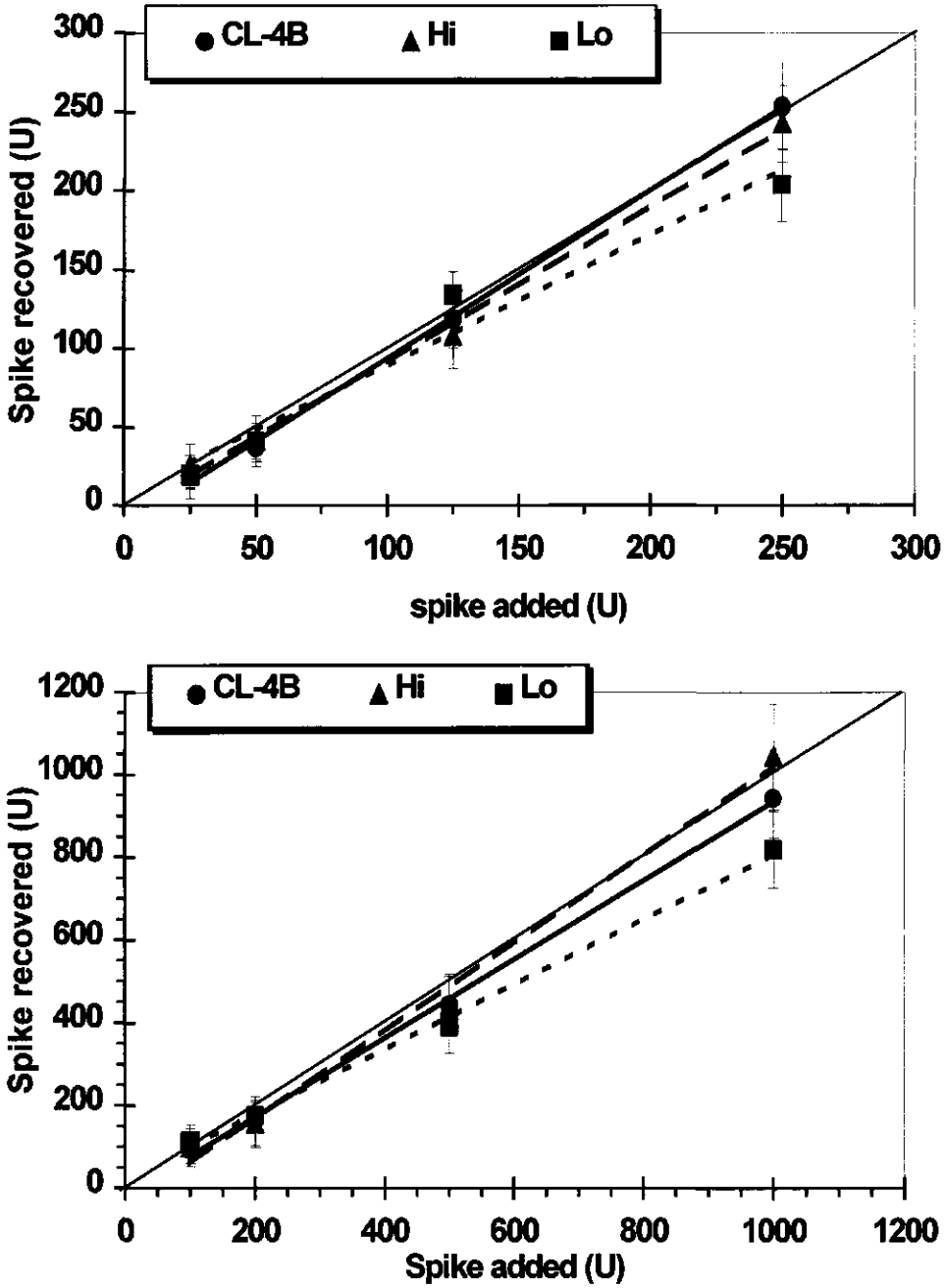


Figure 5 Spiking recovery of partly purified calpains.
Top: μ-calpain; Bottom: m-calpain. CL-4B: Phenyl-Sepharose CL-4B; Hi: Phenyl-Sepharose Fast Flow (High substitution); Lo: Phenyl-Sepharose Fast Flow (Low substitution).

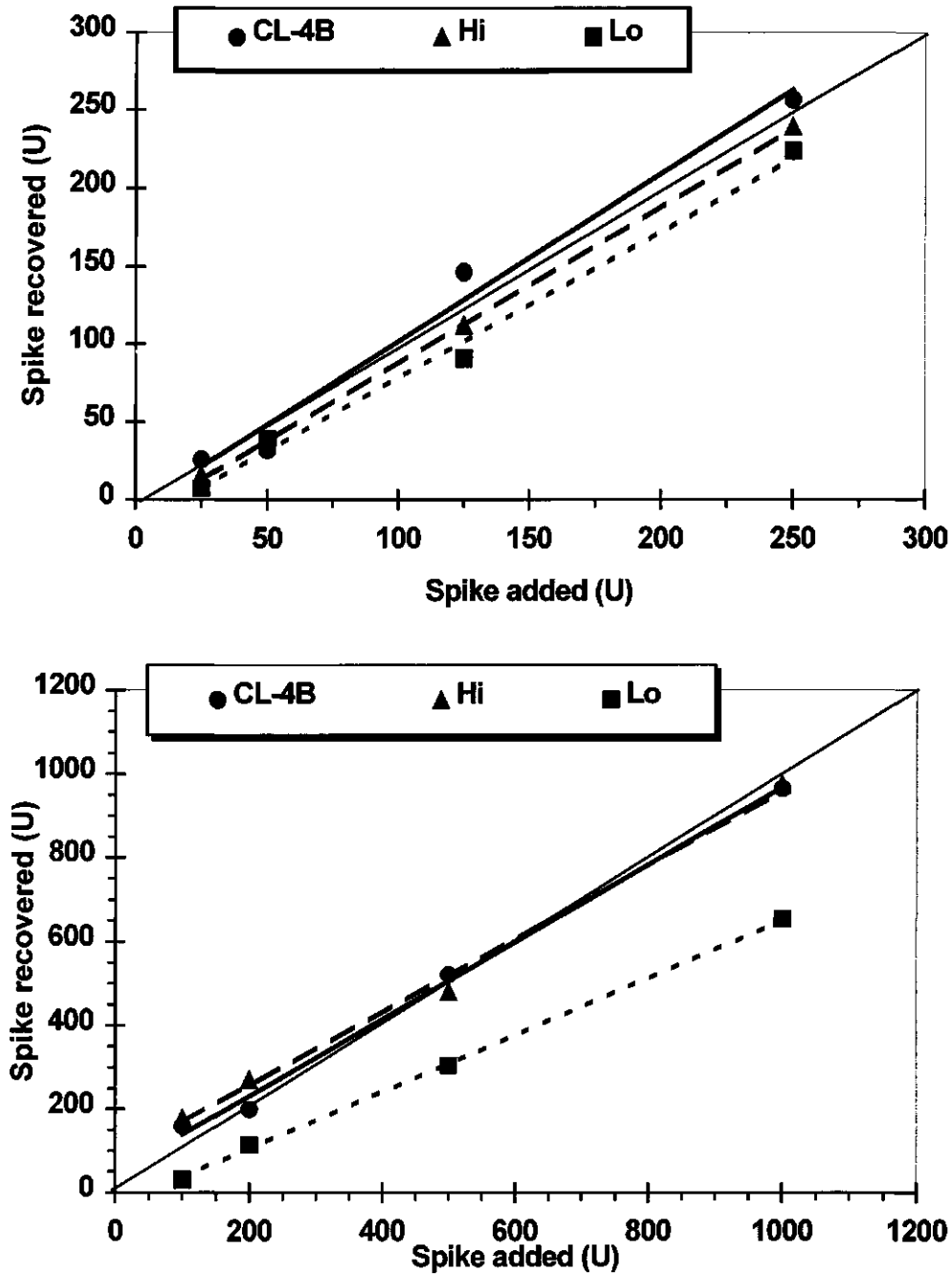


Figure 6 Spiking recovery of calpains in tissue extract
Top: μ-calpain; Bottom: m-calpain. CL-4B: Phenyl-Sepharose CL-4B; Hi: Phenyl-Sepharose Fast Flow (High substitution); Lo: Phenyl-Sepharose Fast Flow (Low substitution).

115%). For phenyl sepharose fast flow high substitution, recoveries average to 82% (range 69 - 95%). For phenyl sepharose fast flow low substitution, recoveries average to 72% (range 41 - 90%). In all cases, worse recoveries were found with low spikes. Recoveries of spikes in the order of the amount, present in the crude tissue extracts were all between 85 and 115%, which means that amounts of μ-calpain, expected to be found in tissue samples, will give acceptable recoveries. Only when samples are processed with substantially lower μ-calpain concentrations the found activities will be underestimated.

Figure 6(bottom) shows the results from the same experiment for added m-calpain. Both phenyl sepharose CL-4B and fast flow high substitution show recoveries around 100% when amounts of m-calpain are added comparable to crude tissue samples. When small amounts of m-calpain are added, the method tends to overestimate the m-calpain activity. The phenyl sepharose fast flow low substitution underestimates the m-calpain activity over the whole range of additions. This may be explained by the fact that the m-calpain is probably bound to the matrix loosely, so that competing proteins are able to expel the m-calpain from the matrix in the run through fraction. Since this fraction also contains the calpastatin, these losses go undetected.

From experiments 1 through 5 it may be concluded that it is possible to develop a good method using hydrophobic interaction chromatography for the separation of calpains from calpastatin in chicken muscle tissue, with very acceptable recoveries of both iso-enzymes. From the recovery experiments, it may be concluded that the phenyl sepharose CL-4B matrix is best suited to perform with the most accuracy in the method developed in this study.

Ion exchange chromatography

After the calpastatin has been removed from the crude tissue extract, the calpains have to be separated from eachother in order to be able to determine their respective activities. This can be most easily achieved by separation through ion exchange chromatography as illustrated in the preliminary experiments. For a routine assay it is desirable to be able to separate the isoenzymes as quickly as possible without the need for complex gradient elution and fraction collection. When it is possible to collect the μ–calpain in one well known volume and m-calpain in another fraction of which the volume is also well known, the necessity to first determine proteolytic activity in separate fractions and subsequently in pooled fractions is obsolete. It will be very easy and convenient to calculate the activity present originally in the tissue extract.

The low pressure chromatography with the DEAE-sephacel matrix was not pursued any further because the utilization of a low pressure chromatography method would result in long separation times and is therefore unfit to be used in a routine assay method in which large amounts of samples have to be processed in short periods of time. Much quicker runs can be carried out using medium pressure matrices like the mono-Q column. An experiment was carried out to determine the optimal separation conditions for calpains prepurified by hydrophobic interaction chromatography in which high resolution was the primary criterion but also separation time and fraction volume was taken into consideration.

Figure 7 shows a typical elution profile of a calpain separation on mono-Q which was carried out after preseparation of calpains from calpastatin by hydrophobic interaction chromatography as described above. The salt gradient used was developed over approximately 5 column volumes. It is clear that the μ- and m-calpain elute off the column well separated. Compressing the gradient elution to below 5 column volumes resulted in an unacceptable loss of resolution and the calpain isoenzyme activity peaks starting to overlap. It is also clear that when the elution volumes between 40 and 48 ml respectively between 52 and 62 ml are

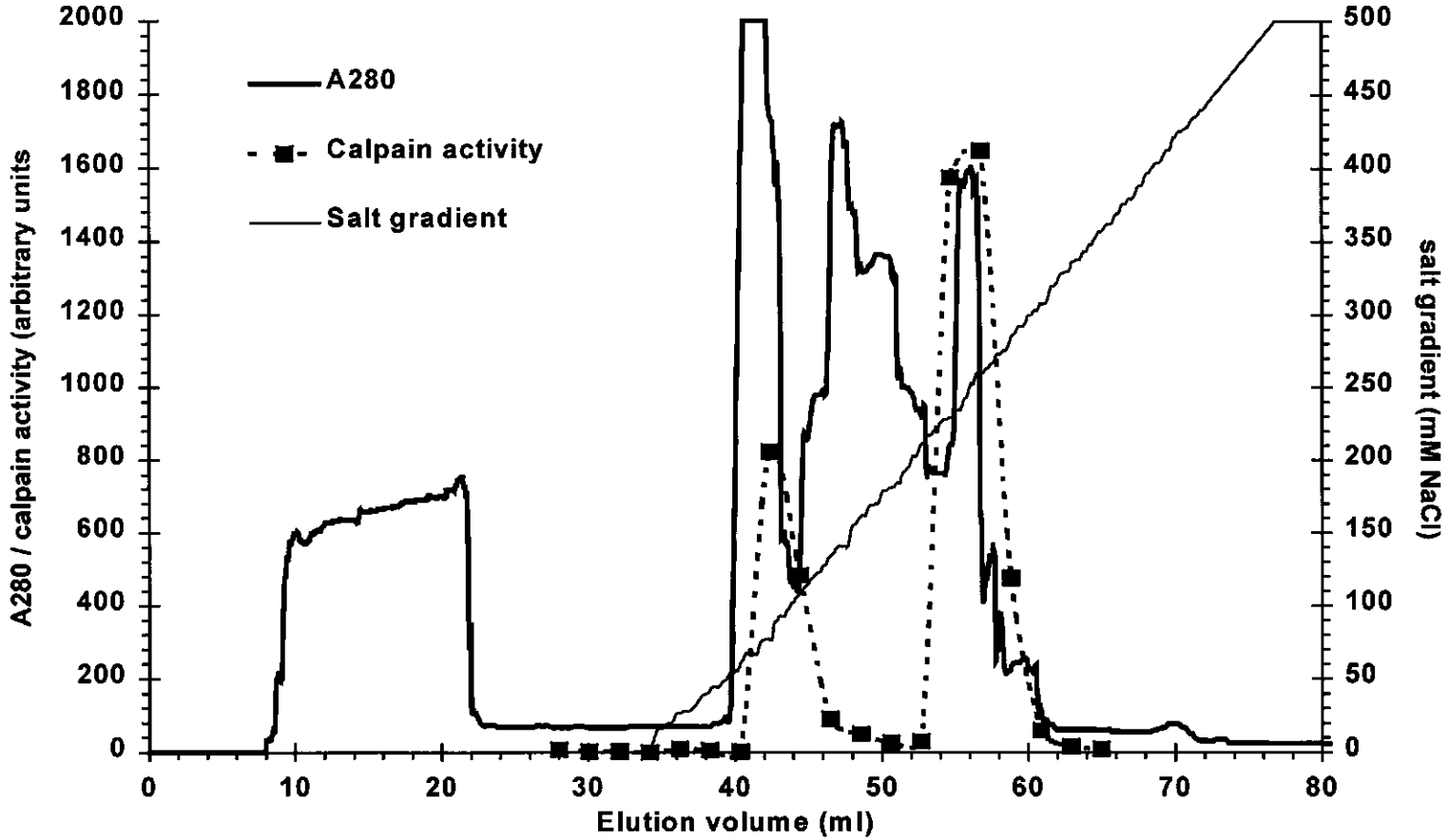


Figure 7: Ionexchange chromatography of calpains after separation from calpastatin by Hydrophobic Interaction Chromatography.

collected, separation of the peaks is sufficient to be able to quantify their activities separately, so there is no need for extensive fraction collection and subsequent screening of some 15 to

		Within groups					
		calpastatin		μ-calpain		m-calpain	
	animal #	Average	St.dev.	Average	St.dev.	Average	St.dev.
Leg	1	781	50.0	92	13.2	2155	176.1
	2	943	49.0	104	16.8	1289	120.6
	3	1005	68.6	143	4.0	3197	375.1
	4	943	20.1	126	7.1	1470	171.7
	5	975	52.2	108	9.6	2046	405.4
	6	945	84.4	71	12.8	1243	115.1
	7	1059	42.7	184	24.1	2529	163.7
	8	895	63.1	84	9.5	2631	199.6
	9	724	28.4	93	9.6	2692	271.5
	10	677	23.2	171	23.3	2527	156.8
breast	1	228	10.4	288	51.9	653	58.4
	2	271	15.4	647	71.9	1313	166.8
	3	277	8.7	278	10.1	1011	124.4
	4	271	20.2	457	30.2	1375	145.7
	5	232	16.7	395	51.9	352	42.7
	6	246	9.1	429	23.7	701	63.6
	7	181	16.8	291	10.1	1033	155.0
	8	158	7.2	268	31.6	1088	123.7
	9	263	25.1	215	23.8	1505	11.1
	10	224	9.1	269	28.5	1191	296.6
		Between groups					
leg		895	281.2	117	84.4	2178	1479.0
breast		235	89.2	354	290.5	1022	803.7

20 fractions and pooling of samples. From the collected volumes, dilution series for activity measurements can be made very easily and subsequent calculation of proteolytic activity is simple.

Measurement of calpains and calpastatin in breast and leg muscles

Table 1 shows the results of the determinations of the isoenzymes and the inhibitory activity in repeated portions of breast and leg meat in 10 different chickens.

This experiment was carried out mainly to determine the within animal and the between animal variance. With leg meat calpastatin averaged over all to 895 U/g wet tissue with a standard deviation (SD) within animals of 52. The SD between animals was 281. For μ-calpain leg meat showed an overall average of 117 U/g wet tissue with a within animal SD of 22 and a between animal SD of 84. M-calpain showed an overall average of 2178 U/g wet tissue with within and between animal SD's of 237 and 1479 respectively. For breast meat calpastatin the average was 235 U/g wet tissue with within and between SD's of respectively 15 and 89. For breast meat μ-calpain the average was 354 U/g with respective SD's of 38 and 291 and finally breast meat m-calpain averaged to 1022 U/g with a within animal SD of 142

and between animal SD of 804. Within animal coefficients of variation (CV_w) were for leg meat 5.8%, 18.7% and 10.9% and for breast meat 6.4%, 10.7% and 13.9% for calpastatin μ-calpain and m-calpain respectively. The between animal CV_b's were for leg meat 31.4%, 72.1% and 67.9% and for breast meat 38.0%, 82.1% and 78.6% for calpastatin μ-calpain and m-calpain respectively. This means that the thus developed assays are capable of picking up differences between individual animals.

Field experiment

Table 2 shows the results of the field experiment. The results were analyzed by full factorial ANOVA. Lean birds showed to be significantly lighter than fat birds ($p<0.05$) while males were significantly heavier than females ($p<0.001$) There were no significant interactions observed between genotype and sex. For calpastatin significant interactions between genotypes and sexes were observed ($p<0.05$). Both lean and fat females were significantly lower in calpastatin than the males. For μ-calpain also significant interactions between genotype and sex were observed ($p<0.01$). The females of both genotypes were higher in μ-calpain than the males ($p<0.001$) while the fat animals were higher than the lean birds ($p<0.001$). For m-calpain much lower significancies were observed. Interactions between genotype and sex were not observed. There were no significant differences between sexes. The lean animals showed significantly higher values than the fat animals ($p<0.05$).

Genotype	Sex		Live weight	calpastatin	u-calpain	m-calpain
Lean	**Male**	**Average**	1541	225	138	1287
		Std.dev.	136	50	29	324
	Female	**Average**	1286	217	317	953
		Std.dev.	67	67	71	308
Fat	**Male**	**Average**	1718	423	221	888
		Std.dev.	99	154	56	362
	Female	**Average**	1362	204	808	682
		Std.dev.	102	56	241	249

The results of these experiments demonstrate that it is possible to develop a good and robust method to determine the activities of the components of the calpain/calpastatin system *in vitro*. It is also shown that it is possible to pick up differences between individual animals as well as (sub)populations. Whether the differences observed between lines and sexes are really meaningful and originate from a physiological basis has to be determined by further (larger) experiments.

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Chapter 3:

Endogenous Proteolytic Enzymes in Chicken Muscles. Differences Among Strains with Different Growth Rates and Protein Efficiencies

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ABSTRACT The theory that net muscle growth is, at least partly, regulated by catabolic factors has been tested in order to set up an animal model to study meat aging and post mortem tenderization. Male and female chickens of a layer strain (White Leghorn), a commercial broiler strain (Ross), and two experimental broiler lines designated GL and FC were used to estimate differences in proteolytic enzyme activities in the breast muscles. The GL and the FC lines were selected for high body weight gain and high feed efficiency respectively. At 6 wk of age the birds were slaughtered and the activities of endogenous proteinases and their specific inhibitors in breast muscles measured.

The Leghorns showed significant differences in all traits compared with the three broiler genotypes. Within the broiler types, FC birds tended in the direction of the Leghorns and GL birds in the opposite direction. Ross birds were intermediate between FC and GL line birds. All types and sexes differed significantly in slaughtering weight. Feed conversion ratio and protein conversion ratio were highest for Leghorns. The FC line birds showed the lowest food conversion. Ross and GL line birds showed intermediate values. The Leghorns showed higher calpain activities and lower calpastatin activity than the three broiler genotypes. The FC line broilers showed intermediate calpain and calpastatin activities but higher cathepsin H and total cystatin values. The GL line broilers showed lower cathepsin B, D, and H activities. In all cases the Ross broilers showed intermediate values.

From these figures it is concluded that the strains of birds used in this study can be used as a natural source of variability to study the mechanisms involved in post mortem proteolytic degradation and thus in the study of muscle tenderization and meat aging. It is also concluded that it could be very interesting to study the behavior of the different proteolytic systems more carefully in relation to muscular growth characteristics, and compare them to anabolic factors involved in muscle growth.

(Key words calpains, calpastatin, cathepsins, cystatins, meat aging)

INTRODUCTION

Accretion of (muscular) tissue is a dynamic process (Grant and Helferich, 1991). Anabolic and catabolic processes are counteracting, their equilibrium being regulated by the demand of the organism (Goldberg, 1969a,b). Changes in muscular growth, occurring as a result of a changing demand by the organism, are caused by a coordinated change in the kinetics on both sides of the equilibrium. Relative changes on one or both sides of the equilibrium will result in an increased or a decreased muscle growth (Bergen and Merkel, 1991).

Studies indicate that muscular accretion can be regulated at the catabolic side of the equilibrium (Dayton *et al.*, 1981). In fact, there is a natural limit to the protein synthesis rate (Calzone *et al.*, 1983). A number of physiological, and mainly pathological states exist in which muscle growth is determined solely at the catabolic side, as with muscular atrophy after denervation and tenotomy in rats (Goldberg 1969b). Chemically and hormonally induced changes in catabolic activity have also been described. Administration of cortisone to rats (Goldberg 1969b) resulted in an increased protein degradation in muscular tissue. Administration of β-agonists like clenbuterol to rats (Reeds *et al.*, 1986) and lambs (Higgins *et al.*, 1988), cimaterol or clenbuterol to steers, sheep, chickens, and rats (Bardsley *et al.*, 1992), and cimaterol to chickens (Morgan *et al.*, 1989) and lambs (Wang and Beermann, 1988) resulted in muscle hypertrophy and a decrease in proteolytic activity. The study by Reeds *et al.* (1986) showed no response to clenbuterol administration at the anabolic side of the equilibrium. It can therefore be concluded that the degradation of muscular proteins constitutes an important regulatory mechanism for muscle growth (Dayton *et al.*, 1981, Goll *et al.*, 1992).

Biochemical processes occurring in living tissue do not stop at the moment of death. Therefore, these ante- and post mortem processes are of great influence on meat quality. In the post mortem state the essentially de-regulated enzyme systems contribute towards meat tenderization (Koohmaraie, 1988,1992, Koohmaraie *et al.*, 1991, Ouali, 1992). Reports of increased toughness in meat from β-agonist treated animals also suggest a reduced post mortem proteolytic capacity (Kretchmar *et al.*, 1990) resulting in decreased meat quality. The activity of endogenous proteolytic enzymes in muscle tissue is a major factor in the process of conversion of muscle to meat called aging (Goll *et al.*, 1983, Ouali, 1992). Not only tenderness, but also color and waterholding capacity and probably also taste, are at least partly determined by proteolytic processes in muscular tissue after slaughtering of the animal and during storage of the meat. The activity of the proteinases is of major importance in the development of post-rigor meat quality (Ouali 1990, 1992), and is mainly determined by the intramuscular pH and temperature, and the amount of active proteinases and inhibitors present.

According to Etherington (1984), the major endogenous proteinases responsible for post mortem proteolytic degradation of myofibrillar proteins are generally regarded as being represented by two systems. The first of these is the calcium-dependent neutral proteolytic system, consisting of two isoenzymes with different activation concentrations for calcium ions and a specific proteinaceous inhibitor, called the calpain-calpastatin system (Murachi, 1983). The system exhibits optimal activity at a neutral pH. It physiologically controls the disassembly of intact myofibrils and thus is involved in the metabolic turnover of the myofibrillar proteins (Dayton *et al.*, 1976). The main active proteinase in this group is the so-called μ-calpain (Dransfield, 1992). This calpain is activated at micromolar calcium concentrations (Dayton *et al.*, 1981; Goll *et al.*, 1990) and is capable of degrading the cytoskeletal proteins

responsible for the lateral and longitudinal structural integrity of the muscle, such as desmin, titin and nebulin. However, it does not degrade any of the major myofibrillar proteins like myosin and actin. The proteinases of this system are only capable of limited proteolysis, breaking up the intact proteins into relatively large fragments (Goll *et al.*, 1992). The second isoenzyme from this group, called m-calpain, needs a higher Ca^{2+}-ion concentration than the μ-calpain. However, the concentration required for half maximal enzyme activity is much lower for chicken m-calpain than for the same enzyme in mammalian species (Wolfe *et al.*, 1989).

The second system composes the lysozomal acid proteinases called cathepsins and a group of inhibitors called cystatins (Mikami *et al.*, 1987; Calkins and Seideman, 1988; Ouali, 1992). Under physiological conditions these proteinases are compartmentalized inside the lysosomes, where they act as a cellular disposal system for damaged and otherwise obsolete proteins and peptides. These enzymes exhibit their maximal proteolytic activities at low pH and are capable of degrading myosin and actin and a large number of other myofibrillar proteins into relatively small fragments (Matsukura *et al.*, 1981; Mikami *et al.*, 1987; Pearson and Young, 1989; Ouali, 1992). They are also capable of further breaking down degradation products of other proteolytic systems (Elgasim *et al.*, 1985). In regard to post mortem proteolysis in muscular tissue, the main enzymes of this system are cathepsin D, an aspartic proteinase, and cathepsins B, L and H, three cysteine proteinases (Goll *et al.*, 1983)

The activities of the different endogenous proteolytic enzymes and their inhibitors depend on both genotypic and phenotypic factors. By using these factors in studies on the influence of proteolysis on meat quality, it is possible to develop a model to study the aging of meat by using the natural variation in proteolytic capacity of the muscular tissue.

Purpose of our studies is to investigate the relationships between conditions at farm level and subsequent meat quality in broiler production and therefore the purpose of this study was to quantify the differences in proteolytic capacity of the calcium-dependent neutral and catheptic systems in chicken breast muscle from birds with different growth rates and feed conversions. The hypothesis tested by this study was that the activity of the endogenous proteolytic enzymes and their inhibitors relevant to post mortem proteolysis depends on genotypic factors.

MATERIALS AND METHODS

Materials

All chemicals used were of analytical grade and obtained from Merck unless otherwise stated. Water was always of milli-Q quality. T-61 was obtained from Hoechst. Triton X-100 and BRIJ-35 were obtained from Pierce. N-carbobenzoxy-L-arginyl-L-arginine-7-amido-4-methyl-coumarin (Z-Arg-Arg-NMec), N-carbobenzoxy-L-phenylalanyl-L-arginine-7-amido-4-methyl-coumarin (Z-Phe-Arg-NMec), and L-arginine-7-amino-4-methyl-coumarin (Arg-NMec) were obtained from Bachem. Hemoglobin, papain, pepstatin A, aminomethylcoumarin, and *p*-nitrophenyl-N-acetyl-β-glucosamide were obtained from Sigma. The phenylsepharose and the mono-Q column were obtained from Pharmacia.

Methods

An experiment with a 2 x 4 factorial arrangement of treatments was performed to investigate the effects of selection line and sex on proteolytic enzyme activities in chicken breast muscle. The complete experiment consisted of two completely randomized blocks (hatches) of 16 100 x 70 cm floor pens with litter. For each block, 160 one-day-old chicks, 80 males

and 80 females, of the four different lines, hatched at the Spelderholt Center, were reared up to 6 wk of age. Within a hatch, each experimental treatment was randomly allocated to two floor pens. Ten chickens were placed in each floor pen, resulting in 20 male and 20 female birds per line and per hatch. The birds were given unrestricted access to feed and water. The birds were fed with standard Spelderholt broiler feed containing 21% crude protein and 3,050 kcal ME/kg. A light regimen of 1 h light and 3 h darkness was applied throughout the rearing period. The air temperature was gradually decreased from 31 C on the 1st d to 18 C on the 40th d.

The four chicken lines used were: 1) White Leghorns: slow growing (males approximately 650 g; females approximately 550 g at 6 wk) with a feed conversion ratio of approximately 2.5. 2) Ross broilers: commercial broilers with a high growth rate (males approximately 2,400 g; females approximately 2,100 g at 6 wk) and a feed conversion ratio of approximately 1.8. 3) GL line broilers: experimental broiler line with an extremely high growth rate (males approximately 2,500 g; females approximately 2,250 g at 6 wk) and a feed conversion ratio of approximately 1.8. 4) FC line broilers: experimental broiler line with average growth rate (males approximately 1,800 g; females approximately 1,650 g at 6 wk) and a feed conversion ratio of approximately 1.6. Background and selection history of the GL and FC lines are described by Leenstra (1988).

Birds were individually weighed on the 1st d and subsequently weekly throughout the rearing period. Feed consumption was measured per pen at the same time as the birds were weighed.

At 6 wk of age, five birds per pen were randomly allotted to be slaughtered by standard processing as described below. Three birds per pen were randomly allotted to be killed by lethal injection with T-61 and subsequent determination of average crude carcass protein according to ISO-937 (Anonymous, 1992).

Slaughtering took place in the following standard manner: Birds were stunned in a water bath stunner (approximately 10 s, 100 V, 50 Hz) and killed by neck cut. The birds were allowed to bleed out for approximately 90 s. Immediately after bleeding, one half of the large breast muscle (*Pectoralis major*) was removed, wrapped in aluminum foil, frozen in liquid nitrogen, and subsequently stored at -80 C until assayed for calpain and calpastatin activity as described below. A muscle sample was taken from the other breast half, for isolation of lysozomes and subsequent determination of cathepsin- and cystatin-like activities as described below.

lysozomal enzymes and cystatin like activities.

Isolation of lysozomes. Preparation of lysozomal extracts was carried out according to Béchet *et al.* (1986) with some minor modifications. Five grams of muscle tissue were homogenized in 45 mL of homogenization buffer (10 m*M* potassium phosphate, pH 7.4, containing 1 m*M* EDTA, 50 m*M* sodium chloride, and 250 m*M* sucrose) with a Polytron homogenizer at maximum speed for 60 s. One milliliter of the homogenate was mixed with 10 µL of a 10% solution of Triton X-100 and frozen at -20 C and subsequently stored at -80 C until analysis of N-acetyl-β-glucosaminidase activity. Prior to the assays, the tubes were quickly thawed in a water bath at 30 C, after which they were centrifuged at 12,000 x g and 4 C for 10 min. The supernatants, hereafter referred to as total cell extracts, were used in the N-acetyl-β-glucosaminidase assays.

The rest of the homogenate was centrifuged at 2 C and at 1,000 x *g* for 10 min and subsequently for 10 min at 4,000 x g. The pellet containing the myofibrillar fraction was discarded and the supernatant containing the majority of the intracellular organelles was centrifuged at 20,000 x *g* for 20 min at 2 C. The supernatant, hereafter referred to as soluble fraction, was frozen at -80 C for the analysis of cystatin like activity as described below. The pellet containing the lysosomal fraction was resuspended in 3 mL of lysozome buffer (20 m*M* sodium acetate, pH 5.0) and frozen at -20 C and subsequently stored at -80 C until determination of the activity of cathepsins B, H, L and D and N-acetyl-β-glucosaminidase. Prior to the assays, the tubes were quickly thawed in a water bath at 30 C after which they were centrifuged at 12,000 x *g* at 4 C for 10 min. The supernatants, hereafter referred to as lysosomal extracts, were used in the assays for cathepsins B, H, L, and D and N-acetyl-β-glucosaminidase. Determinations of cystatin like activity, carried out in these lysosomal extracts as described below did not show any detectable inhibitory activity.

Determination of Activity of Cathepsins B, H, and L. The cysteine catheptic enzymes were assayed fluorimetrically using methods according to Barret (1980) with some minor modifications. Activity of cathepsin B was assayed with Z-Arg-Arg-NMec (Kirschke *et al.*, 1983). Cathepsins B and L were assayed together using the common substrate Z-Phe-Arg-NMec (Barret and Kirschke, 1981) and cathepsin H was assayed with Arg-NMec (Barret, 1980). For each assay, 40 µL of lysosomal extract were diluted with 300 µL of incubation buffer (100 m*M* sodium acetate, pH 5.5 containing 1 m*M* EDTA, 5 m*M* dithiothreitol, and .1 % BRIJ-35). Fifty microliters of this dilution were pipetted, in triplicate, into a white opaque 96-well fluorescence microtiter plate. Assys were started by adding 20 µL substrate solution (40 µ*M* in incubation buffer) to each well followed by incubation at 37 C for 20 min. The reactions were stopped by the addition of 150 µL of 33 m*M* sodium acetate, pH 4.3, containing 33 m*M* sodium chloroacetate to each well, after which the trays were read in a Perkin Elmer LS 50B spectrofluorimeter with a plate reader accessory attached to it. An excitation wavelength of 360 nm and an emission wavelength of 460 nm and split widths of 10 nm each were used, with aminomethylcoumarin solutions as standards. Each plate also contained the appropriate assay- and reagent blanks. One unit of proteolytic activity is defined as the amount of enzyme capable of releasing 1 µmol of aminomethylcoumarin from the substrate/min.

Determination of Activity of Cathepsin D. The aspartic proteinase cathepsin D, was determined according to Takahashi and Tang (1981) with minor modifications. Twohundred microliters of each lysosomal extract were pipetted, in duplicate, into 1.5-mL microcentrifuge vials. Fivehundred microliters of 15% trichloroacetic acid (TCA) were added to one of the tubes that would serve as the sample blanks. Fivehundred microliters of substrate solution (3% hemoglobin in 200 m*M* acetic acid) were added to all tubes, which were then incubated at 37 C for 30 min. The reaction was stopped by the addition of 500 µL of TCA to the sample tubes, after which the tubes were kept at 4 C for at least 30 min. The tubes were then centrifuged at 12,000 x *g* for 5 min and the optical densities of the supernatants were read spectrophotometrically at 280 nm.

One unit of proteolytic activity is defined as the amount of enzyme capable of increasing the optical density of the supernatant relative to the blank by .01 absorbance units.

Determination of Activity of N-Acetyl-β-Glucosaminidase. The determination of the activity of N-acetyl-β-glucosaminidase was carried out in both the total cell extract and

the lysosomal extract. Because there is no known endogenous inhibitor for this enzyme, the ratio of the activities in the two extracts can be used to calculate the effectiveness of the lysosomal isolation. Using the resulting value, the activities of the other lysosomal enzymes present in the starting material can be calculated.

The assays were carried out according to Béchet *et al.*(1986) with minor modifications. Of the serially diluted samples (either total cell or lysosomal extract) in reaction buffer (.05 *M* Na-citrate, pH 4.4) 30 µL together with 30 µL substrate (*p*-nitrophenyl-N-acetyl-β-glucosamide, 5 m*M* in reactionbuffer) were pipetted into a 96-well microtiter plate and incubated at 37 C for 30 min. The reaction was stopped by the addition of 150 µL of .5 *M* glycine/NaOH, pH 10.4 and the optical density at 420 nm was recorded. After correction for dilution the efficiency of the lysosome isolation could be calculated. The average recovery of the lysosomal isolation determined according to this method was 29.5% (SD 9.9%).

Determination of Total Cystatin-Like Activity. The cystatin assays were carried out according to Bige *et al.* (1985) with minor modifications. To 10 mL of the soluble fractions, obtained as described above, 5 *N* NaOH was added until a pH of 10 was reached, in order to destroy any residual proteolytic activity. The mixture was incubated in a water bath at 37 C for 60 min after which the pH was readjusted to 6.0 by addition of 5 N HCl. The cloudy suspension was centrifuged for 15 min at 20,000 x *g* and at 4 C, after which the pH was increased to 7.6 with 5 *N* NaOH. Serial dilutions of the prepared extracts were made in incubation buffer (100 m*M* sodium acetate, pH 6.0, containing 1 m*M* EDTA, 5 m*M* dithiothreitol, and .1% BRIJ-35) and these were incubated with 10 n*M* papain and 20 µ*M* Z-Phe-Arg-NMec, in a total volume of 80 µL, in fluorescence microtiter plates. After an incubation time of 10 min, reactions were stopped and evaluated as described under the cathepsin B, H, and L determination. One unit of cystatin activity was defined as the amount of inhibitor capable of totally blocking one unit of papain activity.

Lysosomal extracts, prepared as described above and submitted to inactivation at high pH as described here did not show any detectable proteolytic activity.

Calpains and Calpastatin

Tissue extraction. The determinations of the activities of the proteins comprising the calpain-calpastatin system were essentially carried out according to Etherington *et al.* (1987) with some modifications based on methods by Gopalakrishna and Barsky (1985), Karlsson *et al.* (1985), and Iversen *et al.* (1993). Ten grams of the frozen muscle samples were minced and homogenized for 1 min in 40 mL of homogenization buffer (50 m*M* Tris.HCl, pH 8.0 containing 5 m*M* EDTA, 10 m*M* β-mercaptoethanol (MCE), .05% sodium azide, and 150 n*M* pepstatin A) in a Polytron homogenizer at maximum speed. After an incubation period of 1 h at 0 C with occasional shaking, the homogenates were centrifuged at 100,000 x *g* for 1 h at 2 C. The supernatants were filtered through glass wool to remove contaminating lipids. The extracts thus obtained will be further referred to as calpain extracts.

Determination of Calpastatin Activity. Calpastatin activity was determined in the calpain extracts. The method of Geesink (1993) was used.

Determination of Activity of μ- and m-Calpains. Twenty milliliters of the calpain extracts were adjusted to .4 *M* with solid NaCl and pH 6.0 with acetic acid. The samples were applied to small (10 mL gel bed volume) phenyl sepharose columns equilibrated in HIC-application buffer (20 m*M* Tris.HCl, pH 6.0 containing .4 m*M* EDTA, 10 m*M* MCE and .02% sodium azide) containing .4 M NaCl (buffer A). The columns were washed with 20 mL of the

same buffer to remove the calpastatin, after which the calpain isoenzymes were eluted with 20 mL chromatography buffer (20 m*M* Tris.HCL, pH 7.5 containing .4 m*M* EDTA and 10 m*M* MCE) containing 50% ethyleneglycol (buffer B). The obtained eluates containing the calpain isoenzymes did not show any inhibitory (calpastatin) activity when determined as described above. Twenty milliliters of the eluate were diluted with 10 mL diluent (.4 m*M* EDTA and 10 m*M* MCE) and 25 mL of this mixture were applied to a Mono-Q® HR 10/10 column that was equilibrated with chromatography buffer. The column was developed with a linear gradient of 0 to .6 M NaCl in chromatography buffer. The calpain isoenzymes were eluted well separated and the fractions containing the proteolytic activity could be pooled to serve as the samples in which the activities of the calpain-isoenzymes were determined. None of the obtained fractions showed any detectable calpastatin activity when determined as described above.

To 400 µL of serial dilutions of the pooled fractions, 400 µL of substrate solution (.5% casein in 50 m*M* Tris.HCl containing 10 m*M* MCE and .02% sodium azide) were added. To one of the duplicates, 100 µL of a .5 *M* $CaCl_2$ solution were added to start the reaction. The same amount of EDTA was added to the other tube which served as a sample blank. After 30 min incubation at 30 C, 400 µL of a 10% TCA solution were added to each tube to stop the reaction. After incubation at 0 C for 30 min, the tubes were centrifuged at 12,000 x *g* for 10 min and the supernatants read at 280 nm. The sample blank extinctions were subtracted from the measurements and the optical densities obtained corrected for dilution. The values obtained from the linear part of the dilution curve were used. One unit of calpain activity is defined as that amount of enzyme that is capable of increasing the optical density of the sample with .001 absorbance units per minute.

Statistical Analysis

Errors were assumed to be independent and normally distributed with mean of zero and a constant variance. Because, in the case of slaughtering weight, the experimental errors for White Leghorns were substantially lower than for the other three lines, data for these traits were split and analyzed separately. Due to heterogeneity of the error variance, data for proteolytic capacities, µ-calpain:calpastatin ratio and m-calpain:calpastatin ratio were analyzed after logarithmic transformation. These transformations were only performed on the calpain:calpastatin data because the cathepsin:cystatin data showed a homogenous error distribution. Effects with $P<.05$ were considered to be significant. All data were subjected to ANOVA (GENSTAT 5). For the enzyme data, means by floor pen were calculated. Ratio figures were calculated on an individual bird basis. Subsequent ANOVA was performed on floor pen means.

$$Y_{ijkl} = \mu + H_i + S_j + L_k + (S*L)_{jk} + e_{ijkl}$$

The means were analyzed according to the following ANOVA:

where µ = general mean, H = Hatch (i=1,2), S = Sex (j = male, female), L = Line (k=White Leghorn, Ross, GL line and FC line), SxL = interaction between line and sex, and e is the experimental error.

RESULTS AND DISCUSSION

Table 1 shows some of the results on live bird performance obtained during growth of the birds. The first column shows the live body weight on the day of slaughter. All lines and sexes differ from each other ($P<.001$). In all cases males were heavier than females. For the FC line

birds, sex differences were smaller ($P<.05$) than for the other three lines ($P<.001$). There were no interactions between lines and sexes. The White Leghorns showed the lowest weights ($P<.001$). The FC line birds were much lighter than the other two broiler lines ($P<.001$). The differences between Ross and GL line birds were slightly smaller than between the other lines ($P<.01$).

These data reveal a large variation in slaughter weights. It is obvious that the White Leghorns grow much more slowly than the broiler lines. The fact that the three broiler lines all differ significantly in growth rate was expected (Leenstra and Pit, 1987; Leenstra 1988).

Feed conversion ratio data only showed differences between sexes in the GL line birds ($P<.05$) where females showed higher values than males (Table 1). Averaged over the sexes the differences between lines were significant ($P<.001$). There were no signs of interaction between lines and sexes. White Leghorns showed the highest feed conversion followed by the Ross and GL line broilers. The FC line birds showed a lower feed conversion ratio compared to the other lines ($P<.01$).

TABLE 1: Live bird performance results[1]

Strain of bird	Sex	Slaughter weight	Feed Conversion Ratio	Protein Conversion Ratio
		(g)	(g : g)	(g : g)
Leghorns	Males	651	2.527	2.441
	Females	550	2.581	2.445
Ross	Males	2237	1.758	1.952
	Females	2114	1.815	1.961
GL line	Males	2512	1.701	1.892
	Females	2258	1.907	2.080
FC line	Males	1788	1.611	1.750
	Females	1647	1.634	1.745
			SED [2]	
Effects	Sex	28.0 ***	.0413 *	.0454 NS
		8.4 *** [3]		
	Line	34.3 ***	.0583 ***	.0642 ***
	Sex x Line	48.6 NS	.0825 NS	.0908 NS

[1] n = 20 birds per group

[2] SED = Standard error of the differences

[3] SED for sex differences of Leghorns only

Significance levels: *** $P<.001$

* $P<.05$

The protein conversion ratio data showed no differences between sexes (Table 1). There were no signs of interactions between lines and sexes. Averaged over the sexes, the White Leghorns showed higher values than the other three lines ($P<.001$). The FC line birds differed from the two other broiler lines ($P<.01$) and from the White Leghorns ($P<.001$).

That layers are inefficient in feed and protein utilization for body growth and synthesis of

body protein is a well-known fact. Selection for feed conversion efficiency has resulted in birds with a high efficiency for both feed and protein utilization. However, these birds grow considerably more slowly than the commercial Ross broilers and birds especially selected for growth rate. The FC line birds show protein conversion efficiencies well over 57% (100% / Protein Conversion Ratio), but the other broilers show feed protein incorporation percentages between 40 and 53%.

Total feed utilization percentages show the same picture. Although the FC line broilers show a percentage of 61 to 62% (100%/Feed Conversion Ratio) the other lines show percentages between 38 and 59%.

The fact that the slaughter weights and the feed conversion figures were more divergent in this study than in the earlier work by Leenstra (1988) can be explained by the ongoing selection of these lines for growth rate and feed conversion.

TABLE 2: Proteinases of the calpain / calpastatin system[1]

Strain of bird	Sex	μ-calpain	m-calpain	calpastatin
			(Units x g wet tissue^{-1})	
Leghorns	Males	708	1499	104
	Females	1058	1598	102
Ross	Males	185	707	285
	Females	334	611	297
GL line	Males	283	678	282
	Females	275	728	317
FC line	Males	347	600	129
	Females	471	710	122
			SED[2]	
Effects	Sex	34.6 ***	30.9 NS	6.37 NS
	Line	48.9 ***	34.7 ***	9.01 ***
	Sex x Line	69.2 ***	61.8 NS	12.75 NS

[1] n = 20 birds per group

[2] SED = standard error of the differences

Significance levels: *** $P<.001$

Table 2 shows the activities of the proteinases from the calpain-calpastatin proteolytic system. For μ-calpain, differences between sexes and between lines as well as the interactions between sexes and lines were highly significant ($P<.001$). The White Leghorn ($P<.001$) and Ross ($P<.05$) males showed higher values than the female birds of the respective lines. The White Leghorn females, the slowest growing birds, showed the highest μ-calpain activity ($P<.001$). For m-calpain only the differences between lines were significant ($P<.001$). White Leghorns differed from the broiler line birds (P<.001).

The calpastatin activities are shown in the last column of Table 2. There are no differences between sexes and no interactions between sexes and lines. The Ross and GL line chickens differ from the White Leghorns and the FC line birds ($P<.001$). The FC line broilers show differences from the White Leghorns ($P<.05$).

The enzyme activities of the calpain-calpastatin system indicate that birds with either a low protein utilization or a highly efficient protein metabolism show high calpain activities and low calpastatin activities, whereas fast-growing birds show a reversed ratio of the same components. This indicates that the catabolic part of protein metabolism is an important element in muscle growth. Johari *et al.* (1993) found the same effects when comparing m-calpain and calpastatin values in layers and broilers. Ballard *et al.* (1988) investigated the effects of alteration of growth rate, induced by different diets, on the levels of m-calpain and calpastatin in chicken leg muscle. They reported no significant differences between groups with different growth rates. Furthermore, Ouali and Talmant (1990) reported large differences in calpain and calpastatin distribution in different muscle types in beef, pork and lamb. A mixture of different leg muscles was used in the study of Ballard *et al.* (1988). This indicates that it is important to use well-defined muscles when comparing tissue growth characteristics and metabolic variables.

Although properties of the chicken calpain-calpastatin system are reported to be different from these of mammalian species (Kawashima *et al.*,1984; Ballard *et al.*, 1988), comparisons with studies conducted in lamb, pork, and beef (Koohmaraie *et al.*, 1987, 1991; Calkins and Seideman, 1988; Ou *et al.*, 1991; Ouali, 1992; Whipple and Koohmaraie, 1992; Dransfield, 1993) emphasize the importance of this proteolytic system in post-mortem meat aging.

Table 3 shows the results of the activity assays of the enzymes of the lysosomal system. For the Cathepsin B values the differences between lines ($P<.001$) were much larger than the differences between sexes ($P<.05$). There were significant interactions between lines and sexes ($P<.05$). Differences between sexes were only found in the FC line group ($P<.01$). Male GL line birds differed from the White Leghorn and Ross birds ($P<.001$) and from the FC line ($P<.05$). The female GL line broilers differed from the FC and White Leghorn birds ($P<.001$). The White Leghorn females differed from the Ross ($P<.01$) and the GL line ($P<.001$).

Because a specific substrate to measure the activity of Cathepsin L is unknown, this enzyme was measured with the common substrate for Cathepsins B and L as indicated by Kirschke *et al.* (1983). These measurements showed interactions between sexes and lines ($P<.01$) (Table 3). The lines showed highly significant differences ($P<.001$). Differences between sexes were found in the White Leghorns and the FC broilers ($P<.05$). The White Leghorn males were different from the male birds of the three broiler lines ($P<.001$). The Ross males differed from the GL line ($P<.0.05$). The female White Leghorns differed from the Ross and GL line females ($P<.001$). FC line females differed from Ross and GL line females ($P<.05$).

To get an indication of the contribution of the Cathepsin L to the measurements obtained with the common substrate, the ratio of the measurements with the common substrate over the measurements of Cathepsin B activity alone were calculated. The differences between sexes ($P<.01$) and between lines ($P<.001$) were significant as were the interactions between sexes and lines ($P<.01$) (Table 3). Sex differences were only observed in the Leghorns ($P<.01$). For the males, the White Leghorns differed from the three broiler lines ($P<.001$). The female White Leghorns differed from the Ross and FC line birds ($P<.01$).

For Cathepsins B and L, comparable activity distributions were found in this study. Both cathepsins showed high values in White Leghorns. In case of the males, the layers differed from the FC line birds. In the case of the females they did not. In all cases the GL line birds differed from the White Leghorns. When the ratio of the common substrate values over the

specific cathepsin B measurement is considered to be a good indication of cathepsin L activity, it can be observed that the Ross birds, as well as the other broilers, differ from the layers. This indicates that for the Ross birds, cathepsin L is responsible for the differences whereas for the GL- and FC line birds, cathepsin B is responsible for the differences observed. The FC-hens show a high cathepsin B level combined with a low cathepsin L level. As the GL line females have a relatively high ratio figure, it may be concluded that these birds show a low cathepsin B level combined with a high cathepsin L level. Etherington *et al.* (1987, 1990) obtained cathepsin B and cathepsin B + L values for chickens comparable to the values found in this study. The specific activity of cathepsin L is ten times higher than the cathepsin B specific activity (Goll *et al.*, 1983). Etherington (1984) further indicated that cathepsin L is much more important for post-mortem proteolytic degradation than is cathepsin B.

TABLE 3: The enzymes of the catheptic system[1]

Strain of bird	Sex	CathepsinB	Cathepsin B+L	Ratio B+L:B[2]	Cathepsin H	Cathepsin D	Cystatins
		(U/g)		(U/U)		(U/g)	
Leghorn	males	1681	6642	4.37	1992	228	6504
	females	1799	5027	3.11	2146	225	6969
Ross	males	1308	3125	2.72	1612	197	7877
	females	1180	2168	2.10	2181	200	7821
GL line	males	611	1459	2.50	501	73	6366
	females	802	2246	2.83	1286	85	7021
FC line	males	1085	2441	2.44	2801	159	8325
	females	1780	3799	2.00	2621	267	10199
Effects				SED[3]			
Sex		92.6 *	319.2 NS	.1665 **	89.4 **	13.44 *	483.9 NS
Line		130.9 ***	451.4 ***	.2355 ***	126.5 ***	19.00 ***	684.4 **
Sex x Line		185.2 *	638.3 **	.3330 *	178.9 **	26.88 *	967.9 NS

[1] n = 20 birds per group

[2] ratio B + L : B = the ratio of cathepsin B + L over cathepsin B

[3] SED = standard error of the differences

Significance levels: *** $P<.001$

* $P<.05$

The Cathepsin H results showed a completely different picture. The differences between sexes ($P<.01$) and between lines ($P<.001$) were significant as were the interactions between lines and sexes ($P<.01$) (Table 3). Ross females showed higher values than the Ross males ($P<.01$) and GL line males showed more Cathepsin H activity than the females of the same line ($P<.001$). For the males, the FC line birds differed from the other three lines ($P<.001$). The White Leghorns differed from the Ross ($P<.05$) and GL line males ($P<.001$). The Ross and GL line birds also differed ($P<.001$). For the females, the FC line birds also differed from the Ross broilers and White Leghorns ($P<.05$) and the GL line broilers ($P<.001$). The Ross females differed from the GL line birds ($P<.001$). The White Leghorn females differed from the GL line birds ($P<.001$).

Goll *et al.* (1983) indicate that cathepsin H is strongly involved in myofibrillar protein turnover. It shows a specific activity intermediate to cathepsins B and L. The results from this study support these observations. Birds with a very efficient use of feed protein, e.g. the FC line birds, show a potentially high protein breakdown, possibly devised for amino acid reuse, whereas fast growing birds generally show little proteolytic activity. As slow-growing birds like White Leghorns show high rates of proteolytic activity, this indicates that the degradation of proteins plays a major role in net body- and muscle growth.

The last proteolytic enzyme shown in Table 3 is Cathepsin D. For this proteinase, the differences between lines ($P<.001$) were larger than the differences between sexes ($P<.05$). There were also signs of interactions between lines and sexes ($P<.05$). The differences between sexes were only found in the FC line broilers where the females showed higher values than the males ($P<.01$). For the males, the White Leghorns differed from the FC line ($P<.05$) and GL line ($P<.001$). The FC line males differed from the GL line ($P<.01$) birds. The Ross birds differed from the GL line males ($P<.001$). For the females, the FC line birds differed from the Ross ($P<.05$) and GL line broilers ($P<.001$). The White Leghorns differed from the GL line birds ($P<.001$). The GL line females showed lower cathepsin D values than the Ross birds ($P<.001$). These values show that the GL-birds show very low cathepsin D activity compared to the other three lines. Again, this could indicate that fast growth rate is combined with low proteolytic activity. This is confirmed by Rososchacki (1985), who reported that induced muscular hypertrophy results in a lower cathepsin D activity than in normal muscles. Etherington *et al.* (1990) found comparable values for cathepsin D activities in chickens. Because this proteinase does not have an endogenous inhibitor in muscular tissue, it plays a major role in later post-mortem proteolysis, as was indicated by Rico *et al.* (1991).

The total cystatin-like activity measurements showed differences between lines ($P<.05$) but not between sexes (Table 3). There were no signs of interactions between lines and sexes. The FC line broilers showed differences with the White Leghorns, the GL line broilers ($P<.01$) and the Ross birds ($P<.05$).

To have some indication of the proteolytic potential in post-mortem muscle, it is necessary to combine the activities of the different proteinases with their specific inhibitors. This can be accomplished by taking the ratios of the calpains over calpastatin and the cysteine-Cathepsins B, L and H with cystatin. Table 4 shows the results of these calculations. The proteolytic capacities of the calpain-calpastatin system are indicated in the first two columns (ratios A and B). Due to the heterogenous distribution of the residuals, it was necessary to transform the calculated ratios to a logarithmic form in order to be able to estimate the significance of the differences. For the μ-calpain:calpastatin ratio the differences between lines ($P<.001$) were larger than between sexes ($P<.05$) (Table 4). There were no signs of interactions between lines and sexes. Between sexes, the only differences were found in the Ross birds. Averaged over the sexes, the White Leghorns differed from the broiler lines ($P<.001$). The FC line broilers differed from the other broiler lines ($P<.001$). For the m-calpain:calpastatin ratio the only differences observed were between lines (table 4). There were no signs of differences between sexes and interactions between lines and sexes. Averaged over the sexes, the White Leghorns differed from the broiler lines ($P<.001$). The FC line broilers differed from the other broiler lines ($P<.001$).

The differences in the proteolytic potential of the calpain-calpastatin system were quite

dramatic. The difference between the largest and smallest proteolytic capacities for μ-calpain was 12-fold and for m-calpain 8-fold. Concerning the μ-calpain proteolytic potential, it is clear that whereas in the slow-growing types of chickens the proteinase is present in large excess, for the fastest growing birds the inhibitor is present in a slight excess. The m-calpain is present in excess to the inhibitor in all cases.

TABLE 4: Proteolytic capacities of proteinases and their endogenous inhibitors[1]

Strain of bird	Sex	Ratio A[2]	Ratio B[3]	Ratio C[4]	Ratio D[5]	Ratio E[6] (x 10^{-4})	Ratio F[7]
Leghorn	Males	1.957	2.716	.316	1.224	.801	.366
	Females	2.364	2.797	.304	.902	.577	.408
Ross	Males	-.997	.859	.181	.425	.403	.243
	Females	-.191	.576	.196	.346	.348	.359
GL line	Males	-.558	.759	.104	.253	.446	.086
	Females	-.753	.790	.125	.345	.449	.203
FC line	Males	.632	1.500	.175	.399	.386	.438
	Females	1.163	1.854	.204	.398	.235	.321
Effects		SED[7]					
Sex		.1393 *		.01478 NS	.0504 NS	.482 *	.0260 NS
Line		.1970 ***	.1115 ***	.02090 ***	.0713 ***	.682 ***	.0368 ***
Sex x Line		.2786 NS	.1576 NS	.02955 NS	.1008 *	.964 NS	.0520 *

[1] n = 20 birds per group

[2] Ratio A = Ln(μ-calpain/calpastatin)

[3] Ratio B = Ln(m-calpain/calpastatin)

[4] Ratio C = cathepsin B/cystatin

[5] Ratio D = cathepsin B+L/cystatin

[6] Ratio E = (ratio of B+L/B)/cystatin

[7] Ratio F = cathepsin H/cystatin

[8] SED = standard error of the differences

Significance levels: *** $P<.001$

* $P<.05$

The catheptic proteolytic capacities are shown in the last four columns of Table 4. The proteolytic capacity of Cathepsin B, shown as ratio C (cathepsin B:cystatins), showed only differences between lines ($P<.001$). The differences between sexes and the interactions between sexes and lines were not significant. The White Leghorns showed higher ratios than the broiler lines ($P<.001$). The GL line showed a lower ratio than the Ross and the FC line birds ($P<.05$).

The proteolytic capacity of the Cathepsins B + L , shown as ratio D, indicated no differences between sexes and no interactions between lines and sexes (Table 4). However, differences between lines were significant ($P<.001$). The White Leghorns showed a higher cathepsin B+L:cystatin ratio than the broilers ($P<.001$). When this figure is corrected for Cathepsin B activity, as shown under ratio E, an impression is obtained of the proteolytic capacity of Cathepsin L. The table shows differences between sexes ($P<.05$) and lines

($P<.001$) but no signs of interactions between sexes and lines. Differences between sexes were only observed in the White Leghorns ($P<.01$). Averaged over the sexes, the White Leghorns differed from the GL line birds ($P<.01$) and from the Ross and FC line broilers ($P<.001$).

The proteolytic capacity of cathepsin H, indicated in the last column of Table 4 as ratio F, shows differences between lines ($P<.001$) and interactions between sexes and lines ($P<.05$). The GL line males showed differences with the Ross ($P<.01$) and with the FC- and White Leghorn males ($P<.001$). The White Leghorn males showed differences with the Ross birds ($P<0.05$). The FC line males differed from the Ross male birds ($P<.01$). For the female birds the GL line broilers also showed lower ratio values than the FC line ($P<.05$), the Ross broilers ($P<.01$) and the White Leghorns ($P<.01$).

Considering the proteolytic capacities of the lysozomal cathepsins B, H, and L found in this study, it is evident that with the sole exception of the cathepsin B+L:cystatin ratio for the White Leghorn males, all ratios are less than one. This might lead to the misleading conclusion that cystatins are always well in excess over cathepsins. It must however be stressed that all activities of the proteinases were assessed with different substrates and that the cystatin like activities were all assessed against papain, so they can not be compared directly. The figures can only be used to assess the differences between animals within one trait. Bearing this in mind, the results can give a good indication of potential post-mortem proteolytic degradation of muscle proteins.

In conclusion this study indicates that differences in growth rate and protein efficiency are reflected in the proteolytic status of the muscular tissue. It must be stressed that the objective of this study was not to establish any relationships between the intracellular proteolytic capacity in the muscular tissue and the net muscle growth observed in the animal. It was merely meant to identify whether the theory indicated in the introduction, that catabolic activity is a factor in the regulation of net muscle growth, can be used as a source of natural variability in model studies in meat aging. In this the presented study was successful because it was shown that the different types of birds developed different proteolytic capacities in their breast musculature. The White Leghorns showed a large proteolytic capacity of the calpain:calpastatin system. The FC line showed an intermediate capacity of the same proteolytic system but also a high activity of the cathepsin H and high cystatin activities. The GL-line birds showed very low cathepsin H and D activities. In all cases the Ross birds showed intermediate activities. From the presented figures, it can be concluded that the strains used in this study are good sources of muscle material to study the mechanisms of myofibrillar protein breakdown, and meat aging. It could be very interesting to study the behavior of the different proteolytic systems more carefully in relation to protein turnover and muscular growth characteristics, and compare them to anabolic factors involved in muscle growth.

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ABSTRACT The activity of endogenous proteolytic enzymes in muscle tissue is a major factor in the process of the conversion of muscle to meat. In earlier work, it was shown that chicken genotypes selected for very fast growth show a markedly decreased proteolytic capacity in their breast muscles. Chickens growing slower showed a higher proteolytic potential. This suggests that breast meat of fast growing broilers ages slower than the meat of slow growing broilers or layers. In this study, post mortem changes in breast muscle of broiler type chickens from different lines selected for growth rate (GL-line) and protein efficiency (FC-line) were compared to the changes in layer type chickens from a slow growing and very protein-inefficient line (White Leghorn) and a normal commercial broiler type (Ross). Shear force values from breast muscle of leghorns and, to a lesser extent, FC-broilers decreased more quickly post mortem than those of the faster growing birds (Ross and GL-line). The data also indicated that aging in the fast growing lines was not yet completed at 48 hrs post mortem, while in the slower growing lines, shear forces leveled off after 24 hrs post mortem. Myofibrillar fragmentation was shown to be higher in the muscles of White Leghorns than of the three broiler lines at 6 hrs post mortem, suggesting that the myofibrillar degradation is faster in the layer type than in the broiler lines. The course and onset of rigor mortis as reflected in the maximum obtained in the shearforce measurements coincided well with the events related to the energy metabolism.

(*Key words: Meat quality, tenderness, post mortem metabolism, meat aging*)

INTRODUCTION

Chicken meat production is characterized by an ongoing demand for increased production efficiency. That is, for higher growth speeds of the birds and higher breasts meat percentages of the carcasses. However, along with the drive for cost reduction and thus lower feed and protein conversion ratios this may lead to impaired meat quality as well as animal welfare and health problems (Scheele, 1996).

Growth of muscular tissue is the result of anabolic and catabolic processes which are counteracting, their equilibrium being regulated by the demand of the organism (Goldberg, 1969a,b). Changes in muscular mass, due to a changing demand by the organism, are caused by a coordinated change in the kinetics on both sides of the equilibrium. Relative changes on one or both sides of the equilibrium will result in an increased or decreased muscle mass (Bergen and Merkel, 1991).

As there is a natural limit to the protein synthesis rate (Calzone *et al.*, 1983), one might argue that in extremely fast growing animals, net muscle accretion is mainly regulated on the catabolic side of the equilibrium, e.g. regulated by the activity, *in vivo*, of the diverse proteolytic systems present in the cell.

The activity of endogenous proteolytic enzymes in muscle tissue is a major factor in the process of the conversion of muscle to meat called aging. Not only tenderness, but also color and waterholding capacity and probably taste are at least partly determined by proteolytic processes in muscular tissue after slaughtering of the animal and during storage of the meat (Goll et al., 1983, Koohmaraie et al., 1991, Ouali, 1990, 1992). The intramuscular pH and temperature, and the amount of active proteolytic enzymes and inhibitors present, mainly determine the post mortem activity of endogenous proteinases.

Schreurs et al. (1995) have shown that chickens selected for a very fast growth show a markedly decreased proteolytic capacity of both the calpains/calpastatin as well as some cathepsins/cystatins, compared to chickens growing extremely slow. Chickens with a very efficient protein metabolism showed intermediate calpain/calpastatin values, but increased cathepsin H and cystatin activities.

Many data are available on the *post mortem* changes in breast muscle, especially those related to the energy metabolism, of chickens slaughtered under, simulated, practical processing conditions. Very little is known about the changes taking place in breast muscle of chickens undergoing an undisturbed *post mortem* metabolism, especially the *post mortem* myofibrillar degradation, usually regarded as the aging process of the meat.

Purpose of the experiments described here was to investigate the undisturbed *post mortem* processes in breast muscles of chickens from different lines selected for fast growth and high protein efficiency, and to compare these changes to those in chickens from an extremely slow growing and very protein-inefficient line and chickens of a normal commercial broiler type.

MATERIALS AND METHODS

All chemicals used were obtained from Merck and of analytical quality. Enzymes used for the metabolite measurements were obtained from Boehringer Mannheim. All water used in the analyses was of Milli-Q® quality

Animal material

Eggs from four lines were hatched in the in-house hatching facility. Lines used were (Schreurs *et al.*, 1995):

- commercial White Leghorns as a slow growing layer line with high feed conversion ratios
- commercial Ross broilers with fast growth and low feed conversion ratios

- FC-line broilers, a line selected at ID-DLO for 14 generations, with extremely low feed conversion ratios and moderate growth rates
- GL-line broilers, a line selected at ID-DLO for 14 generations, with moderate feed conversion ratios and extremely high growth rates.

The one-day-old chicks were sexed at the day of hatching and 10 males and females from each line were housed separately in 4 replicate floor pens resulting in 40 birds per line per sex available at the day of slaughter. The experiment was repeated twice with two separate hatches.

At the day of slaughter, the birds were cooped and transported to the processing plant. They were electrically stunned in a waterbath at approximately 100 V, and bled by neck cut for 90 seconds. The animals were neither scalded nor plucked nor eviscerated. The skin was removed and the breasts including bone were immediately collected in such a manner that the breast muscle and its bone attachments were left completely intact, e.g. the spinal column attachments were left intact and the wing was cut of at the elbow joint. Measurements in breast muscle were carried out and breast muscle samples for further analysis were taken at 0, 1, 2, 6, 24 and 48 hours *post mortem*. After removal, the breasts were wrapped in plastic bags and kept in ice slush until a temperature of ca. 12°C was reached in the center of the breast muscle. This took approximately 30 minutes for the broiler lines and 20 minutes for the leghorns. Subsequently the breasts were stored at 12°C for a maximum period of 6 hours *post mortem*. After 6 hours, samples to be processed 24 and 48 hours *post mortem* were transferred to 0°C. For measurements of pH, R-value, metabolites and myofibrillar fragmentation index (MFI) samples were taken from the inside of the muscle. Care was taken to avoid sampling of tissue that had been exposed to the surface.

pH Measurements

Repeated measurements of the pH were carried out according to Jeacocke (1977), with only minor modifications, in tissue homogenates of breast muscle of four birds of each line and sex. Briefly, 2.0 ± 0.05 grams of breast tissue, stripped of fat and connective tissue, were homogenized in 20 ml of measuring solution (5 mM Sodium-iodoacetate in 150 mM KCl) using an Ultra Turrax homogenizer at maximum speed for 30 seconds cooled in ice slush. The pH of the resulting homogenate was measured using a combination glass pH electrode within 15 minutes after homogenization.

R-value Measurements

Repeated R-value measurements were carried out according to Khan & Frey (1971) as modified by Honikel and Fischer (1977), with only minor modifications, in tissue extracts of breast muscle of 4 birds of each line and sex. Briefly, 2.0 ± 0.05 grams of breast tissue, stripped of fat and connective tissue was homogenized in 20 ml perchloric acid solution (0.85 M $HClO_3$) using an Ultra Turrax homogenizer at maximum speed for 30 seconds, and allowed to stand on ice-slush (0 °C) for 30 minutes. Subsequently the homogenates were centrifuges at 1375 x g (4 °C) for 15 minutes and the supernatants decanted. From these supernatants 100 µl was pipetted into 2.5 ml of a 100 mM sodiumphosphate buffer (pH =7.0) and the extinction was read at 250 and 260 nm.

Measurements of Glycolytic Metabolites

The metabolites of the glycolytic system, glycogen, glucose, lactic acid and ATP were measured according to Passoneau and Lowry (1993) with modifications making microscale measurements in microtiter plates possible. All volumes of sample and reagents were proportionally reduced to reach an end volume of ca. 200 µl, fitting into a microtiterplate well. The measurements were carried out in the perchloric extract, obtained as described under R-value

measurements on 4 birds of each line and sex per replication. Concentration of the metabolites was measured in microtiter plates in a Molecular devices microplate reader equipped with a 340 nm optical filter and evaluated using the Softmax microplate analysis software.

Myofibrillar Fragmentation Index (MFI)

Repeated MFI measurements were carried out according to Olson et al (1976) with minor modifications, on 4 birds of each line and sex. Briefly, 2.0 ± 0.05 g of tissue was homogenized in MFI buffer (20 mM Potassiumphosphate, pH=7.0, containing 100 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, 2 mM phenylmethanesulphonylfluoride, 25 µg/ml leupeptin, 0.1 ng/ml pepstatin A, and 1 mM sodiumazide) using a Polytron blender for 30 sec at maximum speed. Subsequently, the samples were centrifuged at 1500 x g and 4 °C for 15 min. and the supernatants decanted. The subsequent steps used were identical to the method described by Olson *et al.* (1976).

Shear-force Measurements

For each measurement, different birds were used. The intact breasts were heated in a steam cabinet at 0, 1, 2, 6, 24 and 48 hours *post mortem* until a central temperature of 85°C was reached. After heating the samples were deboned and stored at 0°C until measurement, that was carried out within 24 hours after heating. After deboning shear force measurements were done using an Overload Dynamics universal testing machine on three strips from each breast halve, according to Froning and Uijttenboogaart (1988) on 5 birds of each line and sex

Data Analysis

Statistical analysis was carried out using SPSS®/Windows™. Differences between lines and sexes at different times *post mortem* were analyzed with the General Linear Models (GLM) module using repeated measures ANOVA except for the shear force measurements, where general factorial analysis was applied, since these measurements were carried out on different birds each time. The following model was used:

$$Y_{ijkl} = \mu + H_i + P_j + S_k + L_l + (S \times L)_{kl} + e_{ijkl}$$

where:
- Y_{ijkl} = response variable
- μ = population average
- H_i = hatch, (i = 1,2)
- P_j = floorpen, (j = 1…4)
- S_k = sex, (k= male, female)
- L_l = genetic line, (l = Leghorn, FC-line, Ross, GL-line)
- e_{ijkl} = experimental error in al traits

Post hoc pairwise difference tests were carried out on traits showing significant differences using the least significant difference (LSD) as the criterion.

RESULTS AND DISCUSSION

Statistical analysis did not show significant differences between hatches and floorpens for all traits so they were done on pooled data with a simplified statistical model in which H_i and P_j were omitted.

Figure 1 shows the intramuscular pH at different times *post mortem*. There were no significant differences between sexes, except at 48 hours *post mortem*, nor were there any significant interactions between sexes and lines. Immediately after killing, the leghorns showed a lower pH than the other 3 lines ($p<0.05$). At 1 and 2 hours after slaughter no significant differences between lines were established.

At 6 hours after slaughter, the FC-line broilers showed significantly lower pH values than the other 3 lines ($p<0.05$). Twenty-four and 48 hours *post mortem*, the leghorns show higher

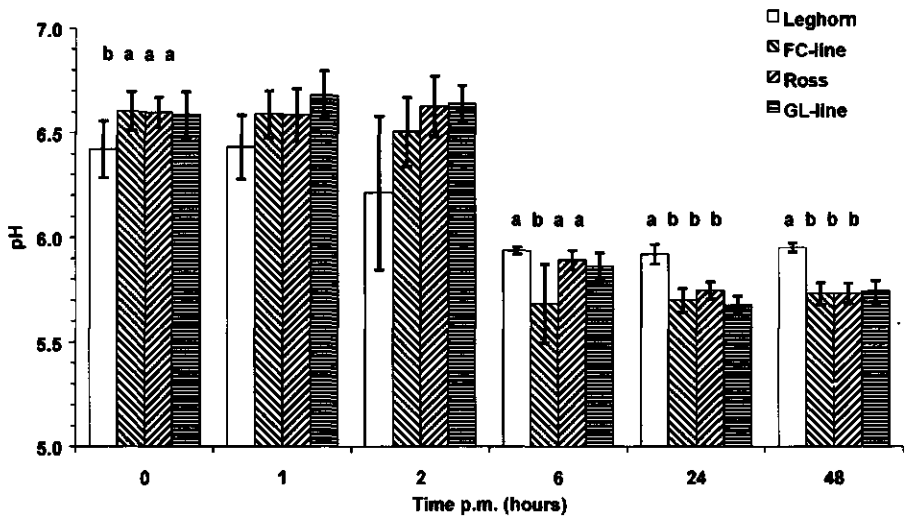

Figure 1 *Post mortem course of pH in breast muscle of chickens from different selection lines. Bars with different superscripts differ significantly (p<0.05).*

pH values than the other 3 lines (p<0.001). The 48-hour *post mortem* samples showed significantly higher pH values for male than for female animals (p<0.05).

Figure 2 shows the *post mortem* course of the R-value. This value is a measure of the breakdown and deamination of energy rich adenosine-phosphates via inosine-mono-phosphate to hypoxanthine and as such mirrors the course of the energy metabolism of the muscle (Kahn

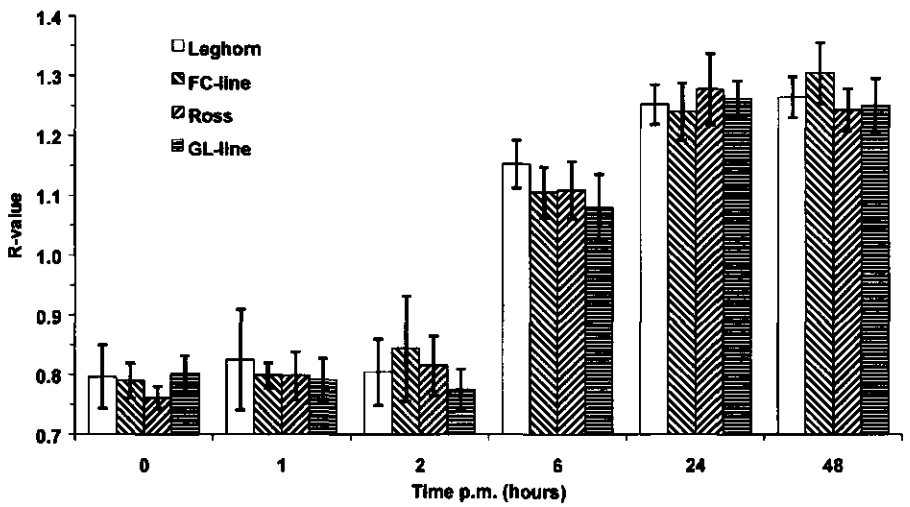

Figure 2 *Post mortem course of R-value in breast muscle of chickens from different selection lines.*

& Frey, 1971; Tsai *et al.*, 1972; Honikel & Fischer, 1977; Roncalés *et al.*, 1989; Watanabe *et al.*, 1989). None of the traits showed any significant differences.

Both the pH and the R-value are a measure of the glycolytic status and hence the rigor-development of the muscle. The pH data may lead to the conclusion that *post mortem*

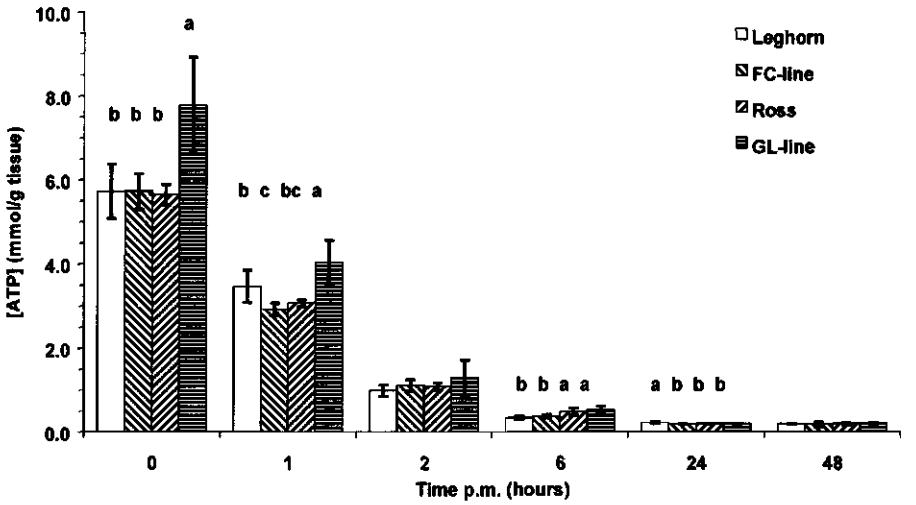

Figure 3 Post mortem course of tissue ATP concentration in breast muscle of chickens from different selection lines. Bars with different superscripts, differ significantly ($p<0.05$).

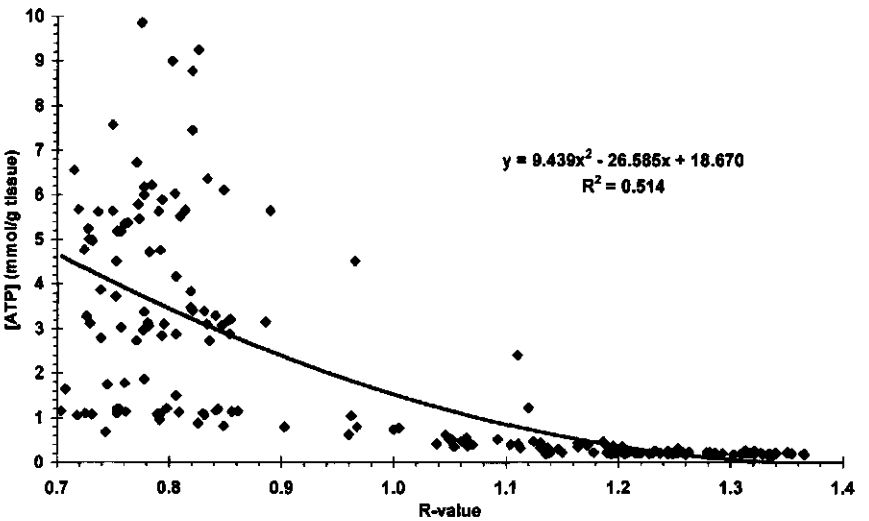

Figure 4 Relationships between tissue ATP concentration and R-value in chicken breast muscle.

glycolysis is faster in leghorns than in the three broiler lines. However, the development of *rigor* mortis as mirrored by the R-value did not differ significantly between different chicken lines. The higher final pH of the leghorns is possibly caused by lower initial glycogen stores in the breast muscle or it might be caused by the fact that glycolytic rate is reduced at lower pH's.

To study this in more detail, analyses of glycolytic metabolites were carried out. Figure 3 shows the *post mortem* degradation of ATP. There were no significant differences between sexes nor were there significant interactions between lines and sexes.

Immediately after slaughter, the GL-line broilers showed higher ATP levels in their breast muscles than the other three lines. One hour *post mortem*, the GL birds still have higher muscle-ATP. The leghorns show higher ATP levels than the FC-line broilers with the Ross birds

cle-ATP. The leghorns show higher ATP levels than the FC-line broilers with the Ross birds giving intermediate values. At 2 hours *post mortem* the ATP values have decreased below 1.5 μmoles per gram of tissue, which constitutes about 20 - 30% of the original amount present, and no significant differences between groups is visible anymore. This percentage signals the onset of *rigor mortis* (DeFremery & Lineweaver, 1962). At 6 hours *post mortem* The GL- and Ross broilers show higher values than the FC-line and leghorn birds ($p<0.001$). At 24 hours *post mortem* significant differences between lines are found but since the ATP concentrations measured are far below 5% of the original amount of ATP, these differences are not very meaningful. From these data, it may be concluded that *rigor mortis* occurs around two hours *post mortem.*

It is clear that the GL-line birds have considerably more ATP in their muscles at slaughter than the other lines. This surplus is maintained up to at least 6 hours *post mortem,* so far beyond the onset of *rigor mortis*. This is not clearly reflected in the R-value measurements as is shown in figure 4. This in contrast to the report of Roncalés *et al.* (1989), who found a good (quadratic) correlation (r^2 ca. 0.92) between ATP concentrations and E_{250}/E_{260} measurements in lamb kept at different temperatures until *rigor mortis* onset. Honikel & Fisher (1977) found a quadratic relationship between the R-values and ATP concentrations in porcine muscles measured at 45-min *post mortem.* Kahn & Frey (1971) found a linear relationship in chicken muscle but used only a small amount of samples (n=12). In our study we only found correlation coefficients of around 0.7 ($r^2 \approx 0.5$) for the total dataset as well as for datasets split by either line, sex or time *post mortem*. The samples in the lower left corner of figure 4, responsible for the lower correlation coefficients, are taken at 2 hours *post mortem,* so around the onset of *rigor mortis*, and showed persistently low R-values and also low tissue ATP. The reason for this probably is the rapid rate of degradation of ATP to ADP but the much slower breakdown of ADP through AMP, IMP, inosine to hypoxanthine. The significance of the established regression lines however was high ($p< 0.001$), and the regression coefficients are comparable to the work of Roncalés *et al.* (1989).

Since ATP is (re)generated from the degradation of glycogen and glucose through the glycolysis, it is interesting to compare the tissue concentrations of these compounds to the above mentioned parameters. Figure 5 shows the post mortem course of glycogen stores in the muscle. There were no significant differences between sexes except at 24 hours *post mortem,* and there were no significant interactions between sex and line throughout the measuring period. Up to 2 hours *post mortem*, no significant differences between lines could be detected. At 6 hours *post mortem*, the GL- and FC-broilers showed higher glycogen values than the leghorns ($p<0.05$) with intermediate values for the Ross birds. At 24 hours *post mortem*, the GL-line animals showed higher values than the Ross broilers ($p<0.01$) and the leghorns ($p<0.001$), while the latter had lower tissue glycogen than the Ross birds ($p<0.05$). The FC-line birds showed no differences with GL-line and Ross birds but were significantly higher in tissue glycogen than the leghorns ($p<0.01$). Over the lines, the females ([glycogen] = 5.7 μmol/g tissue) showed significantly higher ($p<0.05$) values than the males ([glycogen] = 4.3 μmol/g tissue). At 48 hours *post mortem* all three broiler lines showed higher ($p<0.01$) glycogen values than the leghorns while over the lines females ([glycogen] = 5.7 μmol/g tissue) showed higher ($p<0.01$) glycogen values than males ([glycogen] = 4.2 μmol/g tissue).

Figure 6 shows the *post mortem* course of the glucose concentration in the muscle tissue. No significant differences between sexes, nor significant interactions between sexes and lines were detected. Immediately after slaughter and 1 hour *post mortem* no significant differences between lines were observed. At 2 hours *post mortem* the leghorns and FC-line broilers

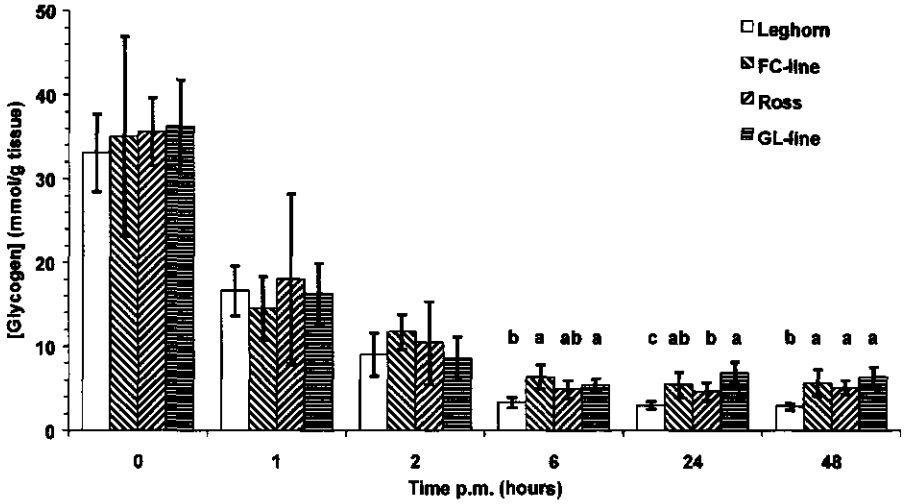

Figure 5 Post mortem course of tissue glycogen concentration in breast muscle of chickens from different selection lines. Bars with different superscripts, differ significantly (p<0.05).

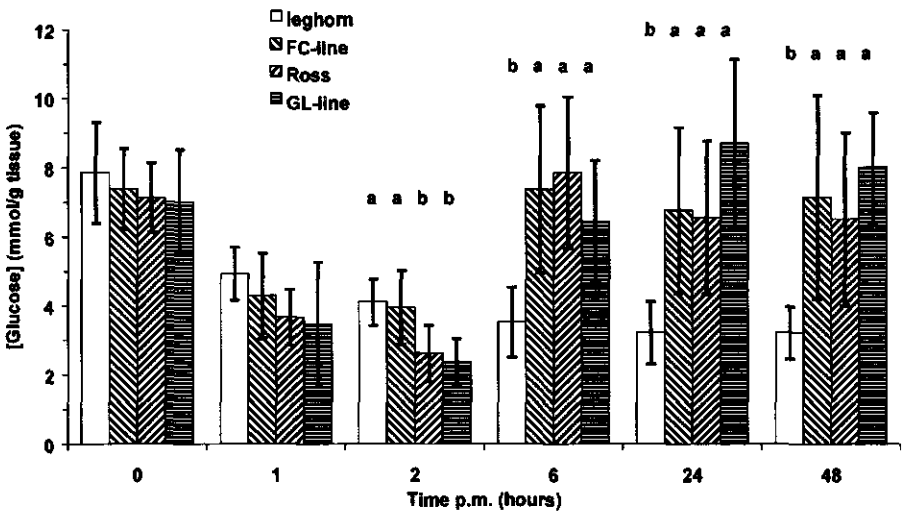

Figure 6 Post mortem course of tissue glucose concentration in breast muscle of chickens from different selection lines. Bars with different superscripts, differ significantly (p<0.05).

showed higher glucose values than Ross and GL-line birds (p<0.05).

From 6 hours *post mortem* on, the leghorns showed significantly lower tissue glucose than the three broiler lines (p<0.05). From the fact that the pH of the leghorns remains relatively high from 6 hours *post mortem* on, while the glycogen levels are low, it may be concluded that the glycogen stores are rapidly depleted in this line. This is supported by the fact that the glucose levels remain low in the breast muscles of the leghorns while in the three broiler lines the glucose concentrations decrease from slaughter until ca. 2 hours *post mortem* after which they increase again to their original level within 24 hours *post mortem*. This minimum coincides with the onset of *rigor mortis*. The leghorns did not start off with a decreased amount of gly-

glycogen due to the increased metabolic rate as reflected by the low pH at slaughter, nor did they reach *rigor mortis* quicker as reflected by R-value and ATP depletion patterns. The increase in tissue glucose in the three broiler lines after 2 hours *post mortem* may be interpreted as a cessation of glucose degradation through the glycolytic pathway while there was still some degradable glycogen present that was hydrolyzed to glucose. This may lead to the conclusion that in chickens the glycolysis cessation occurs at pH between 5.7 and 5.9 even if adequate glycogen stores are present. Kastenschmidt (1970) relates this phenomenon in red meat animals to the inhibition of the glycolytic enzyme phosphofructokinase (PFK) which needs ATP as a substrate in the conversion of fructose-6-phosphate into fructose-1-6-*di*-phosphate. When this conversion stops, the glycolytic pathway is interrupted from fructose-6-phosphate on but the hydrolysis of glycogen into glucose is little impaired by this. This evidence is supported by the onset of rigor mortis, thus the depletion of ATP around 2 hours *post mortem*.
It is generally accepted that the enzyme systems in metabolic pathways involved in energy metabolism are regulated under direct control of several interacting hormone systems (Simonides and van Hardeveld, 1985). Examples of these are the glucokinase in the liver and the muscular hexokinase, enzymes involved in the glycolysis, α-glycerolphosphate dehydrogenase, an enzyme diverting metabolic products of the glycolysis towards the lipogenesis and malic enzyme, an enzyme system diverting an intermediate product of the citric acid cycle towards lipogenesis. Among the most important regulatory systems are the pituitary/liver controlled growth hormone (GH), insulin-like growth factor (IGF-I) axis and the hypothalamic/pituitary/thyroid axis secreting thyroid releasing hormone (TRH), thyroid stimulating hormone or thyrotropin (TSH) and the thyroid hormones thyroxin (T_4) and 3,3',5-triiodothyronin (T_3) of which the latter is the metabolically active form in muscular tissue (Leonard & Koehrle, 1996). Leenstra and Pit (1988) and Decuypere *et al.* (1991) have shown the FC-line birds to exhibit periodic GH secretory patterns with amplitudes twice that of GL-line broilers. Also the overall levels of GH in plasma samples of these FC-birds were substantially higher than of GL-broilers (Leenstra *et al.* 1991) This is supported by the results of Buyse *et al.* (1995) who studied the GH secretory paterns in other lines with high growth rate and favourable feed conversion ratios. Goddard *et al.* (1988) also report the slowest growing lines exhibiting the highest GH levels. Plasma T_3 concentrations in slow growing chickens are generally higher than in lines selected for high weight gain (Goddard *et al.*, 1988; Lauterio *et al.*, 1986; Decuypere *et al.* 1994). This may be caused by the fact that, as in FC-line birds, high endogenous GH levels, probably by increasing the circulating IGF-I (Huybrechts *et al.*, 1992) increase plasma T_3 levels through inhibition of the T_3 degrading type II liver monodeiodinase (Darras *et al.*, 1993).

Scheele *et al.*(1991, 1992) showed that chicken broiler strains, selected for a favorable feed conversion efficiency, often were susceptible to "heart failure syndrome" (HFS) and resulting ascites. It was also suggested that this susceptibility resulted from the impossibility of the animal to adapt to environmental circumstances, due to a decreased thyroid hormone availability. Wehtli and Wessels (1973) reported a tendency towards lower thyroid activity in chickens selected for a low feed conversion ratio. A negative correlation between plasma T_3 and deposition of body fat (Dewil *et al.*, 1996), and a decreased growth in hypothyroid chickens (Herremans *et al.* 1992) support these findings. The evidence presented here, suggest that modern fast growing broiler chickens, especially those lines exhibiting a favorable feed conversion ratio, all show relatively low circulating T_3, close to hypothyroidism. This may have profound consequences for muscular energy metabolism and contractility (Simonides and van Hardeveld; 1985). As the intramuscular activity of several enzyme systems, responsible for

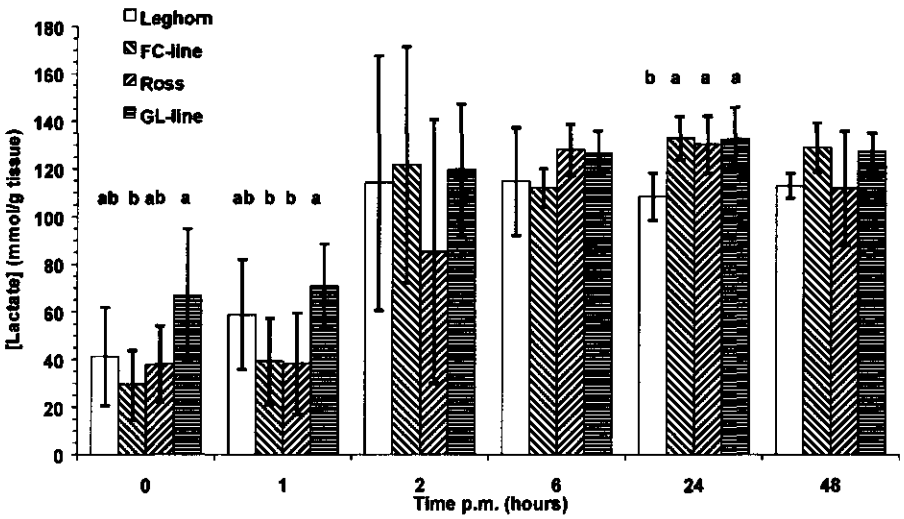

Figure 7 Post mortem course of tissue lactate concentration in breast muscle of chickens from different selection lines. Bars with different superscripts, differ significantly ($p<0.05$).

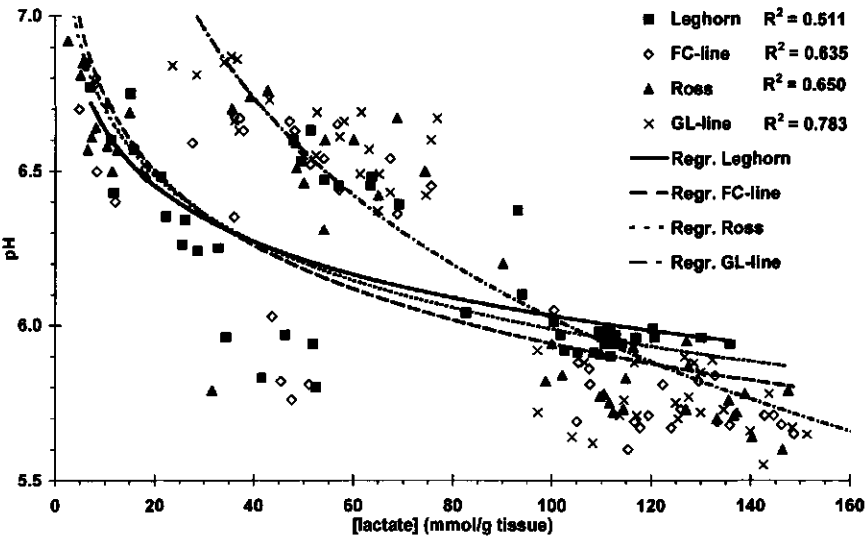

Figure 8 Differences in acid buffering capacity of breast muscle of chickens from different selection lines.

the generation of energy rich compounds from the degradation of glucose are down regulated by low T_3 values, it is probable that glycogen degradation is not finished after cessation of glycolysis due to ATP depletion.

Figure 7 shows the *post mortem* course of the lactate contents of chicken tissue at different times *post mortem*. There were no main effects of sex nor were there any significant interactions of sex and line. The variances of the data obtained in the early *post mortem* period and especially around the onset of *rigor mortis* were very high. At death, the GL-line broilers show the highest lactate values and the FC-line animals significantly lower tissue lactate. The leghorns and Ross birds show intermediate values. One hour *post mortem*, the GL-line birds still show high lactate concentrations in their muscular tissue while the FC-line birds and the Ross

Ross broilers are significantly lower. The leghorns show intermediate values. At two and six hours *post mortem,* no differences between lines were detected. At 24 hours *post mortem* the leghorns show lower lactate values than the three broiler lines while these differences disappeared at 48 hours *post mortem.* The course of the lactate concentrations in the tissue shows large variations. This may be due to the sampling. Excision of pré-rigor tissue is a well-known cause of lactate formation in the samples. The differences in lactate between lines are not very well reflected in the pH values found in the same animals. Figure 8 shows the logarithmic relationship between tissue lactate concentration and the resulting pH. This figure illustrates a possible explanation for the above-mentioned phenomenon. These data show clearly that the pH decline related to increasing amounts of tissue lactate, run virtually parallel in the leghorns, the Ross as well as the FC-line birds, the pH resulting from a certain lactate concentration is different in the breast muscle of GL-line birds. At a relatively high pH, so early *post mortem*, the GL-line birds show a much higher tissue lactate than the other three lines at equal pH values. Honikel & Hamm (1974) investigated the different components responsible for pH buffering in bovine muscular tissue. Immediately after slaughter, at a pH of approximately 7, the main buffering substances are the myofibrillar proteins and ATP and inorganic phosphate. At a pH of approximately 6.2 the main buffering substances are the myofibrillar proteins together with inosine-mono-phosphate, glucose-6-phosphate and inorganic phosphates. At a pH of 5.6, the main buffering substances are myofibrillar proteins, inosine-mono-phosphate and glucose-6-phosphate. This means that the higher ATP concentration in GL-line muscles may account for the higher buffering capacity early after death of the animals. This results in a larger amount of inosine-mono-phosphate and glucose-6-phosphate later *post mortem.* The increase in inorganic phosphate due to the degradation of these components is only effective in buffering at a pH above 6.2. The relative importance of myofibrillar proteins increases at pH values below 6.0. This explains the fact that at a pH below 6.0, lower tissue lactate concentrations as are shown for the leghorns, result in a slightly higher pH.

In order to study the *post mortem* degradation of myofibrillar proteins, the myofibrillar fragmentation index of the breast muscle at different times after slaughter was determined. Figure 9 shows the results of these measurements. There were no significant effects of sex nor were there any significant interactions between line and sex. Immediately after slaughter, the leghorns showed higher MFI values than the Ross and GL broilers ($p<0.01$) with the FC-line birds showing intermediate values. At 1 and 2 hours *post mortem* no significant line effects were detected. At 6 hours *post mortem,* the leghorns showed higher MFI values than the Ross ($p<0.05$) and the two non-commercial broiler lines ($p<0.01$). At 24 and 48 hours *post mortem* there were no significant line effects. From these data, It can be argued that the leghorns, due to their much larger proteolytic capacity (Schreurs *et al.*, 1995) show a much quicker development of myofibrillar fragmentation than the other types, especially in the early *post mortem* phase. However, the MFI is a very crude measure for these events, especially when applied to chicken breast muscle. Probably only very large differences in myofibrillar degradation can be detected. Takahashi *et al.*(1967) and Sayre (1970) studied the myofibrillar fragmentation in chicken *Pectoralis* muscle using phase contrast microscopic methods. These methods are inherently more accurate but very laborious and thus impractical to be applied to the amounts of samples used in this study. The method used in this study (Olson *et al.*, 1976) was developed in beef. In this species the measured fragmentation index is closely related to the microscopic observations. Olson & Parrish (1977) and Culler *et al.* (1978) showed that the MFI has a high correllation with shearforce measurements and some sensory meat quality parameters of beef.

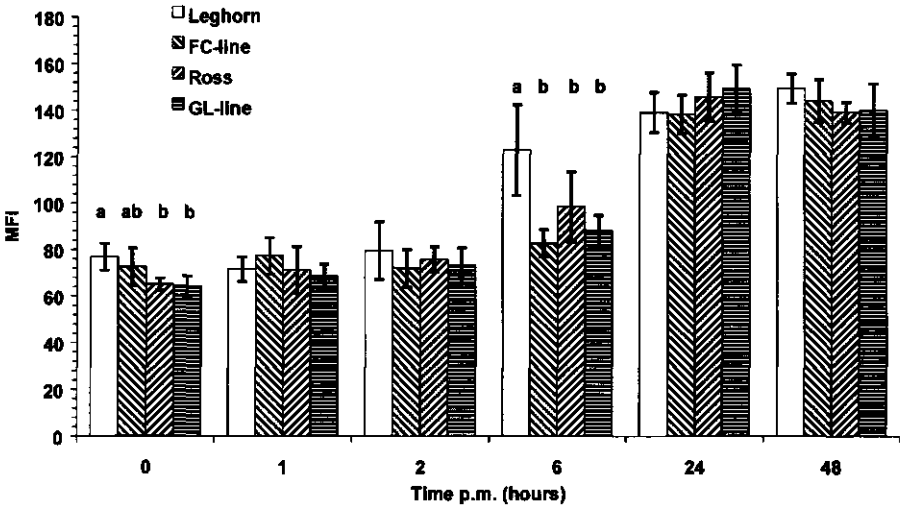

Figure 9 Post mortem course of myofibrillar fragmentation in breast muscle of chickens from different selection lines. Bars with different superscripts, differ significantly (p<0.05).

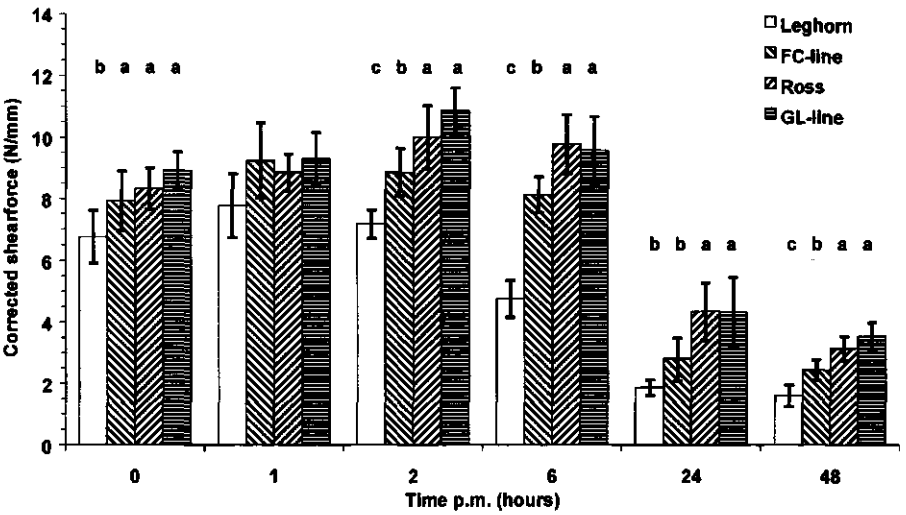

Figure 10 Post mortem course of shear force in breast muscle of chickens from different selection lines. Bars with different superscripts, differ significantly (p<0.05).

In the future it is important to investigate the *post mortem* proteolytic events chicken breast muscle in more detail using more sensitive methods like electrophoresis and immunochemical techniques to verify these data.

Figure 10 shows the course of the Warner-Bratzler shear force measurements. Breast musles of leghorns, and to a lesser extent, of FC-line birds are smaller and markedly thinner than of the Ross and GL-line birds, so a correction of shear force values was applied according to Froning and Uijttenboogaart (1988) and the results were expressed as Newton per millimeter tissue. Immediately after slaughter, the leghorns showed a lower corrected shearforce (CSF) than the three broiler lines ($p<0.05$). The males showed higher CSF values than the female birds

birds ($p<0.05$). At 1 hour *post mortem* no significant differences between lines or sexes or any significant interactions between lines and sexes were detected. At 2 hours *post mortem*, the male birds showed higher CSF than the female animals ($p<0.05$). The GL- and Ross birds did not show significant differences. The FC-broilers showed lower values than the GL- ($p<0.001$) and the Ross ($p<0.05$) broilers but higher ($p<0.01$) values than the leghorns. At 6 hours *post mortem*, the FC-line birds showed lower CSF than the Ross ($p<0.01$) and GL-broilers ($p<0.05$) but higher values than the leghorns ($p<0.001$). At 24 hours *post mortem* the males showed higher CSF than the females ($p<0.05$). The leghorns and FC-line broilers did not show significant differences but they showed lower CSF than the GL-line and Ross broilers ($p<0.01$). The latter two lines did not show significant differences. At 48 hours *post mortem* differences between lines are the same as at 24 hours *post mortem*.

It is clear that with the exception of 1 hour *post mortem*, the leghorns show significantly lower shearforce values than the three broiler lines. The FC-line birds show lower values than the GL and Ross broilers from 2 hours *post mortem* on. At no time *post mortem* did the GL-line and Ross broilers differ significantly.

These data are in agreement with the results of Schreurs *et al.* (1995). The birds with the largest proteolytic potential show the fastest aging while the GL and Ross broilers, with a low proteolytic capacity, age slowest. The FC-line birds show intermediate aging rates. Although it is generally accepted that tenderization in chicken breast muscle is complete after 24-48 hrs *post mortem (*Pearson & Young, 1989*)*, these data indicate that in the fast growing and thus slow aging broilers these processes do not always have to be completed within 48 hours after slaughter. The CSF values still decrease between 24 and at 48 hours *post mortem,* in breast muscle of animals from these lines, and at 48 hours *post mortem* they are still higher than in the FC-line and leghorn birds.

Concluding remarks

From this study the conclusion can be drawn that the four lines used here show marked differences in both *post mortem* energy metabolism and *post mortem* myofibrillar fragmentation. The course and onset of rigor mortis, generally known as conditioning of meat, as reflected in the maximum obtained in the shearforce measurements coincide quite well with the events related to the energy metabolism, although some discrepancies are noted. Moreover, the post rigor course of the corrected shearforce curves can partly be explained by the myofibrillar fragmentation measurements. The aging rate of the breast meat of the different chicken lines is determined by the proteolytic capacity, as was studied by Schreurs *et al.* (1995). However, to be able to detect the processes taking place during meat aging in chicken *Pectoralis* muscle, more sensitive methods are needed to selectively study the changes taking place during *post mortem* myofibrillar degradation. More extensive research is needed in order to generate more knowledge about the impact of genetic selection programs on post mortem processes involved in the conversion of muscle into meat, in order to avoid serious meat quality problems during processing and at the retail level.

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Chapter 5:

Post Mortem Shear Force and Sensory Attributes of Breast Muscle from Chickens Selected for Growth or Protein Efficiency.

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ABSTRACT Fast growing broilers show low proteolytic potential in breast muscle. Aging of breast meat of four strains of chickens, selected for fast bodyweight gain (GL-line), feed conversion (FC-line), a layer strain (Leghorns) and a commercial broiler strain (Ross).

Animals were killed in a standard manner and subsequently, the meat was aged on carcass during 0, 1, 2, 6, 24 and 45 hours under controlled circumstances. After the prescribed aging period, the carcasses were heated, and the cooked meat was removed for further analysis. Strips of meat were subjected to shearforce measurements and pieces of meat were presented to a trained sensory panel for pairwise comparisons and line scale scoring of the attributes tenderness, toughness, juiciness and dryness. Data were analyzed by ANOVA to reveal differences between selection lines. The sensory data of the line scales experiments were analyzed for panel consonance on the terms tenderness and toughness on one hand and juiciness and dryness on the other hand.

The shearforce measurements showed a starting tenderization around 6 hours post mortem. The course of the tenderness as detected by pairwise comparisons showed similarities with the shearforce measurements in the pre rigor period. The differences found post rigor did not compare with the pairwise comparisons. Pairwise comparisons on the basis of juiciness did not show a clear line of progress during 45 hours after slaughter.

Quantitative measurements of sensory meat quality showed comparable progress with time for tenderness and toughness as the shear force measurements. There were no differences between lines. Juiciness showed a decrease with time for the three broiler lines tested. The layer line showed only a marginal decrease in juiciness with time post mortem. Dryness showed a reciprocal course with time although the size of the time effect was almost twice the time effect of juiciness.

Generalized Procrustes analysis revealed a strong uni-dimensionality of the terms tender and tough. The terms juicy and dry showed to be at least tri-dimensional with the panel used for this experiment.

Key words: shear force, tenderness, tougness, juiciness, dryness.

INTRODUCTION

During the last 50 years, meat production, white as well as red, is characterized by an ongoing demand for more efficient production systems. Cost reduction and improvement of efficiency have been the tools to reach this goal. Especially in poultry, the results of genetic selection programs were remarkable. On the one hand, these programs have led to a tremendous increase in growth speed. Commercial broiler strains now grow up to four times faster than layer strains, not selected for body growth but for egg production capacity, and show a breast muscle growth of up to 8 times larger than of layer strains (Griffin and Goddard, 1994). On the other hand, the same selection programs have effected severe reductions in feed conversion and thus a better feed and especially protein utilization. During the forties, broiler chickens were grown to a slaughter weight of 2 – 2.5 kg at 16 weeks of age (Griffin and Goddard, 1994). During the sixties, broilers reached a body weight of approximately 1.1 to 1.4 kg in 10 weeks time utilizing approximately 2 kg of feed per kg of body weight gain (Chambers *et al.*, 1981). Modern broiler chickens reach slaughter weights of approximately 2500 g within 6 weeks of age utilizing approximately 1.7 kg of feed per kg of body weight gain and selection programs are driving for even more (Schreurs *et al.*, 1995). The other side of the medal however, are the animal welfare and health problems resulting from this "growing at the edge of what is metabolically possible" (Scheele, 1996) and a possibly impaired meat quality.

Based on muscular degradation studies, it was suggested that the rapid growth of muscles in broiler strains is facilitated by a low rate of protein degradation (Hayashi *et al.*, 1985; Saunderson and Leslie, 1988). Based on this, Schreurs *et al.* (1995) studied the proteolytic potential of different endogenous proteolytic enzyme systems related to meat quality. They found the fastest growing animals to posses the least proteolytic capacity of some proteinases in the breast muscle, especially the calpain/calpastatin system. This suggests that meat from these extremely fast growing animals would age slower than of less fast growing broilers. Along with this, animals with a very high protein conversion efficiency showed increased proteolytic potential for cathepsin L, a proteinase probably effective in the later stages of meat aging (Ouali, 1992).

Purpose of the experiments described here was to investigate the differences in mechanical shear force, sensory tenderness and juiciness during the first 48 hours of meat aging between groups of birds selected for high growth rate (GL-line) and birds selected for favorable protein efficiency (FC-line), and compare these results with slow growing, protein inefficient chickens (Leghorns) and birds from a normal commercial broiler type (Ross). History and background of the FC- and GL-line are described by Leenstra (1988)

MATERIALS AND METHODS

Animal and handling

Eggs from 4 different selection lines, Leghorns, FC-line, GL-line and Ross broilers were hatched and sexed at the Spelderholt institute and male animals were brought up under standard circumstances (Schreurs *et al.*, 1995; This thesis: chapter 3) to 42 days of age. The birds were housed in separate floorpens for each line. Eighty animals per selection line were randomly divided over 8 floorpens containing 10 birds each. At the day of slaughter, the animals were cooped and transported to the in-house processing plant at the Spelderholt institute. The birds had free access to feed and water until cooping. The experiment was repeated twice.

The birds were hanged in shackles at slaughter and were electrically stunned with 100 Volts, 50 Hz, during approximately 10 seconds after which they were killed by neck cut and

bled for exactly 90 seconds. The animals were neither scalded nor plucked nor eviscerated. After bleeding, the carcasses were decapitated, and breasts were harvested immediately in such a manner that the attachment sites of the *M. Pectoralis major* to the carcass were left intact. After skinning, the spinal cord was cut at the neck and below the ribs and the wings were cut at the elbow joint. The organs inside the breast case were removed after which the breast pieces were wrapped in plastic and packed in ice slush until the core of the *M. Pectoralis major* reached a temperature of approximately 12 °C. Core temperatures were monitored randomly in some breasts using small thermocouple sensors.

After cooling to 12 °C, which took typically between 20 and 30 minutes, the breast pieces, still wrapped in plastic, were stored in a climate room at 12 °C for up to 6 hours. After 6 hours *post mortem*, samples to be processed at 24 and 45 hours *post mortem* were transferred to a 0 °C cold-room.

At 0 (uncooled), 1, 2, 6, 24 and 45 hours *post mortem*, breasts were removed from storage and unwrapped. The breast pieces were subsequently heated to a core temperature of 85 °C and cooled to 0 °C in a cold-room. Subsequently, the cooked breast muscles were deboned and vacuum-sealed in plastic bags and stored until analysis. From each animal, one breast halve was randomly submitted to shear force measurements, while the other breast half was submitted to sensory analysis. Samples for shear force measurements were stored at 0 °C and processed within 24 hours after heating. Samples for sensory analysis were stored at –20 °C and processed within 2 weeks after heating.

Shear force measurements

The vacuum packed breast muscles were brought to a temperature of 25 °C in a water bath. Subsequently, three 10 mm wide strips of muscle tissue were removed from the cooked breast parallel to the muscle fibers, according to Froning and Uijttenboogaart (1988). Thickness of the muscle strips in a direction perpendicular to the 10 mm wideness, was measured with a sliding gauge. Measurements were carried out in triplicate on an Overload Dynamics universal testing machine equipped with a Warner Bratzler shear blade (Bratzler, 1932), with a cross head speed of 400 mm/min and a load cell of 100 N. Force data were collected and processed by a computer attached to the load cell. From the thus obtained shear force curves, corrected shear force values, adjusted for thickness, were calculated according to Pool and Klose (1969).

Sensory analysis

Measurements were carried out using a trained 21-member test panel. Samples were thawed overnight at 4 °C and allowed to reach room temperature before presentation to the panel. Samples presented to the panel consisted of a piece of breast meat from the middle part of the breast. Top and bottom parts were discarded.

Pairwise comparisons. Each presentation consisted of 9 paired samples per panel member, presented in a random order to the panel members. One sample of breast muscle of Ross broilers (standard) was always compared to one sample of breast muscle of one of the other three lines. Panel members were asked to point out the most tender and the most juicy sample.

Line scaling method. Panel members were asked to score samples on a line scale with anchor points 100 mm apart, extending 15 mm beyond the anchors on both sides of the line. Attributes to be scored were: tenderness, toughness, juiciness, and dryness. The anchors were labeled "very" at one side and "little" on the other side. Scores were measured as mm distance from the left anchor.

Statistical analysis

Statistical analyses were carried out using SPSS®/Windows™. The shear force data were analyzed with the General Linear Models (GLM) module using general factorial ANOVA. Post hoc pair wise differences were analyzed using the least significant difference (LSD) as the criterion. The ANOVA model used was:

$$Y_{ijkl} = \mu + H_i + P_j + L_k + T_l + e_{ijkl}$$

Where: Y_{ijkl} = response variable
μ = overall mean
H_i = effect of hatch (i = 1, 2)
P_j = effect of floorpen (j = 1, 2,…., 8)
L_k = effect of line (k = Leghorn, FC-line, Ross, GL-line)
T_l = effect of time post mortem (l = 0, 1, 2, 6, 24, 45 hours)
e_{ijk} = random effect over all traits (error)

Sensory measurements were analyzed using the χ^2 –test for pairwise comparisons and ANOVA and Generalized Procrustes Analysis (Dijksterhuis & Punter, 1990; Dijksterhuis & Gower, 1991; Dijksterhuis, 1995a,b; 1996; 1997) for line scale measurements.

RESULTS AND DISCUSSION

Shear force measurements

Figure 1 shows the results from the shear force measurements in breast muscle at different times *post mortem*. There were no significant differences between hatches nor were there any differences between floorpens, so statistical analysis was carried out on the pooled data sets. In ANOVA with time as a covariate, the Leghorns showed lower shear force values than the other lines ($p<0.001$) while the FC-line birds were significantly lower than the GL-line and the Ross broilers ($p<0.001$), but higher than the Leghorns ($p<0.001$). The GL-line and the

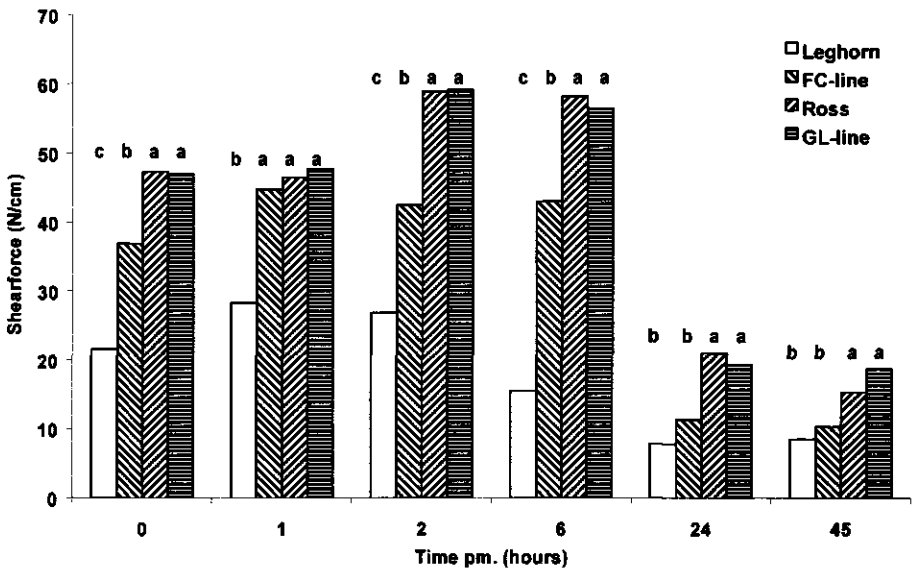

Figure 1 *Shearforce of breast muscle of different broiler lines at different times post mortem. Within times post mortem, columns with different superscripts differ significantly ($p < 0.05$).*

Ross broilers did not show significant differences. ANOVA with line and time as fixed effects showed significant differences between lines ($p<0.001$), time ($p<0.001$) and significant interactions between time and lines ($p<0.001$). At slaughter (0 hours *post mortem*) and at 1, 2 and 6 hours the Ross and GL line birds showed no significant differences in corrected shear force. The FC-line birds showed lower values at 0, 2 and 6 hours. The leghorns showed lower shear force values than the FC-line birds and the other two broiler lines at 0, 1 2 and 6 hours. At 24 and 48 hours, the Leghorns and the FC-line birds showed no significant differences. The Leghorns and the FC-line birds were lower than the GL-line and Ross broilers The GL-line and Ross broilers did not show significant differences.

In general, the course of the shearforce shows the same changes with time as earlier reported (Schreurs & van der Heide, chapter 4: this thesis). The reduction in shear values as time post mortem increases is well documented in the literature (Dawson et al., 1987; Lyon *et al.*, 1992). The two fast growing broiler lines, GL and Ross show the highest shear forces throughout the time studied. The leghorns show the lowest shear force while the FC-line birds show intermediate results. Decrease of shear force starts between 2 and 6 hours post mortem in Leghorns while in the three broiler lines shear force decrease starts between 6 and 24 hours post mortem. This is in accordance with the findings of Schreurs *et al.*(1995; this thesis: chapter 3) where the faster meat aging of slow growing strains was predicted on the basis of higher proteolytic capacity in the breast muscles of these strains.

Sensory analysis

Pairwise comparisons. Figure 2 shows the results of pairwise comparisons made on 30 pairs of samples per selection line per time *post mortem*. Immediately after slaughter the FC- and GL-line birds ($P<0.001$) and the Leghorns ($P<0.05$) show significantly more tender meat than the standard (Ross). At 1 ($P<0.001$), 2 ($P<0.01$) and 6 hours ($P<0.05$) the Leghorns show more tender meat than the standard. At 6 hours, the GL-line shows significantly less ($P<0.01$) tender meat than the standard. The FC- ($P<0.01$) and the GL-line ($P<0.001$) at 24 hours and the FC-line at 45 hours ($P<0.01$) show significantly more tender meat than the standard.
During the pre-rigor period, up to 6 hours, the Leghorns show the most tender meat, together with the FC-line birds immediately after slaughter. Post rigor, at 24 and 45 hours, the FC-line birds show the most tender meat, when compared with the standard. Around rigor, 6 hours post mortem, the GL-line birds show tougher meat than the other lines. This is in accordance with the findings of Schreurs *et al.*(1995; this thesis: chapter 3). Faster growing birds show slower aging, caused by a reduced proteolytic potential in their breast muscles. Pre rigor, the tenderness of the Leghorn breast muscles follows the trend of the shear force measurements. Post rigor, there seems to be no agreement between the shear force measurements and the sensory perception of tenderness of the taste panel. Probably, the differences found in the shear force measurements are too small to be detected by sensory analysis.

Figure 3 shows the results of the juiciness comparisons of the same 30 pairs of samples per selection line per time post mortem. The FC-line shows significantly juicier samples than the standard at 2 ($P<0.05$) and 24 hours ($P<0.001$), while the Leghorns ($P<0.05$) and the FC-line broilers ($P<0.01$) showed significantly less juicy samples at 45 hours. There is no clear line of progress in the results of the juiciness measurements by pairwise comparisons against the Ross breast meat.

Line scales.

Figure 4 shows the results of the line scales measurements for the term tenderness averaged over panel members per line and time *post mortem*. The average tenderness scores show a clear increase at 24 and 45 hours. At 1 hour the average tenderness score for Leghorn

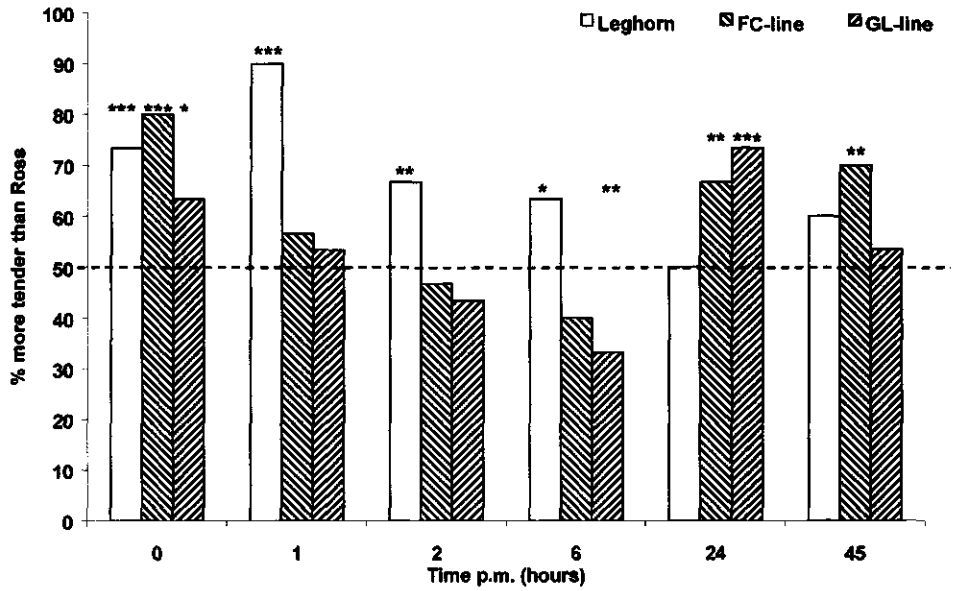

Figure 2 *Sensory tenderness: pairwise comparisons of chicken breast muscle of different broiler lines at different times post mortem.*
*Significance levels: * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$.*

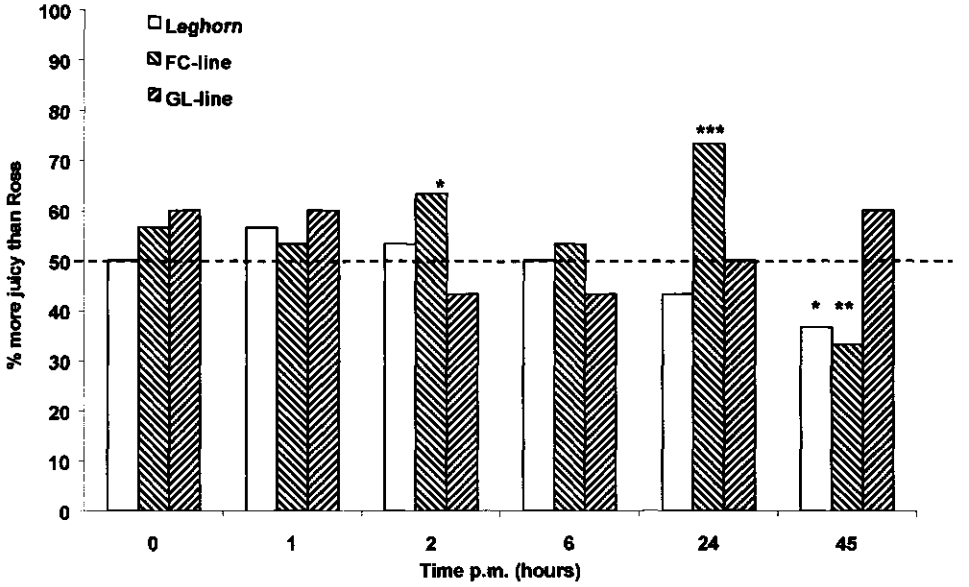

Figure 3 *Sensory juiciness: pairwise comparisons of chicken breast muscle of different broiler lines at different times post mortem.*
*Significance levels: * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$.*

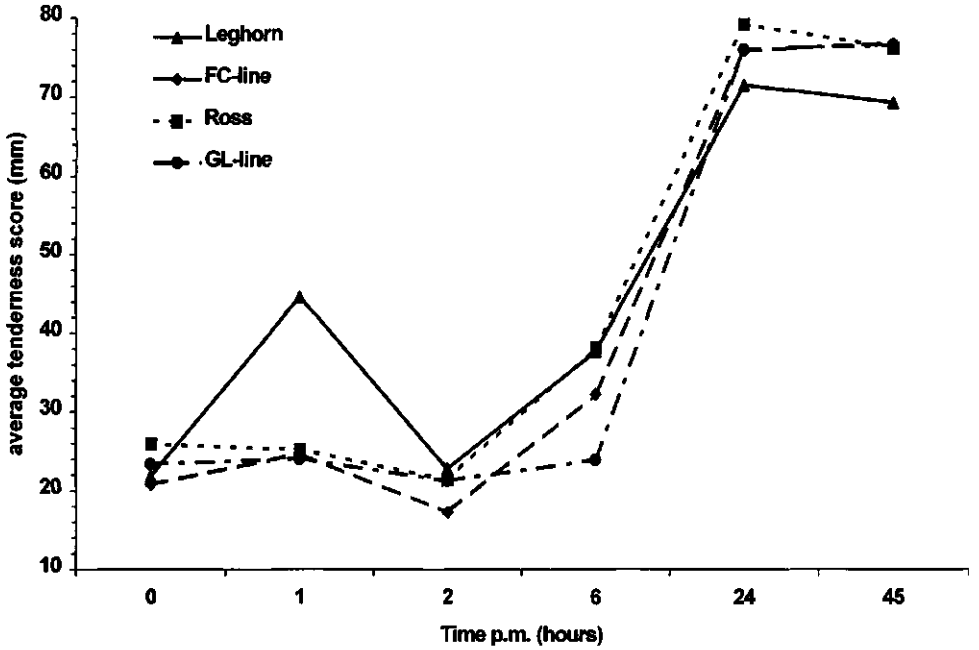

Figure 4 *Sensory tenderness: line scales measurements in breast muscles of different broiler lines at different times post mortem. Points are averages over total panel.*

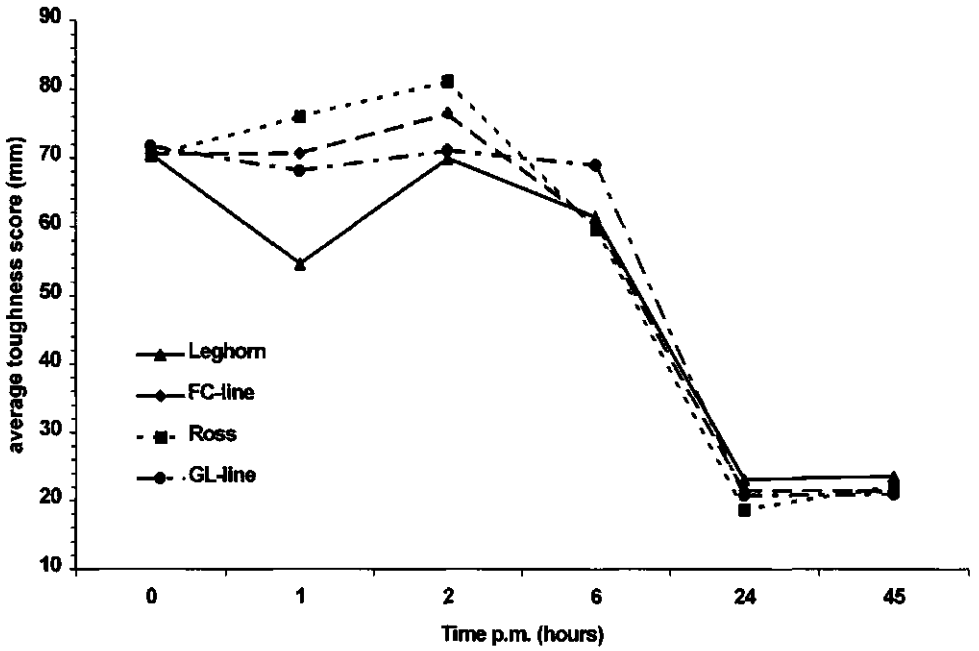

Figure 5 *Sensory toughness: line scales measurements in breast muscles of different broiler lines at different times post mortem. Points are averages over total panel.*

shows a marked increase, followed by a decrease at 2 hr .

Figure 5 shows the results of the line scales measurements for the term toughness averaged over panel members per line and time *post mortem*. The average toughness scores show a decrease at 24 and 45 hours and a marked decrease is seen for Leghorn ad 1 hour. These toughness scores are almost reciprocal to the tenderness scores, suggesting that the terms tender and tough show a close inverse correlation. This in turn means that line scales can run from very tender to not tender or reciprocally from not tough to very tough or from tender at one end to tough at the other end. The marked decrease in toughness and inversely the increase in tenderness in the samples at 24 and 48 hours compared to the samples at earlier times suggests that sensory tender- and toughness show the same relationship to rigor mortis development and post mortem proteolysis as the shear force measurements. However, the sensitivity of this sensory method is lower than the instrumental method due to the higher variance of the sensory measurements. Differences between lines cannot be detected with these measurements.

Figure 6 shows the results of the line scales measurements for the term juiciness averaged over panel members per line and time *post mortem*. Perceived juiciness is higher at 24 and 45 hours than at earlier deboning times, with the exception of Leghorn for which juiciness increases only marginally. Ross shows a low juiciness just after slaughter, higher scores at 1, 2 and 6 hours, and a dramatic decrease at 24 and 45 hours.

Figure 7 shows the results of the line scales measurements for the term dryness averaged over panel members per line and time post mortem. The dryness scores seem reciprocal to the juiciness scores. A clear increase in sensory dryness is noted at 24 and 45 hours, with the exception of the Leghorn samples. However, for perceived dryness in the three broiler lines, the increase at 24 and 48 hours is about double the increase of perceived juiciness. This indicates a difference in perception of the two terms by the panelists.

Table 1 presents the significance of ANOVA's performed on each attribute separately, with the two factors line, time post mortem and their interaction. It shows clearly that time has an effect on the sensory attributes, for tenderness the interaction of line and time was significant. An effect of line is not found in these results. The above mentioned reciprocity of tenderness/toughness and juiciness/dryness is reflected to some extent in the ANOVA results in table 1, be it with a borderline significance of the interaction term for tough

Sensory attribute	Line	Time p.m.	Interaction
Tender	0.174	< 0.001	0.001
Tough	0.268	< 0.001	0.060
Juicy	0.158	< 0.001	0.937
Dry	0.262	< 0.001	0.454

Table 1 *Significance levels of ANOVA of different meat quality attributes with line and time post mortem as effects.*

. The general increase in tenderness, respectively decrease in toughness, reflects the average changes in shear force quite well. However, the differences between lines as reflected in the results of the shear force measurement are not picked up by this sensory line scale method. The pairwise comparisons result in a better distinction between lines, while line scaling methods reflect changes in time more accurately.

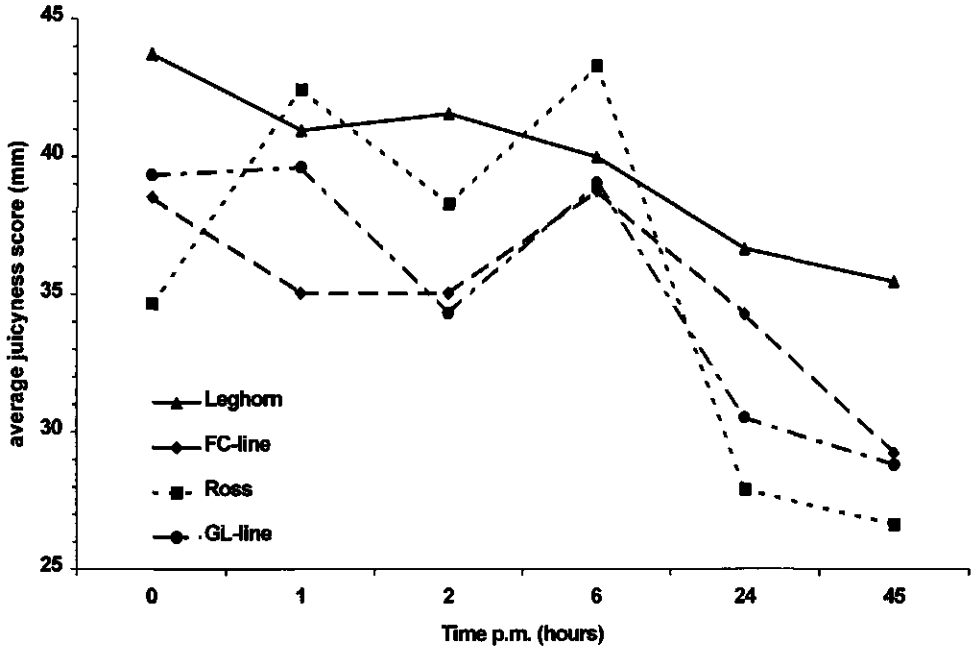

Figure 6 *Sensory juiciness: line scales measurements in breast muscles of different broiler lines at different times post mortem. Points are averages over total panel.*

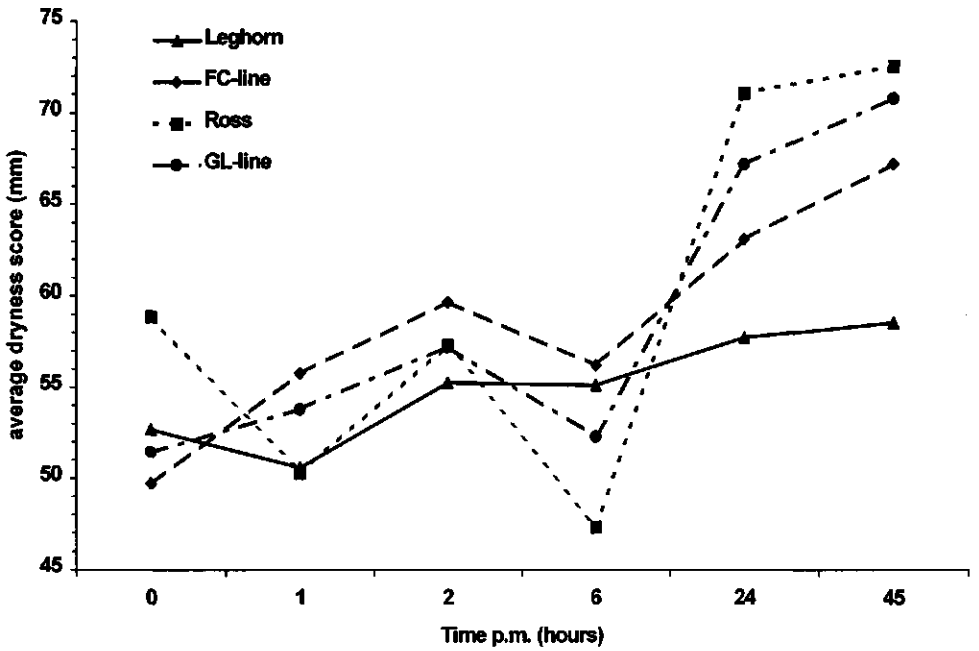

Figure 7 *Sensory dryness: line scales measurements in breast muscles of different broiler lines at different times post mortem. Points are averages over total panel.*

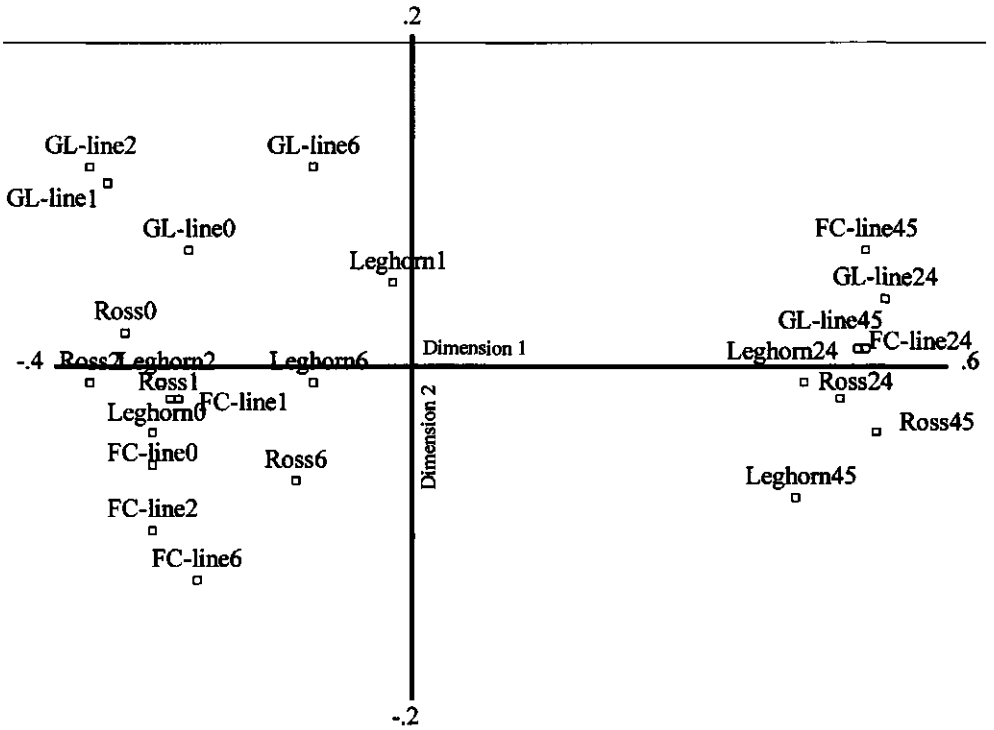

Figure 8 *GPA group average biplot showing the 4 lines by 6 times post mortem. The digits behind the line names, signal the elapsed time post mortem.*

Consistency of the sensory panel

The sensory panel is an important instrument in studying the perceived levels of tenderness, juiciness, etc. The results in the previous sections are based on scores averaged over panelists. In order to check the consistency of scoring of individual panelists, in addition to correcting for individual differences a Generalized Procrustes Analysis (GPA) (Gower 1975, Dijksterhuis & Punter, 1990; Dijksterhuis & Gower 1991; Dijksterhuis, 1995a,b; 1996; 1997) was carried out on the line scale scores of the individual panelists.

The plot in Figure 8 contains 64 and 2 percent variance accounted for in the 1st and 2nd dimension respectively, this is a highly one-dimensional result. It signals clear differences between the products, hence a homogeneous panel. Figure 8 shows two groups of 'products', at the left all the pre-rigor samples, 0 up to 6 hours *post mortem*, reside, at the right side all the post-rigor samples, 24 and 45 hours, are shown. The distinction along the first dimension axis in Figure 8 probably corresponds to the directions of increasing tenderness to the right and toughness to the left. The pre-rigor GL-line samples are situated higher in dimension 2 than the pre-rigor samples of the other three lines, while the FC-line pre-rigor samples show a trend towards lower dimension 2 values, although to a lesser extent than the GL-line samples. This signals some distinct sensory difference between lines, however, from these experiments no identification of the differences can be made. Possibly, a texture profile analysis or a free choice profiling experiment would make clear the precise sensory differences between lines. Post-rigor samples did not show distinct differences in the second dimension.

Figure 9 shows the directions of average tenderness scores for all individual panelists projected over the data as shown in figure 8. This figure also illustrates the reciprocal relation between tender and tough. These terms are clearly negatively correlated in the panelists' perception of the attribute. There is one panelist with a different perception of the term tough,

panelist 9, in both toughness directions, as is shows in the bottom panel of figure 9. Panelist 1 is a little different from the other panelists concerning tenderness perception, in the second (minor) tenderness dimension, as can be seen from the top panel of figure 9.

Figure 10 shows analogous pictures for the sensory attributes juicy and dry. Clearly, there is not as much agreement between panelists on the attributes juicy and dry as there is on tender and tough. The general direction of juicy seems to be to the left in juicyness dimension 1 (except for panelists 14, 1 and 7), and of dry to the right in dryness dimension 1 (except for panelists 7 and 1). There is no clear direction in either juicyness or dryness in the second attribute's dimension. The heterogeneous results of juicy and dry, when compared to tender and tough, may have resulted in the ANOVA of the former terms showing no significant results, and the lack of a clear trend in figures 3 respectively 6 and 7. This illustrates the importance of training, for a sensory panel.

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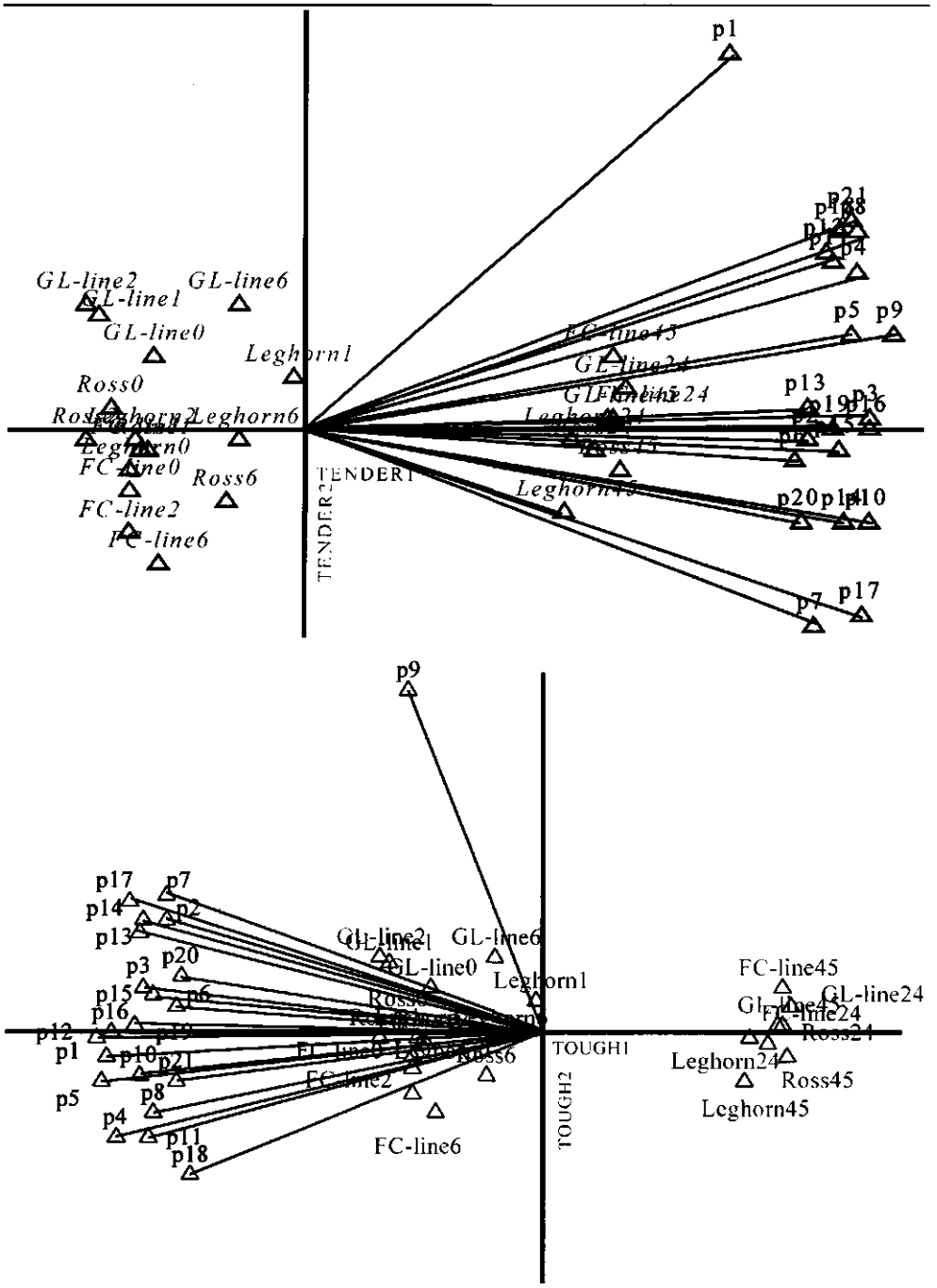

Figure 9 *Average individual panelists' directions of tenderness (top) and toughness (bottom) superimposed over biplot of figure 8. The number indicates individual panelist.*

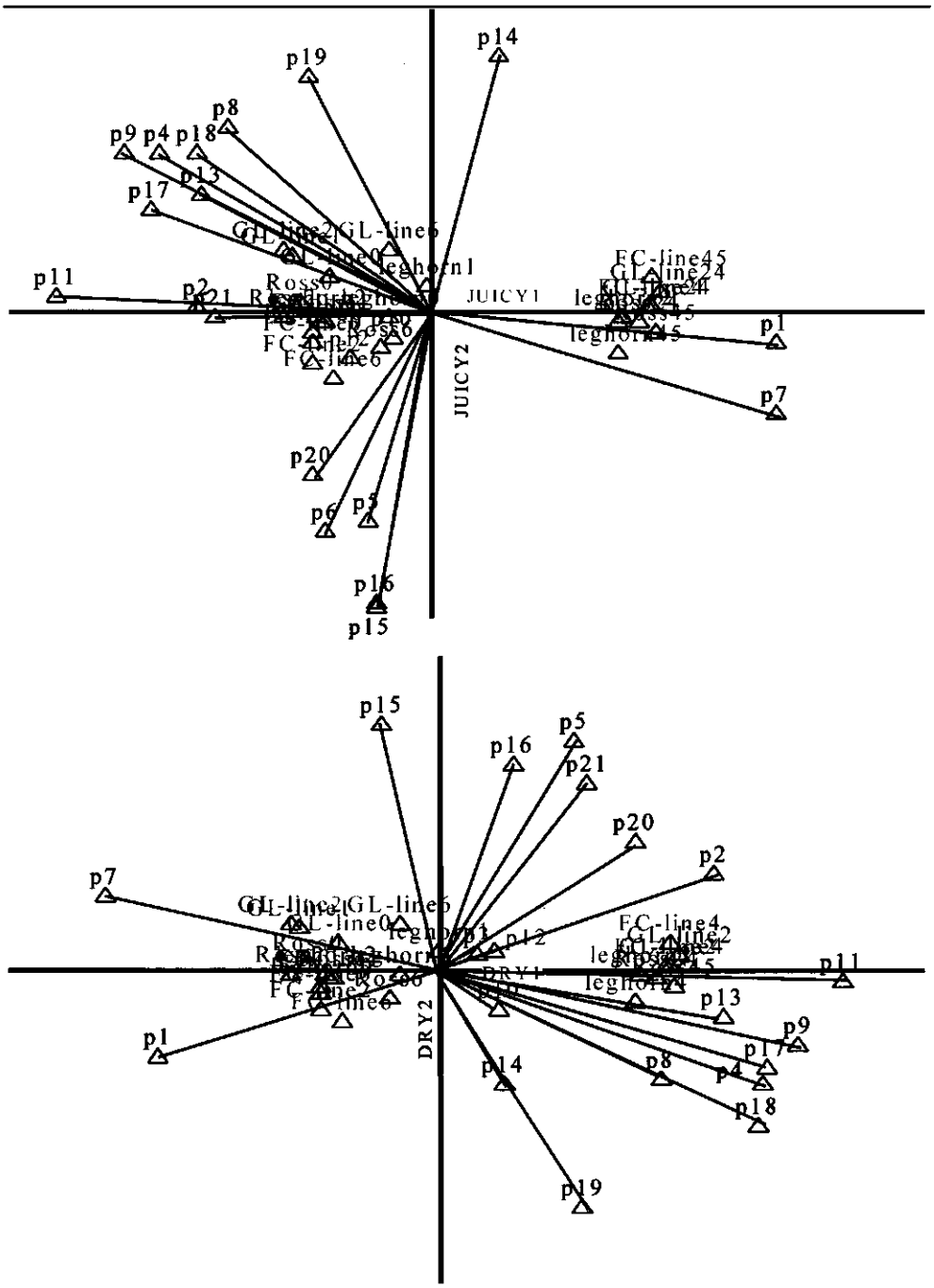

Figure 10 *Average individual panelists' directions of juiciness (top) and dryness (bottom) superimposed over biplot of figure 8. The number indicates individual panelist.*

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Chapter 6:

Post Mortem Development of Meat Quality as Related to Changes in Cytoskeletal Proteins of Chicken Muscles.

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ABSTRACT A procedure was developed, to analyze the degradation process of titin, nebulin, vinculin and desmin with high resolution in chicken breast and leg muscle during meat aging, by flat bed sodium-dodecyl-sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and subsequent detection by either a general protein stain or western blotting. Degradation of titin, nebulin, and desmin started at 3 h post mortem. Both in breast and leg muscle, titin and nebulin disappeared within one day. In leg muscle, nebulin seemed to be degraded slightly faster than in breast muscle. In breast muscle, both desmin and vinculin were not detectable after three days post mortem. Desmin degradation was substantially slower in leg than in breast muscle.

The changes observed in cooking loss and shear force were compared to myofibrillar fragmentation (MFI) and to changes in cytoskeletal proteins. The MFI, as developed for measurements in beef, was found to be unsuitable to measure myofibrillar degradation in chicken muscle. Degradation products of titin, nebulin and desmin were identified as potential markers for monitoring the development of meat aging.

(Key words: poultry meat aging, electrophoresis, SDS-PAGE, western blotting)

INTRODUCTION

The development of meat quality is dependent of the post mortem degradation of myofibrillar proteins. First studies of post mortem changes of myofibrillar proteins have been reported in the sixties and seventies (Davey & Gilbert, 1967; Stromer *et al.*, 1967; Hay *et al.*, 1973; Olson *et al.*, 1976, 1977; Penny, 1976; Samejima & Wolfe, 1976). Structural myofibrillar proteins are responsible for the integrity of muscular tissue (Robson & Huiatt, 1983). Meat aging is characterized by loss of this structural integrity, caused by the post mortem proteolytic degradation of these proteins (Young *et al.*, 1980; Ouali, 1990).

During post mortem aging of meat, the major contractile proteins of the muscle, actin and myosin, together comprising approximately 80% of the myofibrillar mass, seem not to be affected by proteolysis (Stromer *et al.*, 1974; Samejima & Wolfe, 1976; Yates *et al.*, 1983; Bandman & Zdanis, 1988). One of the most noticeable changes during post mortem aging is the disintegration of the sarcomeric Z-disks (Stromer *et al.*, 1967; Davey & Dickerson, 1970; Henderson *et al.*, 1970). The most important structural components involved in meat aging are considered to be the cytoskeletal proteins among which there are titin, nebulin, and desmin (Koohmaraie *et al.*, 1984; Fritz & Greaser, 1991, Robson *et al.*, 1995) and vinculin (Taylor *et al.*, 1995). Titin, also called connectin, is an extremely large monomerous protein of approximately 3000 kDa, found in striated muscle (Maruyama *et al.*, 1977, Wang *et al.*, 1979), that forms a set of longitudinal filaments, spanning from the M-line region to the Z-line (Robson *et al.*, 1995). Nebulin is considered to be a long molecule of molecular weight of approximately 800 kDa which runs parallel, and in close association with the thin filaments (Robson *et al.*, 1995). Both proteins, titin and nebulin, are largely responsible for the longitudinal integrity of the muscular fiber (Robson *et al.*, 1984). Desmin is a protein of approximately 53 kDa present in intermediate filaments connecting adjacent myofibrils at their Z-line levels (Robson *et al.*, 1995) and one of the many proteins composing the cell membrane's skeleton named costameres (Taylor *et al.*, 1995). Desmin is considered to be the most important protein responsible for lateral integrity of the muscle fiber (Robson *et al.*, 1984). Vinculin is one of the proteins composing the costamere structure and has a molecular weight of approximately 126 kDa (Taylor *et al.*, 1995). Vinculin belongs to a chain of proteins involved in the connection of the myofibrils to the sarcolemma and thus for the transfer of contraction forces of the myofibrils to the anchorsites of the muscle. The post mortem loss of longitudinal and lateral integrity of the muscular tissue as a result of proteolytic degradation of the cytoskeletal proteins resulting in tenderization of the meat (Ouali, 1990) is called aging.

The kinetics of the proteolytic degradation of these key cytoskeletal proteins is of crucial importance for meat quality development. Early studies of post mortem changes in the myofibrillar structure of bovine (Stromer *et al.*, 1967) and chicken (Hay *et al.*, 1973a) skeletal muscle used electron microscopy. Z-line degradation in the sarcomere of aging meat was discovered, however the way in which this degradation was related to changes in particular proteins composing the muscle could not be understood. After development of electrophoretic techniques such as sodium-dodecylsulphate-polyacrylamide gel-electrophoresis (SDS-PAGE), to separate and visualize individual myofibrillar proteins, it became possible to study the degradation of the structural proteins comprising the muscle fiber (Hay *et al.*, 1973b). In the eighties, most of the SDS-PAGE studies were focused on titin and nebulin and their breakdown (Lusby *et al.*, 1983; Paterson and Parrish, 1987). Because of the limited sensitivity and separation of proteins by SDS-PAGE, it was necessary to search for a new technique to detect proteins and their degradation products, present in the muscle in very low concentration.

Bandman and Zdanis (1988) successfully used western blotting to study protein breakdown in beef with antibodies to myosin and titin. This immunochemical method has also been used in studies on titin and nebulin breakdown in bovine meat by Fritz and Greaser (1991) and in studies on the breakdown of desmin in chicken muscle by Schreurs (1991). Taylor et al. (1995) studied meat aging in beef using Western blotting in combination with electron microscopy and demonstrated that the first changes occurring in post mortem muscle was disintegration of the N_2-line and of costameres, corresponding to degradation of proteins mainly constituting those structures i.e. titin, nebulin, desmin and vinculin.

Depending on the molecular weight of myofibrillar proteins, different percentages of total acrylamide and cross-linker have been used throughout literature, to produce gels of various porosity and with good separating capabilities. For the extremely large myofibrillar proteins like titin and nebulin, with a molecular weight of approximately 2800 and 800 kDa respectively, highly porous polyacrylamide gels have been utilized. These gels are extremely difficult to handle, especially during western blotting or staining, due to their fragile nature. The gels described in literature for the separation of these high molecular weight proteins, without exception, were of the 'vertical slab-gel type' (Studier, 1973; Porzio & Pearson, 1977; Paterson & Parrish, 1987; Fritz *et al.*, 1989; Granzier & Wang, 1993). Cytoskeletal proteins and some of their degradation products separated on these gels can be detected very sensitively and specifically by western blotting (Burnette, 1981), however, chicken myofibrillar proteins are very difficult to separate (Chou *et al.*, 1994). Generally, resulting gels prove to be of very low resolution.

Presently, little is known about the changes in cytoskeletal proteins of poultry meat. The purpose of this study was to investigate degradation processes of cytoskeletal proteins in chicken meat. Sample preparation, gel casting and Western blotting were optimized for chicken muscle tissue. Gels utilized were ultra-thin, highly porous polyacrylamide gels on a plastic backing film, to be used in a flat-bed gel-electrophoresis set-up. This procedure resulted in very high-resolution electropherograms for high molecular weight proteins. The procedure was adapted to medium molecular weight myofibrillar proteins like vinculin and desmin using precast, commercially available SDS-PAGE gels of 7.5 and 12.5 % total acrylamide, resulting in comparable high resolution.

In order to be able to relate the changes observed in the cytoskeletal proteins to meat quality characteristics, myofibrillar fragmentation as well as cooking loss and shear force was measured in both breast and leg muscles.

MATERIALS AND METHODS

Materials

Water was always Milli-Q quality. Standard laboratory chemicals were obtained from Merck and of analytical grade unless stated otherwise. Commercial antibodies against nebulin, desmin and vinculin, anti-mouse and anti-rabbit alkaline phosphatase conjugates, and β-galactosidase were obtained from Sigma. The polyclonal anti-titin antibody was produced in rabbits at the Institute for Animal Science and Health. Precast ExcelGel SDS Homogenous 7.5 % and gradient 8-18% gels, ExcelGel SDS buffer strips and Coomassie brilliant blue were obtained from Pharmacia. Acrylamide/Bisacrylamide and tetraethylenemethylene-diamine (TEMED) were obtained from BDH. Reinforced nitro-cellulose membranes, pore size 0.45 µm, for Western blotting were obtained from Schleicher and Schuell. Kerosene was obtained from SERVA.

Animals

Commercial broilers, brand Ross, at 6 weeks of age were obtained alive from a local slaughterhouse, transported to the institute in normal commercial broiler crates and subsequently slaughtered at the in-house processing plant of the Institute for Animal Science and Health. Birds were stunned in a waterbath stunner set at 100 Volts and 50 Hz for approximately 10 seconds after which the jugular vessels were severed with a rotating knife. Animals were neither scalded nor plucked, nor eviscerated. After approximately 3 minutes of bleeding, the birds were skinned and deboned. The 0 hour samples for electrophoresis were taken immediately after removal of the muscles and frozen in liquid nitrogen after which they were stored at –80 °C. The skinned breast and leg parts were wrapped in paper towels saturated with an antimicrobial solution (10 mM sodiumazide, 100 ppm chloramphenicol, 10 ppm riframycin) and stored in a cold room at 4°C for sampling at 1, 3, and 6 hours and further after 1, 2, 3, 5, and 7 days of post mortem storage. Samples for measurement of shear force and cooking loss were wrapped in plastic and stored at 4 °C for at least 24 hours. Samples for electrophoresis and western blotting as well as for measurement of the myofibrillar fragmentation index were taken repeatedly from 9 carcasses at the above mentioned times post mortem. The cooking loss and shear force measurements were carried out on 12 carcasses per sampling time (108 animals total)

SDS-PAGE

Sample preparation. Whole muscle samples for SDS-PAGE and Western Blotting were taken at the above times post mortem, frozen in liquid nitrogen and stored at –80 °C until analysis. At the day of analysis, the samples were again cooled in liquid nitrogen and subsequently pulverized using a liquid nitrogen-cooled mortar and pestle. The resulting powder was dissolved into a volume of sample buffer according to Fritz & Greaser (1991) containing 25 mM Tris-HCl pH=6.8, 8 M urea, 2 M thiourea, 3% (w/v) SDS, 75 mM dithiothreitol (DTT), and bromophenol blue, to make a protein concentration of 1 mg/ml. Samples were heated for 20 minutes at 50°C and subsequently cooled in tap water to room temperature and centrifuged at 12.000 x g for 5 minutes.

Polyacrylamide gel preparation. Commercially available precast ExcelGels (12.5 x 260 x 0,5 mm) with homogenous 7.5% and a gradient of 8 to 18% total acrylamide were used. For the separation of high molecular weight proteins like titin and nebulin, special gels of 3.2% total acrylamide (bisacrylamide:acrylamide, 1:37) were cast in gel cassettes 12.5 x 260 x 1.0 mm with sample wells of 5 x 5 x 1.0 mm (= 25 µl) containing a GelBond PAG plastic backing film obtained from Pharmacia. The casting solution was composed of 3.2 % Acrylamide, 0.08 % *N, N'*-methylene-bisacrylamide, 375 mM Tris.HCl pH 8.8, 0.1% SDS, 0.03 mg/ml ammonium persulphate and 0.4% TEMED. After polymerization and disassembly of the gel cassettes, the gel was covalently bound to the plastic backing film and could be easily removed from the gel casting moulds and positioned on top of the cooling plate of the Multiphor II electrophoresis unit, on a layer of kerosene for good thermal contact.

Electrophoresis. Flat bed SDS-PAGE was performed with a horizontal Pharmacia LKB Multiphor II System. Muscle samples were loaded on 7.5% and 12.5 % gels (10 µg of protein) and on 3.2% gel (20 µg of protein). On the 7.5% and 12.5% gels, several aliquots of a mixture of Pharmacia low molecular weight (LMW) and high molecular weight – SDS (HMW-SDS) calibrators were applied to estimate the molecular weight of the different bands. On the gels, used for subsequent western blotting, a mixture of the above mentioned standards was applied as well as a sample prepared from muscle tissue that was frozen immediately after death of the bird. As anodic and cathodic buffer, ExcelGel buffer strips (245 x 4,5 mm)

were used. The buffer system in the strips forms, together with the gel buffer, a discontinuous buffer system. High- and low- molecular weight standards from Pharmacia were used to identify the protein molecular weights. Electrophoresis was performed at constant current of 50 mA with the power supply set at a maximum voltage of 600 V and a maximum power of 30 W. The power supply was equipped with a volthour integrator and electrophoresis was extended until 500 volthours per gel had been recorded. The cooling equipment was set at 15°C.

Fixation and staining. Immediately after electrophoresis, the gels were immersed in a fixing solution (40% ethanol, 10% acetic acid) for 30 minutes. Subsequently the gels were incubated in staining solution (0.05% Coomassie brilliant blue R-250 in 25% ethanol, 8% acetic acid) for 30 minutes at 60 °C, after which the electropherograms were destained in de-staining solution (25% ethanol, 8% acetic acid) until a clear background was obtained. De-stained gels were soaked in preserving solution (10% acetic acid, 10% glycerol) after which they were dried covered with a clear cellophane preserving film.

Western blotting & detection

Immediately after electrophoresis as described above, gels were separated from the backing film and transferred to a nitro-cellulose membrane, soaked in transfer buffer (25 mM Tris - 192 mM glycine pH=8.3, 0.1% w/v SDS and 10% v/v methanol). Subsequently, the proteins were transferred from the gel onto the nitro-cellulose membrane by a semi-dry transfer method according to Pharmacia (1990) using a constant voltage setting 500 V for 120 min. Subsequently, the blotted membranes were incubated with blocking solution (Phosphate buffered saline (PBS) containing 0.1% Tween-20 and 5% non-fat dry milk) for 30 min at 37°C in order to saturate the protein binding places on the nitro-cellulose. The saturated membranes were subsequently immersed in diluted primary antibody in incubation buffer (PBS containing 0.1% Tween-20 and 0.1% non-fat dry milk) and incubated overnight at 4 °C in a moist environment. Antibodies and dilutions used were:

- Rabbit polyclonal antibody raised against titin at the Institute for Animal Science and Health diluted 1:2500
- Rabbit polyclonal antibody to desmin, Sigma D-8281, diluted 1:400
- Mouse monoclonal antibody to nebulin, Sigma N-9891, diluted 1:400
- Mouse monoclonal antibody to vinculin, Sigma V-9131, diluted 1:200

After the overnight incubation, the membranes were washed 3 times with incubation buffer to remove excess antibody. Subsequently, the membranes were incubated with secondary antibody alkaline phosphatase conjugate (either anti-rabbit (Boehringer Mannheim No 1214-632; diluted 1:2000) or anti-mouse (Sigma A-3688; diluted 1:1000)) for 3 hours at room temperature. Detection of the alkaline phosphatase and thus of the antigen-antibody complexes was carried out using a 0,45 mg/ml solution of 5-bromo-4chloro-3-indolyl phosphate/ nitro blue tetrazolium (Sigma No. B-5655) chromogenic substrate, during 5 minutes. The reaction was stopped by quickly rinsing the membrane in water.

Densitometry

The gels and the blots were scanned on a Hewlett Packard 6100C tabletop scanner with 400 dots per inch which is comparable to a resolution of 63.6 μm. The image scans were subsequently processed as densitometer traces on a tabletop computer equipped with the Quantiscan program from Biosoft. From the analysis of the lanes containing the calibrator samples, a standard molecular weight calibration curve was prepared. For analysis of the high molecular weight proteins titin and nebulin, a molecular weight calibration curve was prepared using the

bands appearing in the fresh muscle sample lane. For this purpose, molecular weight of intact titin was set to 3000 kDa, intact nebulin to 800 kDa and myosin heavy chain to 212 kDa.

Myofibrillar fragmentation index (MFI).

Repeated MFI measurements were carried out according to Olson et al (1976).

Cooking loss and shear force measurements

The breast halves and legs designated for shear force measurement were weighed, vacuum sealed and subsequently heated to a core temperature of 85 °C in a boiling water bath. Immediately after heating the samples were brought back to 25 °C in a water bath. Before shear force measurement the samples were removed from the sealing bag and blotted dry with a paper towel. Subsequently the samples were weighed again and the weight differences before and after heating were recorded as cooking loss.

Shear force measurements were carried out on the *M. Pectoralis major, M Semitendinosus, M. Biceps femoris* and *M. Quadriceps femoris* according to Froning and Uijttenboogaart (1986). Corrected shear force values, adjusted according to thickness, were calculated according to Pool and Klose (1969).

Statistical analysis

Repeated measures ANOVA was carried out on the MFI data using the general linear models module of the SPSS/Windows® statistical package. For the cooking loss and shear force measurements, the simple one way ANOVA was used.

Correlation coefficients of the relationships of MFI, cooking loss and shear force with the optical density classification of the different protein bands on the western blots was carried out with the Spearman rank correlation module after the classification were converted to scores in the following manner: - = 0; ± = 1; + = 2; ++ = 3; +++ = 4.

RESULTS AND DISCUSSION

Typical SDS-PAGE and Western blotting results are shown in figures 1 through 7. Figures 1, 2 and 3 show results of SDS-PAGE of meat proteins on 3.2%, 7.5 % and 12.5% total acrylamide gels respectively. Figures 4 and 5 show results of Western blots from 7.5% total acrylamide gels with electropherograms of meat proteins developed with polyclonal anti-titin and monoclonal anti-nebulin antibodies respectively. Figures 6 and 7 show results of western blots from 12.5% total acrylamide gels with electropherograms of meat proteins developed with polyclonal anti-desmin and monoclonal anti-vinculin antibodies. Table 1 shows the results of the semi-quantitative scoring of the banding patterns as will be discussed below.

The migration of different proteins was comparable to electrophoretic patterns as shown earlier for bovine muscle (Wang, 1982). All gels and blots showed an increasing degradation of all proteins analyzed during aging of chicken muscle. Additionally, two well-known changes were observed in the 7.5% and 12.5% total acrylamide gels (figures 2 and 3). The widely reported appearance of a 30 kDa component as well as the disappearance of the α-actinin band with a concomitant increasing amount of a 98 kDa component, also observed by Koohmaraie et al. (1984), and Hwan and Bandman (1989) can be clearly seen in these gels.

Analysis of titin degradation was carried out on the basis of SDS-PAGE with a 3.2% gel (figure 1), a 7.5% gel (figure 2) and Western blotting (figure 4). Both in breast as well as in leg meat the results from the 3.2% SDS-PAGE gel at death showed that titin was visible as a closely-spaced doublet T_1 and T_2. This is in agreement with other studies (Wang, 1982; Kimura et al., 1984). With increasing time post mortem, the amount of titin T_1 decreased and simultaneously the amount of titin T_2 increased in all analyzed samples (Fig.2). In breast meat, this phenomenon was most noticeable 6 hours, when the T_2 band was much more

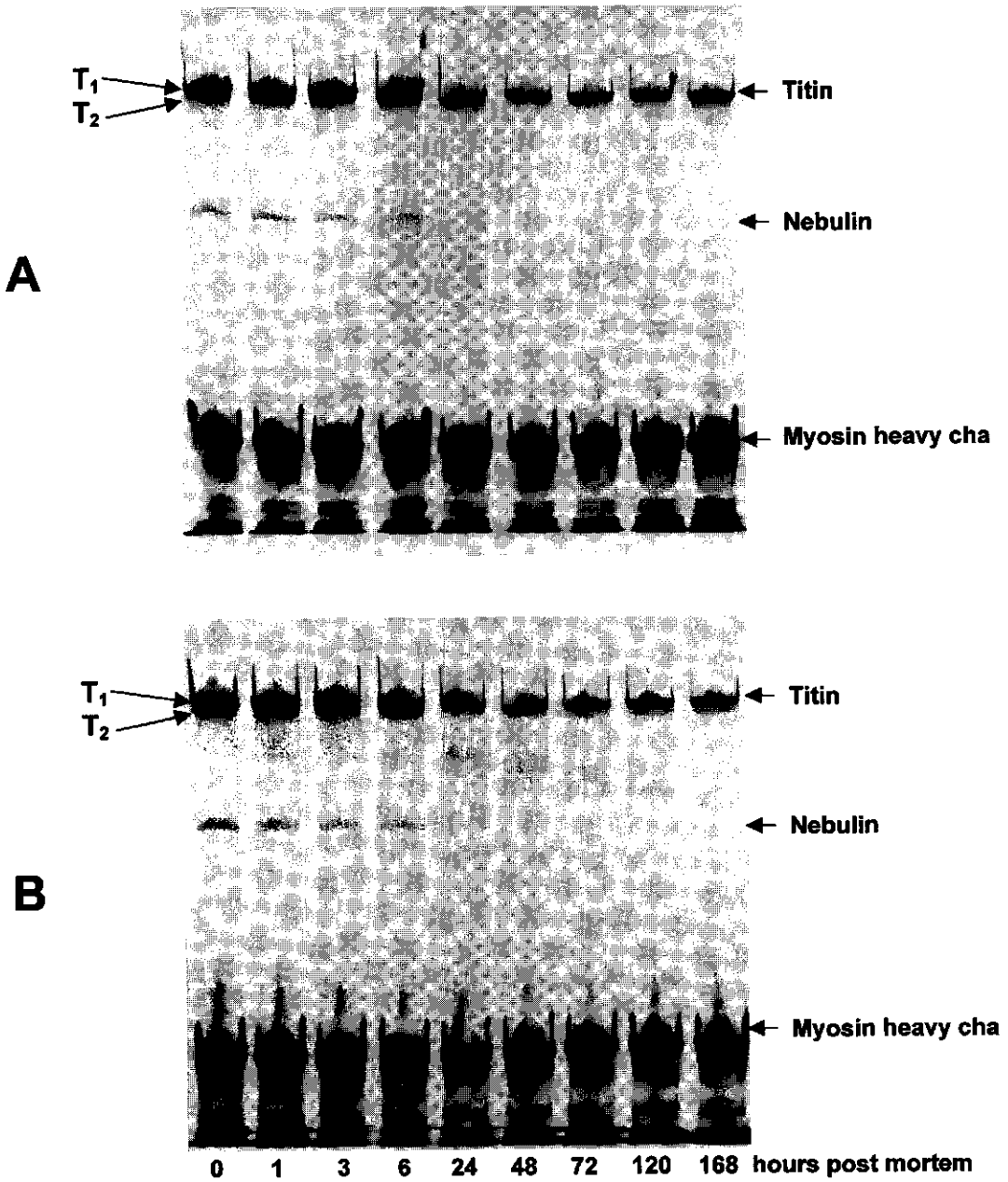

Figure 1 SDS-PAGE of myofibrillar proteins at different times post mortem on 3.2% polyacrylamide gels. A: breast muscle; B: leg muscle.

	Fragment	Mw	Time post mortem (hours)								
			0	1	3	6	24	48	72	120	168
Breast	Titin	2800	++	++	++	++	++	++	++	++	+
	Tba	1700	+	++	++	++	+++	+++	+++	++	+
	Tbb	1400	-	-	-	-	-	-	±	++	++
	Tbc	1100	+	++	++	++	+	±	-	-	-
	Tbd	900	+	+	++	++	++	+++	++	++	+
	Tbe	700	-	±	+	+	++	+++	++	+++	+
	Desmin	53	+++	+++	+++	+++	++	+	±	-	-
	Da	47	-	-	±	+	++	++	+	±	+
	Db	40	-	-	-	±	+	+	++	+	+
	Dc	30-37	-	-	-	-	±	±	+	+	+
	Nebulin	800	++	++	++	+	+	-	-	-	-
	Nba	500	-	-	-	±	±	+	++	±	±
	Nbb	400	-	-	-	-	-	-	±	+	+
	Vinculin	126	±	+	+	+	+	+	±	-	-
Leg	Titin	3000	+	++	++	++	+	+	+	+	±
	Tla	1900	++	++	++	++	++	+	+	+	+
	Tlb	1400	-	-	-	-	±	+	+	+	+
	Tlc	1100	±	±	±	±	±	+	+	+	±
	Tld	900	-	±	±	±	+	+++	+++	+++	+++
	Tle	700	±	+	+	+	+	++	++	++	++
	Tlf	500	-	-	-	±	±	+	+	+	+
	Desmin	53	+++	+++	+++	+++	+++	++	+	±	±
	Da	47		±	±	±	±	+	+	+	±
	Db	40	-	-	-	-	-	+	+	+	±
	Dc	30-37	-	-	-	-	-	±	+	+	+
	Nebulin	800	+++	++	++	+	+	-	-	-	-
	Nla	500	-	-	-	-	±	+	±	+	±
	Nlb	400	-	-	-	-	-	-	±	±	±
	Vinculin	126	++	++	++	++	++	++	+	+	±

Table 1 Relative optical densities of bands on western blots.

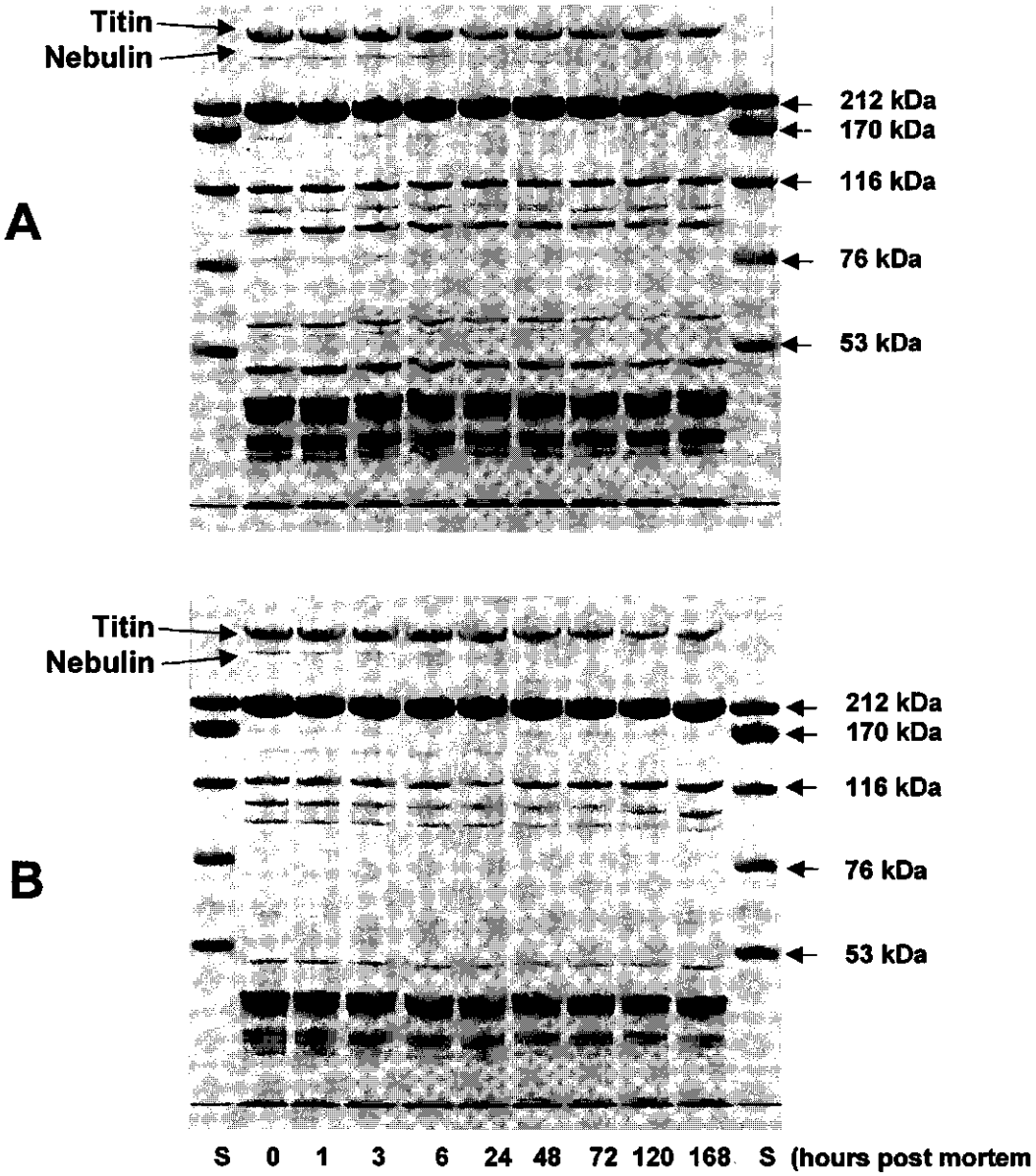

Figure 2 SDS-PAGE of myofibrillar proteins at different times post mortem on 7.5% polyacrylamide gels. A: breast muscle; B: leg muscle; S: molecular weight standards.

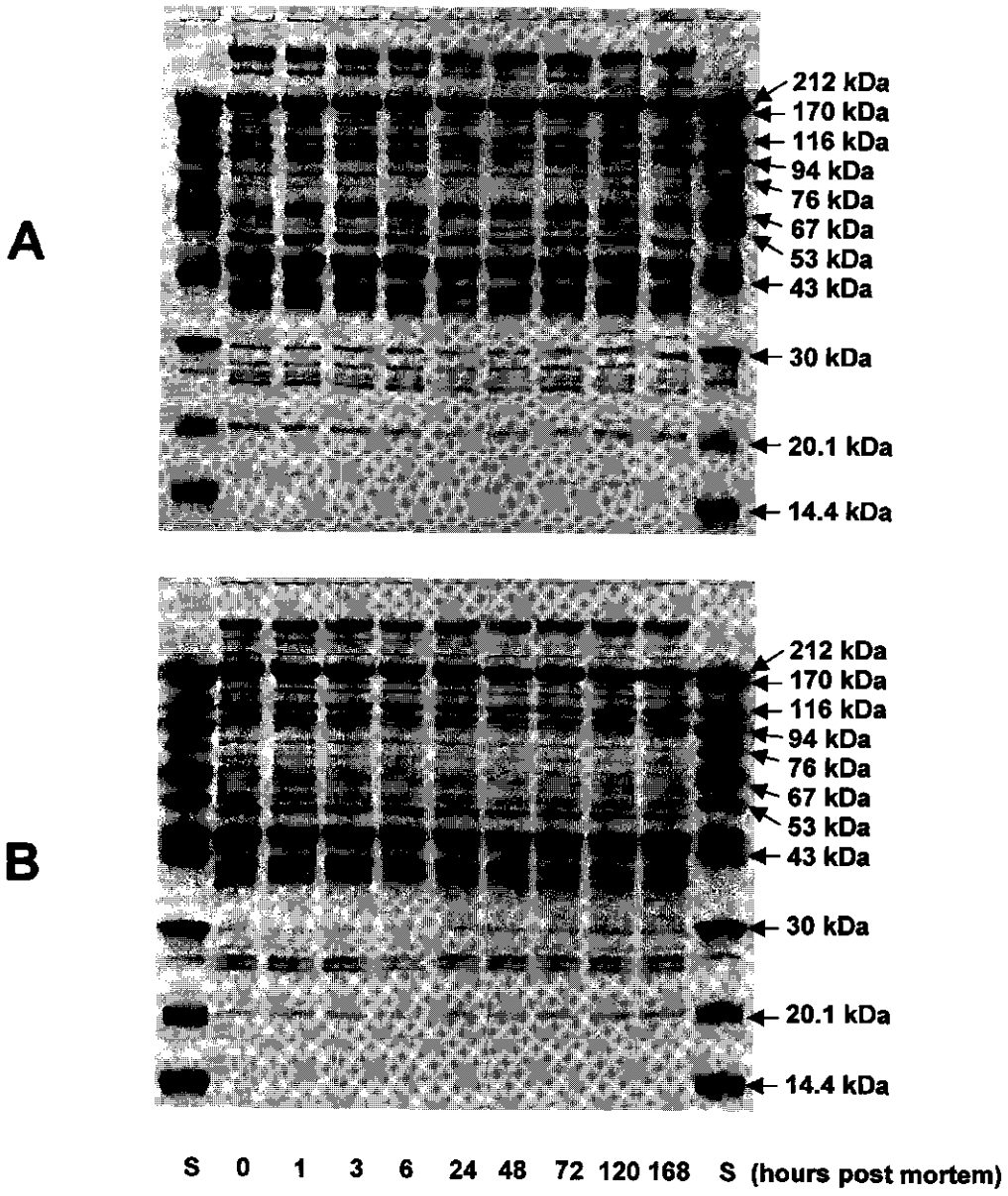

Figure 3 SDS-PAGE of myofibrillar proteins at different times post mortem on 12.5% polyacrylamide gels. A: breast muscle; B: leg muscle; S: molecular weight standards.

intensive than in the 0-time sample. This suggests that the T_2 was raised from the degradation of T_1. The T_1 to T_2 conversion was completed in all 24 hours samples, when the T_1 was no longer detectable while T_2 remained present for 7 days. The leg muscle samples show a comparable conversion of T_1 into T_2. Although the same amounts of protein were applied to the gels as with the breast muscle samples, the leg muscle samples show more, smaller degradation products from titin, appearing as faint banding patterns between titin and nebulin, than the breast muscle samples.

Western blotting analysis with polyclonal antibodies to titin was used to detect the degradation products of titin. On the titin blot (figure 4) of the breast muscle, five clearly visible degradation products of titin were identified, referred to as Tba, Tbb, Tbc, Tbd and Tbe respectively. Components Tba, Tbc, Tbd and Tbe with a molecular weight of approximately 1700,

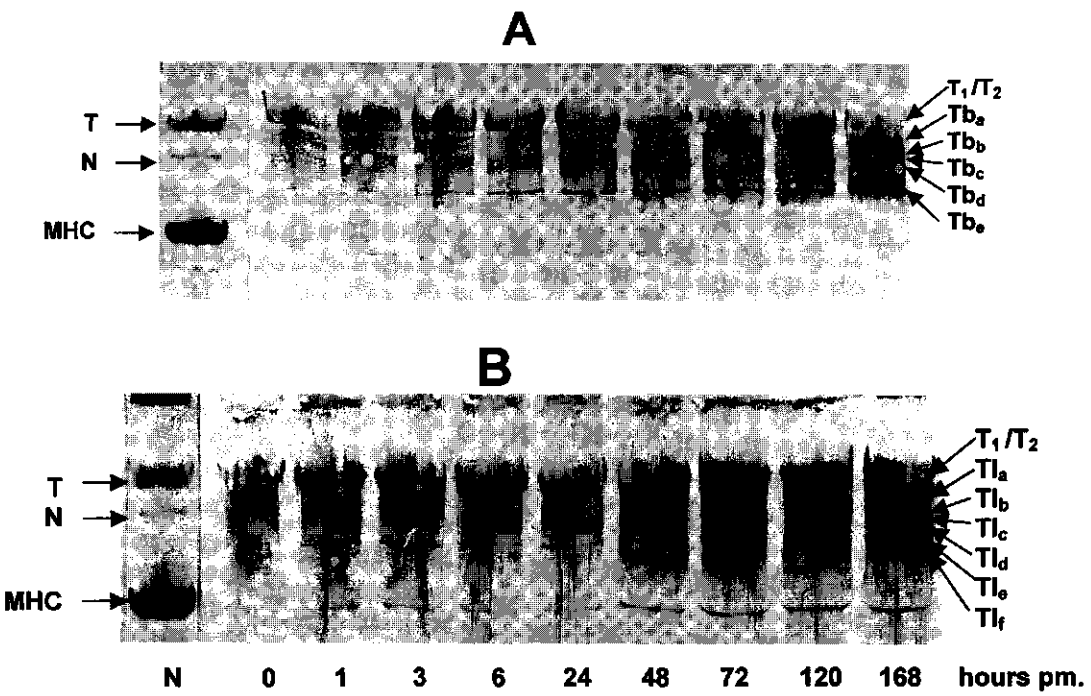

Figure 4 *Western blotting of myofibrillar proteins at different times post mortem using polyclonal anti titin antibodies. A: breast muscle; B: leg muscle; N = total protein stain of muscle at 0 hours post mortem; T = titin (3000 kDa); N = nebulin (800 kDa); MHC = myosin heavy chain (212 kDa).*

1100, 900 and 700 kDa respectively, already appeared early post mortem as very faint bands. The relative amounts of these components increased gradually until the second day, when the highest concentration was noted except for Tb_c of which the largest relative amounts were present during the first day after slaughter. The fifth component Tb_b, with a molecular weight

of approximately 1400 kDa, appeared after 3 days. In leg muscle a more complex pattern of titin degradation products appeared with some very distinct bands. At least 5 bands could be identified, designated Tl_a, Tl_b, Tl_c, Tl_d, Tl_e and Tl_f with approximate molecular weights of 1900, 1400, 1100, 900, 700 and 500 kDa respectively. The Tl_a as well as the intact titin band gradually decreased during the measuring period. The lower molecular weight bands Tl_c, Tl_d, Tl_e and Tl_f all gradually increase until an intensity plateau is reached after approximately 72 hours. No disappearance of degradation bands is observed within 7 days with exception of the Tl_c band which was faintly present from 0 hours on and increasing in optical density during aging but almost disappeared in the 7 days sample. The Tl_b component appeared in the 24 hours samples and stayed as a faint band up to 7 days post mortem. The presence of 1700 and 400 kDa degradation bands in chicken breast muscle was reported earlier (Kimura *et al.*, 1984), the first probably corresponding with the Tb_a band. Chou *et al.* (1994) identified a 1200 kDa component in chicken leg meat but not in breast meat. In our work a corresponding 1100 kDa band was identified in both muscle types. Tanabe et al. (1992) suggested that this component originates from T_1, and is composed of the Z-line side part of the T_1 molecule.

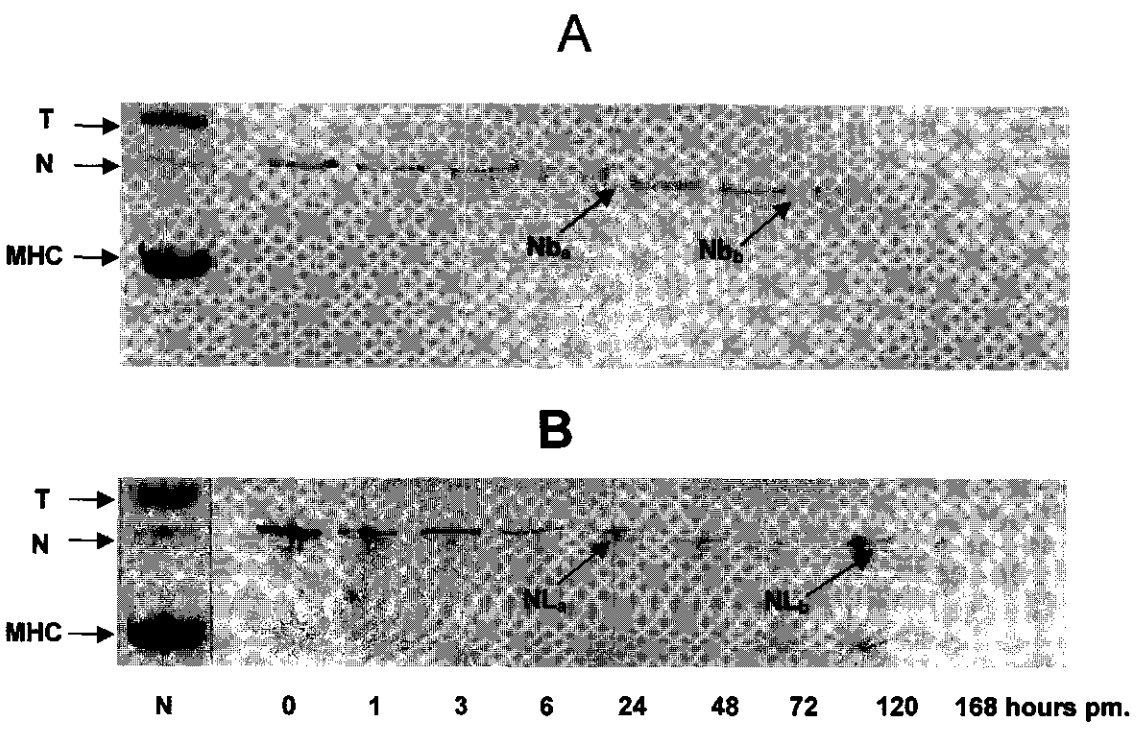

Figure 5 *Western blotting of myofibrillar proteins at different times post mortem using monoclonal anti nebulin antibodies. A: breast muscle; B: leg muscle; N = total protein stain of muscle at 0 hours post mortem; T = titin (3000 kDa); N = nebulin (800 kDa); MHC = myosin heavy chain (212 kDa).*

SDS-PAGE and Western blot results prepared from 7.5% gels with monoclonal antibodies to nebulin (figure 5), demonstrated that in breast muscle the degradation process of nebulin

started after 3 hours as a little, diffuse smearing in the nebulin band and became more intense with increasing storage time. After 6 hours post mortem cleavage of nebulin into two bands, intact nebulin with an apparent molecular weight of approximately 800 kDa, and an immunoreactive degradation product with an apparent molecular weight of approximately 500 kDa, designated Nb_a, occurred. After 72 hours post mortem a smaller degradation product, designated Nb_b, occurs on the blots with a molecular weight of approximately 400 kDa. After 5 and 7 days these two bands remained visible. In leg muscle, after 6 hours only a small fraction of the original intact nebulin is left. After 24 hours most of the nebulin is degraded into a smaller cleavage product, designated Nl_a, with a molecular weight of 500 kDa. Within 72 hours post mortem, virtually all nebulin is degraded into smaller, no longer immunoreactive, fragments. Only small amounts of two fragments, the Nl_a fragment and a new cleavage product designated Nl_b with an apparent molecular weight of approximately 400 kDa, remained visible. On some blots, the latter proteolytic fragment appeared as a closely spaced doublet.

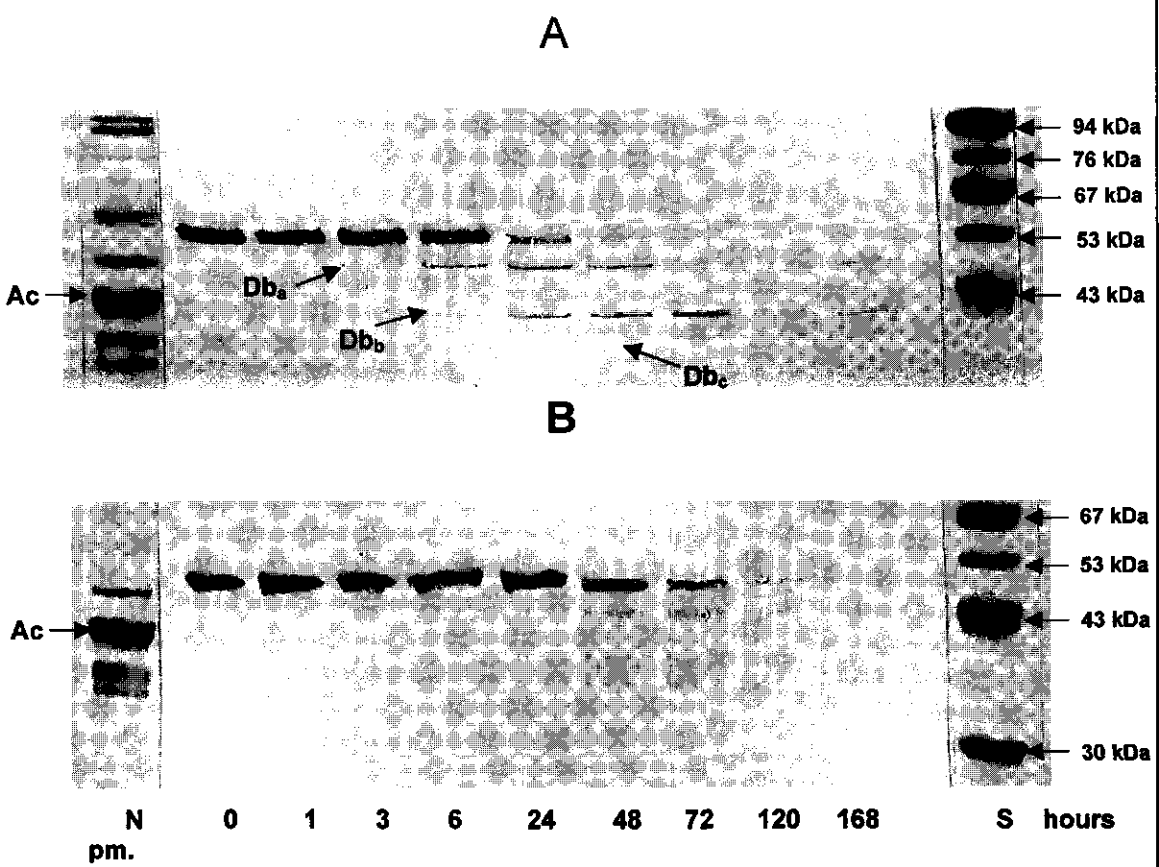

Figure 6 *Western blotting of myofibrillar proteins at different times post mortem using polyclonal anti desmin antibodies. A: breast muscle; B: leg muscle; N = total protein stain of muscle at 0 hours post mortem; Ac = actin (43 kDa); S = molecular weight calibrators.*

From the blots it can be concluded that the breakdown of nebulin was most remarkable between 3 and 48 hours, when intact nebulin was degraded and disappeared. This fact is con-

sistent with previous reports, where degradation of nebulin was analyzed only by SDS-PAGE (Paxhia and Parrish, 1988; Chou *et al.*, 1994).

Figure 6 shows the results of western blotting with polyclonal antibodies to desmin. The results showed that in breast muscle, the degradation process of desmin started within 3 hours of storage. Below desmin, a faint band of approximately 47 kDa appeared, referred to as component Db_a. After 6 hours of aging a new band was visible on the blot, designated Db_b with a molecular weight of approximately 40 kDa. These two bands remained very strong until the second day. After 7 days Db_a and Db_b were still visible as faint bands.

After 24 hours, not very well defined cleavage products in the area of 30-37 kDa became visible on the blots, here referred to as component Db_c. The most remarkable degradation of desmin in breast muscle occurred between 6 and 72 hours post mortem. After this time, intact desmin had disappeared completely. In leg muscle, the degradation of desmin was much

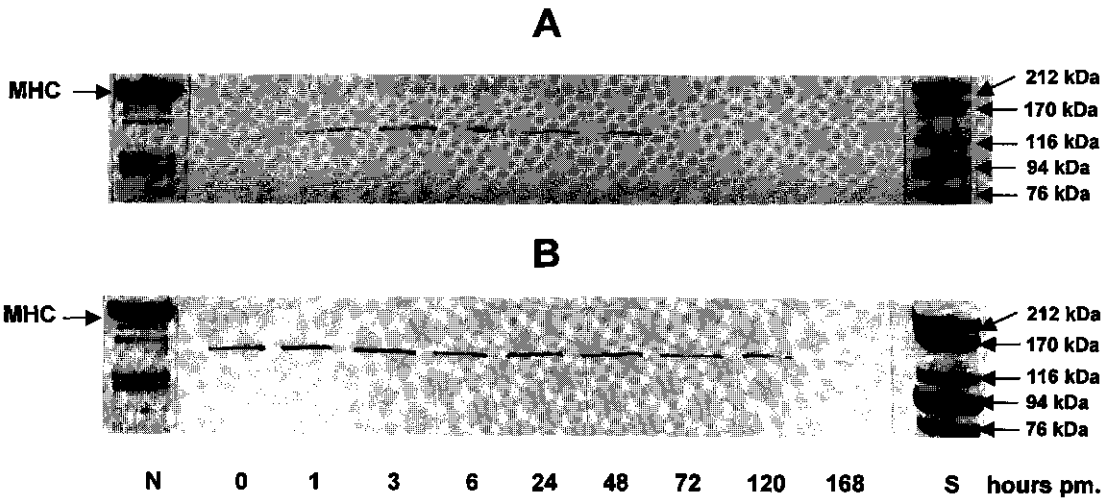

Figure 7 *Western blotting of myofibrillar proteins at different times post mortem using monoclonal anti vinculin antibodies. A: breast muscle; B: leg muscle; N = total protein stain of muscle at 0 hours post mortem; MHC = myosin heavy chain (212 kDa); S = molecular weight calibrators.*

slower. Only after 24 hours, very faint degradation was visible. After 48 hours the density of the native molecule starts to faint and clearly defined degradation products start to occur. The degradation pathway seems to be equal to breast muscle although much retarded. In other studies of desmin degradation by Western blotting analysis, bovine meat was used (Hwan & Bandman, 1989; Taylor *et al.*, 1995). It has been established that degradation of desmin in bovine Semimembranosus muscle, a fast aging muscle type, occurred between 24 and 72 hours, while in bovine Biceps femoris, a slow aging muscle type, the degradation of desmin was much slower.

Degradation of vinculin had completed in the breast muscle within 3 days post mortem, when intact vinculin was invisible on western blots (Figure 7). In the studies on vinculin degradation, it was difficult to establish the time when degradation started due to the use of a monoclonal antibody. The antibody is too specific to be able to pick up degradation products. In leg muscle, degradation of vinculin was much slower, as is shown by the intact molecule

still being present vaguely after 7 days post mortem. Comparison of chicken muscle with the fast aging bovine Semimembranosus muscle (Taylor *et al.*, 1995), showed that vinculin disappeared within the same time.

In conclusion it can be stated that on the titin and desmin blots, the intact protein molecule and, more and more immunoreactive degradation products were visible, progressing in time post mortem. The monoclonal antibodies against nebulin also recognized some breakdown, as is shown by the decrease in intact protein. The degradation pathway of nebulin generates some degradation products that are still recognized by the monoclonal antibody. The monoclonal anti-vinculin antibody was not capable of detecting degradation products of the native protein. This means that, in order to detect degradation products, use of polyclonal antibodies remains to be preferred.

Desmin and vinculin bands are not made visible on the gels by Coomassie staining. Desmin constitutes about 0,18% of skeletal muscle protein (Pearson & Young, 1989). Vinculin has been calculated to comprise about 0,005% of breast muscle proteins (Maruyama, 1985). Both these proteins are easily visualized on western blots.

Time post mortem (hours)	MFI	
	Breast (n=20)	Leg (n=20)
0	30.0 ± 7.9^{a}	18.0 ± 3.7^{a}
1	35.3 ± 9.0^{b}	20.5 ± 3.6^{a}
3	40.6 ± 10.7^{c}	23.3 ± 3.5^{b}
6	52.6 ± 9.1^{d}	25.1 ± 3.3^{bc}
24	62.8 ± 6.3^{e}	26.2 ± 4.5^{cd}
48	66.3 ± 4.8^{e}	30.7 ± 5.7^{e}
72	66.0 ± 5.0^{e}	30.1 ± 2.3^{e}
120	65.6 ± 6.8^{e}	28.7 ± 3.8^{de}
168	62.9 ± 9.4^{e}	33.5 ± 4.7^{f}

Table 2 *Changes in myofibrillar fragmentation index (MFI) with time post mortem.*
Group averages and standard deviations are shown.
Group averages with different superscripts differ significantly within column ($p<0.05$).

Table 2 shows the results of the measurement of the myofibrillar fragmentation with time post mortem. Both in breast and leg muscle, the MFI increases during post mortem aging until after 24 hours it reaches plateau values. The values reached in leg muscle are approximately half of the values reached in breast muscle. This indicates a similar rate of myofibrillar degradation in leg and breast muscle. The differences in absolute height of the MFI indicates a scatter behavior of breast muscle myofibrils, different from the scatter behavior of leg muscle myofibrils. These results are inconsistent with the results of the SDS-PAGE and western blotting experiments and results reported in literature. In the present study, the course of the myo-

fibrillar fragmentation is not in compliance with the rate of degradation of desmin, titin, and nebulin. In beef, the MFI increases much faster in predominantly white than in red muscles (Olson *et al.*, 1976). Ultramicroscopic changes related to post mortem proteolytic degradation evolve much faster in chicken breast muscle than in leg muscle (Hay *et al.*, 1973). In chickens, proteolytic processes, studied by SDS-PAGE, proceed much slower in leg than breast muscle (Chou *et al*,.1994). This suggests that the estimation of myofibrillar degradation by means of measurement of the light-scattering behavior of standardized suspensions of myofibrils as originally developed for beef muscle (Olson *et al.*, 1976; Olson & Parrish, 1977; Culler *et al.*, 1978), is not a suitable method to be used in chicken muscular tissue. In literature, alternative methods for the measurement of the MFI in chicken muscle tissue have been described using microscopic estimation of the degree of myofibrillar fragmentation (Takahashi *et al.*, 1967; Fukazwa *et al.*, 1969; Sayre, 1970), but these methods are too laborious to be used in large experiments.

Time *post mortem* (hours)	Cooking loss (%)	
	Breast (n=20)	Leg (n=20)
0	4.8 ± 1.0^{a}	8.7 ± 0.9^{a}
3	6.2 ± 1.1^{b}	9.8 ± 1.7^{b}
6	6.9 ± 0.8^{bc}	11.5 ± 1.9^{c}
24	7.2 ± 1.0^{c}	8.2 ± 1.3^{ad}
48	8.8 ± 1.6^{d}	7.4 ± 1.2^{de}
72	8.8 ± 1.6^{d}	6.9 ± 1.4^{e}
120	9.5 ± 2.0^{d}	7.9 ± 1.5^{ad}
168	9.3 ± 1.8^{d}	8.0 ± 1.5^{ad}

Table 3 *Cooking loss of breast and leg meat with time post mortem. Group averages and standard deviations are shown. Group averages with different superscripts differ significantly within column ($p<0.05$).*

Table 3 shows the results of the cooking loss measurements with time post mortem. The cooking loss observed in breast muscle gradually increases with time. In leg muscle, cooking loss first increases quickly, but after 6 hours, it decreases again. The course of the cooking loss in leg muscle suggests a relationship with rigor mortis development (this thesis, chapter 4). However, the gradual increase of cooking loss in breast muscle suggests that there is a relationship between cooking loss and proteolytic degradation of myofibrillar proteins. It is generally accepted that water-holding capacity of meat is correlated to pH. In the area of practical interest in meat, pH 5.0 – 6.5, any alteration of pH has a great influence on the water-holding capacity of the myofibrillar proteins (Pearson & Young, 1989). However, since the pH decline, as well as other rigor mortis related factors, do not play a role in post rigor

Time post mortem (hours)	Shear force (N/m^2)			
	M. Pectoralis major (n=24)	*M. Biceps femoris* (n=24)	*M. Quadriceps femoris* (n=24)	*M. Semitendinosus* (n=24)
0	35.8 ± 5.3^{c}	$78,5 \pm 11.1^{c}$	116.9 ± 18.0^{a}	83.0 ± 19.0^{b}
3	46.6 ± 9.0^{b}	$104,5 \pm 18.9^{a}$	92.6 ± 9.8^{b}	91.9 ± 19.4^{a}
6	93.3 ± 23.2^{a}	$89,5 \pm 13.5^{b}$	95.7 ± 14.5^{b}	71.8 ± 19.2^{c}
24	17.9 ± 6.8^{d}	$45,4 \pm 6.8^{d}$	56.9 ± 12.1^{c}	41.4 ± 8.6^{d}
48	15.8 ± 3.8^{d}	$44,3 \pm 6.1^{d}$	39.1 ± 6.7^{d}	34.7 ± 6.7^{de}
72	16.3 ± 5.3^{d}	$37,5 \pm 5.7^{e}$	35.4 ± 6.0^{de}	28.5 ± 6.0^{ef}
120	15.6 ± 3.0^{d}	$34,3 \pm 6.7^{ef}$	32.7 ± 6.4^{de}	27.2 ± 3.8^{ef}
168	14.3 ± 3.3^{d}	$30,0 \pm 6.1^{f}$	29.9 ± 7.1^{e}	24.6 ± 5.5^{f}

Table 4 Shear force in different muscles with time *post mortem*.

Group averages and standard deviations are shown.

Group averages with different superscripts differ significantly within column.

meat, the gradual increase of cooking loss has to be explained by some other mechanism. The degradation of the myofibrillar structure may play a role in the increase in cooking loss.

Table 4 shows the results of the shear force measurements in different muscles with time post mortem. It is clear that breast muscle the course of the shear force is different from that in the leg muscles investigated. Within 24 hours, breast muscle exhibits its final shear force, however, the maximum shear force is reached after 6 hours. The leg muscles shear force proceeds much slower, but its maximum is reached earlier, before 3 hours. This indicates a faster rigor development in leg muscle than in breast muscle but a slower post mortem proteolytic degradation in the three leg muscles investigated. This is consistent with the results of the SDS-PAGE experiments and the results reported in literature (Olson *et al.*, 1976; Hay *et al.*, 1973; Chou *et al*,.1994).

	Breast meat				*Leg meat*		
	MFI	Cooking loss	Shearforce		MFI	Cooking loss	Shearforce
Titin	-0.137	-0.415	0.577	Titin	*-0.671**	0.619	0.770*
Tb_a	0.597	0.177	0.013	Tl_a	-0.866**	0.873**	0.873**
Tb_b	0.508	0.830*	-0.784*	Tl_b	0.913**	-0.913**	-0.913**
Tb_c	-0.728*	-0.845**	0.914**	Tl_c	0.548	-0.845**	-0.507
Tb_d	0.671*	0.180	0.069	Tl_d	0.940**	-0.856**	-0.894**
Tb_e	0.898**	0.708*	-0.519	Tl_e	0.913**	-0.809*	-0.861**
Desmin	-0.796*	-0.963**	-0.945**	Tl_f	0.935**	-0.823*	-0.926**
Db_a	0.713*	0.317	-0.210	Desmin	-0.817**	0.728*	0.932**
Db_b	0.896**	0.777*	-0.664	Dl_a	0.671*	-0.782*	-0.574
Db_c	0.802**	0.919**	-0.882**	Dl_b	0.783*	-0.913**	-0.756*
Nebulin	-0.935**	-0.931**	0.823*	Dl_c	0.820**	-0.809*	-0.913**
Nb_a	0.922**	0.617	-0.447	Nebulin	-0.949**	0.805*	0.907**
Nb_b	0.508	0.830*	-0.784*	Nl_a	0.802**	-0.819*	-0.756*
Vinculin	-0.173	-0.384	0.546	Nl_b	0.639	-0.620	-0.845**
				Vinculin	-0.677*	0.550	0.866**

Table 5 *Correlation coefficients of relationships between relative optical density scores of proteins and proteolytic fragments on Western blots and MFI, cooking loss and shear force. Significance levels:* * = *the correlation is significant at the 0.05 level (2-tailed).*
** = *the correlation is significant at the 0.01 level (2-tailed).*

In order to identify possible candidate marker fragments, the semi-quantitative scores for relative intensity of the different proteins and cleavage products on the western blots were converted to numerical scores as described under materials and methods. Subsequently, the Spearman rank correlation coefficients were calculated of the relationships between these density scores and the MFI, the cooking loss and the shear force measurements respectively. For the leg meat, the shear force averaged over the three muscles tested was calculated. This could be done because the shear forces were corrected for sample size according to Poole and Klose (1969). Table 5 shows the results of these calculations.

In breast muscle, the degradation of titin as detected on Western blot did not correlate well with the meat quality attributes. The course of fragment Tb_c however, showed very high correlation coefficients with both cooking loss and shear force measurements. The decline of the

optical density of intact desmin as well as the course of the optical density of the Db_c fragments showed high correlations with the MFI, cooking loss and shear force although the intact desmin with MFI correlation was lower. The Db_b fragment showed a high correlation with the MFI but lower to non-significant correlations with the cooking loss and shear force measurements. The nebulin decline correlated highly with the cooking loss and to a lesser extent with the shear force measurements. Nebulin fragment Nb_a only correlated with the MFI. Fragment Nb_b only correlated at 5% level with both cooking loss and shear force. The decrease of vinculin showed no significant correlation with any of the traits.

In leg muscle tissue, titin fragments Tl_a, Tl_b, Tl_d, Tl_e and Tl_f showed significant correlations with the MFI, the cooking loss as well as the shear force measurements. Only the decrease of the intact titin and the Tl_b fragment bands showed low correlations. The decrease of the intact desmin as well as the increase in fragments Dl_b and Dl_c correlated significantly with the course of the meat quality traits. The decrease of intact nebulin as well as the increase in fragment Nl_a correlated significantly with the meat quality parameters. The Nl_b fragment only correlated significantly with the shear force. The decrease of the intact vinculin molecule correlated significantly with the MFI and was highly correlating with the shear force.

In conclusion it can be stated that the degradation of specific myofibrillar cytoskeletal proteins, as studied in the present work, follows the pattern of post mortem tenderization and as such can be utilized as indicators for meat aging. Titin, nebulin, as well as desmin degradation follow a course that is consistent with post mortem tenderness development as illustrated by the shearforce measurements. However, it has to be taken into account that processes involved in rigor mortis development like pH decrease can interfere with these relationships. More research is needed to identify specific proteolytic fragments of titin, nebulin and desmin, which can serve as specific markers for estimation of the degree of proteolysis. Once these components are identified, specific probes can be developed to quickly and easily monitor the course of the aging of meat. Using western blot analysis we have identified some possible candidate protein fragments that might prove useful for this purpose. However, since western blotting is a technique that can only be used to identify relative differences between different muscle samples it is unsuitable as a method for measuring quantitatively the course of post mortem proteolytic degradation of cytoskeletal proteins. Immunochemical techniques like ELISA however, are of a quantitative nature and future research will thus have to be carried out to develop similar methods, possibly in the form of "dip-stick" methods or biosensors, using the above mentioned specific probes to monitor meat aging in-line at the processing plant.

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Chapter 7:

Effects of Different Methods of Ante-Mortem Stunning on Carcass Quality, Post mortem Muscle Metabolism, and Subsequent Meat Quality.

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ABSTRACT In this work five different stunning methods are compared. Measures of carcass quality, like color, and hemorrhage scores of breast muscle and thighs as well as measures of post mortem metabolism like pH, R-value and glycolytic metabolites, myofibrillar fragmentation and sarcomere length are compared. Finally, waterholding capacity and shear force was measured.

The five different stunning types were two electrical stunning methods, whole-body and head-only, two gaseous stunning/killing methods, CO_2/O_2 and Ar/CO_2 mixtures and one mechanical stunning method utilizing a captive bolt device together with pressurized air driven directly into the broiler's brain.

Captive bolt and argon stunning results in darker breast meat with an increased redness. Carbon dioxide stunned birds show lighter meat. Hemorrhage scores are clearly increased in whole body electrically stunned birds. Head-only stunned birds showed fewer hemorrhages. The lowest hemorrhage scores in breast muscles were found in CO_2 and argon stunned birds. Captive bolt stunning resulted in more hemorrhages.

Both argon induced anoxia and head-only stunning exhibit an acceleration of the glycolytic rate as observed by a rapid initial pH drop, glycogen, and ATP depletion, lactate increase and R-value increase. Whole-body stunning results in deceleration of the glycolytic rate.

No differences related to stunning method utilized were detected in the microstructural measurements as well as in the degradation of cytoskeletal proteins.

Water holding capacity measurements showed an increased moisture loss in head-only stunned animals. Shear force measurements showed the head-only stunned birds to exhibit significantly tougher meat than the other stunning types.

It is concluded that only head-only electrical stunning has negative effects on the conversion of muscle to meat. Both electrical stunning methods show high hemorrhage scores. CO_2 stunning and whole body electrical stunning cause a deceleration of the glycolysis, with as a consequence longer waiting periods before deboning can be carried out without negative effects on meat tenderness. Argon induced anoxia and captive bolt stunning seem to have no negative effects on meat quality. Argon induced anoxia may accelerate glycolytic rate with shorter waiting times to deboning as a consequence.

(Key words: Stunning, post mortem *metabolism, meat quality)*

INTRODUCTION

In modern processing plants it is normal slaughterhouse practice that broiler chickens are stunned prior to killing. This stunning induces a state of unconsciousness, insensibility and immobility before exsanguination through severing the neck blood vessels can be carried out. The primary justification for stunning are (Thomson *et al.*, 1986):

1. Considerations of humane slaughter.
2. Immobilization of the bird for proper presentation to the rotating neck cutting knife.
3. Reduction of struggling during exsanguination to reduce carcass damage.

Stunning is generally accomplished in line by immersion of the birds into a waterbath which is connected to an electric stunning device so that the electrical current runs through the body of the birds, with the shackles in which the animals are hanged functioning as the ground electrode (Bilgili, 1992). Due to animal welfare considerations, high amperage stunning systems are used to achieve instantaneous and irreversible unconsciousness. This high amperage electrical stunning can lead to muscle supercontractions and subsequent hemorrhaging in muscle tissue caused by rupture of blood vessels and damage to muscle fibers (Veerkamp *et al.*, 1987; Gregory and Wilkins, 1989; Hillebrand 1993; Hillebrand *et al.*, 1996a,b; Kranen *et al.*, 1998).

The pre-slaughter stress associated with the handling required to hang the birds onto the slaughterline and the positioning upside down of the animals may lead to severe impairment of animal welfare (Zeller *et al.*, 1988).

Both considerations have lead to several studies into alternative ways of stunning and killing chickens. Gaseous stunning was accomplished by immersion of the bird in an environment with anaesthetic gas (Raj *et al.*, 1990; Poole and Fletcher, 1995) or an environment without oxygen (Raj *et al.*, 1990, 1991; Raj & Gregory, 1991; Poole & Fletcher, 1995). Alternative forms of electrical stunning were explored by Hillebrand *et al.* (1996a). The authors applied different currents, utilizing head-only stunning with or without relaxation current through the whole body. Also a method commonly used in red meat slaughter, captive bolt stunning, was introduced in this study. Different stunning techniques have been shown to affect post mortem processes and poultry meat quality (Thomson, 1986; Kim *et al.*, 1988; Mohan Raj *et al.*, 1990; Hillebrand *et al.*, 1996a,b; Papinaho & Fletcher, 1995a,b,1996). It is a well-known fact that whole-body electrical stunning of broilers is related to a high incidence of hemorrhages, especially in breast meat (Mohan Raj *et al.*, 1990; Hillebrand *et al.*, 1996a, b). Gaseous stunning techniques however, seem to significantly decrease the incidence of bruising (Mohan Raj *et al.*, 1990).

All of the above mentioned studies investigated the impact of some stunning methods on either post mortem muscle metabolism or carcass quality parameters or meat quality parameters, however, there is a need for a comparative, integrating study on the effects of different stunning methods on post mortem muscle metabolism. Purpose of this study was to investigate the effects of two electrical, two gaseous and one mechanical stunning technique on muscle physiology and subsequent meat and carcass quality.

MATERIALS AND METHODS

Stunning and slaughter procedures

Five weeks old Ross broilers were obtained from a local broiler farm and housed in the holding facility of the institute. The birds were housed in 4 separate floorpens holding 66 (3 floorpens) and 77 (1 floorpen) birds each and fed *ad libitum* with standard broiler feed (Schreurs *et al.*, 1995). At six weeks of age, the birds were deprived of access to feed and

water 4 hours prior to slaughter. Subsequently the animals were cooped and transported to the slaughter facility. Time between catching and slaughter never exceeded 2 hours.

Prior to slaughter the birds were stunned in five different ways:

1. Electrical by immersion of the birds hanging in shackles in a waterbath with 100 V, approximately 120 mA and 50Hz during 10 seconds, the current running through the total body (shackles as ground) and without fixation of the birds. This method served as a standard method comparable to a practical slaughterhouse situation in northwestern Europe.
2. Electrical using electrodes situated at both sides of the head with 120 mA, 300Hz during 1 second with the head fixed and the bird hanging in a funnel for fixation as described by Hillebrand (1996a)
3. Immersion in a box (75 x 75 x 75 cm) containing a gas mixture of 30% CO_2, 70% Argon during 3 minutes.
4. Anesthesia in gas mixture: 40% CO_2, 30% O_2, 30% N_2; subsequent killing by anoxia in gas mixture: 80% CO_2, 20% N_2 using a commercial gas stunning tunnel manufactured by STORK PMT.
5. Mechanical using a hollow captive bold stunning device in combination with pressurized air; 0.5 s, 2 bar as described by Hillebrand *et al.* (1996b). Birds were hanged in a funnel for fixation.

After stunning all chickens were killed by venasection of the jugular vein and scalded, plucked and eviscerated according to standard protocol and cooled during 1 hour by 0-4 °C in a chilling tunnel. 0, 1, 3 and 7 hours and 1, 2, 3, 5 and 7 days after this first cooling period, breast and leg muscles were deboned and samples and/or measurements were taken, which are referred to as respectively 1, 2, 4, 8, 24, 48, 72, 120 and 168 hours post mortem hereafter. Samples were frozen in liquid nitrogen and stored in aluminum cups at - 80 °C for later measurement.

Carcass quality measurements

Hemorrhage score. The inner side of the thighs and the part of the *Pectoralis* muscle that had been attached to the carcass were scored by 3 persons for hemorrhages immediately after deboning. This took place on all carcasses used for sampling at 1, 2, 4 and 8 hours and 1 and 2 days post mortem, 16 carcasses per stunning method and time post mortem. The scale used was previously described by Kranen et al. (1998).

Color measurements. Color measurements were carried out using a Minolta chromameter Color meter at 1, 2, 4 and 8 hours and 1, 2, 3, 5 and 7 days post mortem on 16 carcasses per stunning method and time post mortem. Data were recorded in the instrument memory and subsequently downloaded into a personal computer for further analysis. Single measurements on each breast halve were taken (2 measurements per bird).

Measurements concerning the post mortem *metabolism*

pH. The pH measurements were carried out immediately after sampling according to Jeacocke (1977) at 1, 2, 4 and 8 hours and 1, 2, 3, 5 and 7 days post mortem on 16 carcasses per stunning method and time post mortem. Muscle samples were taken from the *M. Pectoralis major* and the *M. Semitendinosus*.

R-value. Samples for R-value measurements were taken and processed according to Honikel & Fisher (1977) as modified by Schreurs and van der Heide (this thesis, chapter 4) from the *M. Pectoralis major* and the *M. Semitendinosus* at 1, 2, 4 and 8 hours and 1 and 2 days post mortem on 16 carcasses per stunning method and time post mortem. Briefly, About 2 gram of frozen muscle tissue was homogenized in 25 ml cold 0.85 M $HClO_4$ solution during 40-60 sec at 10.000 rpm with an Ultra Turrax homogenizer. After mixing they were centrifuged during 30 min. with 3000g. To 100 µl of clear supernatant 2.5 ml of 0.1M Phosphate buffer, pH 7.0 was added. The absorptions were measured at 250 and 260 nm. R-values were calculated as the A_{250}/A_{260} ratio.

The remainder of the supernatant were frozen and stored at -80 °C for later determination of tissue glucose, glycogen, lactate, and ATP. Before using the supernatants for these metabolite measurements, they were neutralized using 5 µl methylorange as an indicator per 1.5 ml of supernatant. Then 10 M KOH was added until the color changed from red to yellow (typically approximately 120-150 µl). Subsequently, the samples were centrifuged in a microfuge during 10 min at 12.000 x g. The neutralized supernatants were used for the measurements of the metabolites of the glycolytic pathway.

Free glucose and glycogen. Glucose measurements were made using a Sigma diagnostics kit (nr 115) with modifications to the original kit protocol. All volumes of reagents and samples were reduced to fit into the wells of a microtiter plate (approximately 200 µl). Volumes used were: 10 µl of neutralized sample, blank or standard and 25 µl of enzyme/color reagent. The standard curve ranged from 0 to 250 µg/ml. Glycogen measurements were carried out identically on samples previously hydrolyzed with amyloglucosidase according to Passoneau and Lowry (1993).

Lactate. Lactate was measured using a Sigma diagnostics lactate kit (No 826B) with modifications to the original kit protocol. All volumes of reagents and samples were reduced to fit into the wells of a microtiter plate (approximately 200 µl). One part of sample was diluted with 2 parts of water prior to analysis. Volumes used were 10 µl diluted sample, blank or standard and 200 µl of enzyme solution. The standard curve ranged from 0- 400 µg/ml.

ATP. Adenosine-tri-phosphate was measured using a Sigma diagnostics ATP kit (No 366-UV) with modifications to the original kit protocol. All volumes of reagents and samples were reduced to fit into the wells of a microtiter plate (approximately 200 µl). Volumes used were 25 µl neutralized sample, blank or standard, 10 µl of GAPD/PGK enzyme solution, priory diluted with four parts of PGA buffer, and 125 µl NADH solution. The standard curve ranged from 0- 500 µg/ml.

Microstructural measurements

Sarcomere length. Sarcomere length measurements were carried out according to Pospiech & Honikel (1987), with minor modifications, on breast muscle samples at 1, 8 and 24 hours post mortem using samples from 4 carcasses per stunning method and time post mortem. Three thin strips (approximately 0.5 mm wide) of tissue were cut from the breast muscle parallel to the muscle fibers. Subsequently the strips were incubated in fixation solution (0.1M Phosphatebuffer pH 7.2, 5% glutardialdehyde) and fixed at 4 °C for at least 24 hours. After fixation the strips were rinsed in saccharose solution (0.1M Phosphatebuffer pH 7.2, containing 0.2M saccharose). The pieces were teased apart with a pair of tweezers until small well-defined fibers were obtained. The fibers were immersed in a drop of saccharose solution on a microscopic slide and covered. Sarcomere length measurements were carried out under a Helium-Neon laser beam (2 mW, 632.8 nm) measuring the distances between maxima in the

thus obtained refraction pattern. The sarcomere length was calculated according to Cross et al. (1980).

Myofibrillar Fragmentation Index. Myofibrillar fragmentation of breast muscle was measured according to Olson et al. (1986) as modified by Schreurs and van der Heide (This thesis: Chapter 4) at 1,2,4 and 8 hours and 1,2 and 3 days post mortem on 16 carcasses per stunning method and time post mortem.

Sodium dodecyl sulphate polyacrylamide gel-electrophoresis (SDS-PAGE). SDS-PAGE was carried out according to Gras and Schreurs (This thesis: Chapter 6) on breast muscle at 1, 2, 4 and 8 hours and 1, 2, 3, 5 and 7 days post mortem on 16 carcasses per treatment. Briefly, a piece of liquid nitrogen frozen breast tissue was pulverized in a liquid nitrogen cooled Teflon capsule fitted with a steel ball using a Retch ball mill homogenizer during 1 min at maximum speed. From the powder 10 ± 0.05 mg was weighed into a 2 ml Eppendorff vial, and 1 ml sample buffer according to Fritz and Greaser (1991) was added. After mixing using a Teflon Eppendorff pestle, the samples were incubated in a waterbath at 50 °C during 20 min. After remixing, to 50 µl of this extract, 10 µl of an internal standard (0.5 mg β-galactosidase in 1 ml sample buffer) and 40 µl bromophenolblue solution (100 µl of 0.2% bromophenolblue solution diluted with 2 ml sample buffer) were added according to Claes et al. (1992). After mixing 10 µl of the processed samples were loaded on Pharmacia ExcelGel Homogenous 7.5 gels fitted with Excel gel SDS buffer strips. Electrophoresis was carried out during 750 Vh with the following limit settings on the power supply: 600 V, 100mA en 30 Watt per gel. De gels were stained using a Coomassie staining protocol according to Pharmacia (1990).

Densitometry. All gels were scanned using a Hewlett Packard 6100C flat bed scanner connected to a personal computer. Files were processed as densitometer traces using the Quantiscan software from Biosoft. Non-linearity corrections were applied according to Tarlton & Knight (1996). Optical densities of the bands of interest were related to the internal β-galactosidase standard to yield relative amounts of titin and nebulin per gram of wet tissue as described by Claes et al. (1992).

Meat quality measurements

Water holding capacity. The water holding capacity of the breast meat was measured by the filter paper method, previously described by Kauffman et al.(1986) at 1, 2, 4 and 8 hours and 1, 2, 3, 5 and 7 days post mortem on 16 carcasses per treatment and immediately after deboning. One measurement per bird was taken.

Shear-force measurements. From 16 birds per treatment one breast halve was designated for shear force measurements. Muscles were stored at 0-4 °C until sampling at 1, 2, 4 and 8 hours and 1, 2, 3, 5 and 7 days post mortem and subsequently frozen and stored at –20 °C until analysis. Measurements of shear force in three samples of *Pectoralis* muscle were carried out as described by Froning and Uijttenboogaart (1988).

Statistical analysis

Statistical analysis was carried out using SPSS/Windows. The general linear models (GLM) module was used to carry out ANOVA using the following model:

$$Y_{ijk} = \mu + S_j + T_k + (S*T)_{jk} + e_{ijk}$$

Where: Y = response variable
μ = population mean
S_j = method of stunning (j = 1, 2, 3, 4, 5)
T_k = time post mortem (k = 1,2,4,8, 24, 48, 72, 120, 168 hours)
e_{ijk} = experimental error

RESULTS AND DISCUSSION

Table 1 shows the results of the hemorrhage scoring in thigh and breast meat after application of five different methods of ante mortem stunning. There were no significant differences between scoring panel members, nor were there any interactions with other traits. Therefore

Stunning method	Average hemorrhage score	
	thigh meat (N = 144)	Breast meat (N = 144)
Whole-body	3.15 ± 1.17^{a}	3.56 ± 1.17^{a}
Head-only	2.42 ± 0.94^{b}	3.07 ± 1.23^{b}
Argon	2.08 ± 0.96^{c}	1.75 ± 0.89^{d}
CO_2	2.07 ± 0.92^{c}	1.66 ± 0.93^{d}
Captive bolt	2.04 ± 0.90^{c}	1.96 ± 0.93^{c}

Table 1 *Average hemorrhage scores in breast and thigh meat related to different stunning methods.*
Data are given as group average ± standard deviation.

the results were pooled over scoring panel members before analysis. In both thigh and breast meat the whole body electrical stunning resulted in significantly higher hemorrhage scores than the other stunning methods. Head only stunning showed significantly lower occurrence of hemorrhages in both breast and thigh than the whole-body electrical stunning. The two gaseous stunning methods and the captive bolt stunning showed lower hemorrhage scores in the breast than the two electrical methods. In breast muscle the captive bolt stunning caused a higher hemorrhages score than the two gaseous stunning methods but lower than the two electrical methods.

The hemorrhage scores in thighs are lower than in breast for the two electrical stunning methods while they were higher in thighs than in breasts for the two gaseous stunning methods. The captive bolt stunning caused no differences in hemorrhage scores between thighs and breast muscle. This signals the fact that the effects of the electrical stunning as opposed to gaseous stunning is much greater on breast muscle than on thigh muscle hemorrhaging.

The vigorous contraction of muscles occurring during whole-body electrical stunning is responsible for the high hemorrhage scores (Hillebrand et *al.*, 1996a, b). Apparently, the convulsions occurring during and after head-only, gaseous and captive bolt stunning are less vigorous, thus resulting in less hemorrhages. A possible explanation would be the fact that during convulsions, muscle contractions, heavy as they may be, are more or less physiological. This meaning that agonists and antagonists do contract in a coordinated manner and not as uncontrolled as during whole body electrical stunning, thus generating less tension in the muscle resulting, in a lower incidence of vessel rupture and subsequent hemorrhaging.

Table 2 shows the results of the color measurements in breast muscle at different times post mortem. There were no significant interactions of stunning method and time for all color values. Averaged over stunning methods, with increasing time, the C.I.E. lightness (L*) gradually increased from approximately 57 at 1 hour to approximately 61 after 48 hours, while the C.I.E redness (a*) gradually increased from 3.9 at 1 hour to 5.3 after 168 hours of storage at 0 °C. This value was maintained throughout the rest of the post mortem period

C.I.E. color value:	L*	a*
Stunning method	(N = 144)	(N = 144)
Whole body	58.9 ± 3.4 [c]	4.7 ± 1.6 [a]
Head-only	59.0 ± 2.9 [c]	4.8 ± 1.5 [a]
Argon	60.5 ± 3.4 [b]	3.9 ± 1.2 [b]
CO_2	61.4 ± 3.7 [a]	3.5 ± 1.1 [c]
Captive Bolt	59.9 ± 3.7 [b]	4.1 ± 1.4 [b]

Table 2 *Color measurements of breast muscle related to different stunning methods.*
L = brightness*
a = redness*
Data are given as group average ± standard deviation

measured (168 hours). Averaged over time, the carbondioxide stunned broilers maintained the lightest meat. The argon and captive bolt stunned birds showed significantly lower L* values while both electrical stunning methods resulted in the darkest breast meat. The C.I.E. redness (a*) values were lowest in CO_2 stunned birds. Argon and captive bolt stunned birds showed significantly more red breast muscle than the CO_2 stunned animals. Both electrical stunning methods resulted in most red breast meat. Color measurements have been reported in turkeys showing lower breast muscle C.I.E. redness (a*) values with carbon dioxide stunning when compared to electrical stunning (Flemming *et al.*, 1991). In earlier work no significant color differences between CO_2, high current and low voltage stunning were reported (Poole & Fletcher, 1998). No effects of stunning amperage on breast meat color were reported also (Papinaho & Fletcher, 1995a). The differences in color may be related to the amount of blood retained in the breast muscle capillaries after bleeding. To rule out this possibility, total heam content of the muscles was measured(results not shown). No differences between stunning treatments were detected. Another possible explanation for the color differences would be the course of the pH, which is closely related to the extent of denaturation of the muscle proteins (Warris & Brown, 1987). Table 3 shows the results of the pH measurement in *M. Pectoralis major*. Significant interactions of stunning by time were detected. Up to 8 hours, whole body electrical stunning resulted in the highest pH values while head-only electrical stunning gave low breast muscle pH values. At 24 and 48 hours post mortem, no significant differences between stunning methods were detected. High pH values generally coexist with dark breast meat while lower pH values result in lighter meat. In this study we only found this relationship for the whole-body electrical stunning. The head-only electrical stunning resulted in lower pH values but darker meat than the gaseous stunning methods. The pH values of the breast muscle of birds stunned with a gaseous stunning method or with captive bolt stunning showed intermediate pH values. Electrical stunning is known for its inhibition of glycolysis up to 6 hours in broilers (Papinaho & Fletcher, 1995a,b; 1996; Poole & Fletcher, 1998) and turkeys (Murphy *et al.*,1988). Gaseous stunning methods have been found to produce a more rapid pH drop early post mortem in broiler (Mohan Raj *et al.*, 1990; Poole & Fletcher, 1998) and turkey breast muscle (Mohan Raj, 1994).

Related to rigor development and thus to pH drop is the R-value, a measure for the conversion of energy rich adenosine nucleotides into inosine mono-phosphate and thus for course of the glycolysis. A high r-value (R > 1.2) is indicative for the post rigor period. Table 3 shows the results of the R-value measurements in breast muscle. There were significant interactions of stunning method by time. Up to 2 hours, the breast muscle R-values of the head-only

	pH				R-value			
Time *post mortem*:	1 hour	2 hours	4 hours	8 hours	1 hour	2 hours	4 hours	8 hours
Stunning method	(N = 16)	(N = 16)	(N = 16)	(N = 16)	(N = 16)	(N = 16)	(N = 16)	(N = 16)
Whole body	6.47 ± 0.39 [a]	6.21 ± 0.26 [a]	6.05 ± 0.11 [a]	5.99 ± 0.06 [ab]	0.87 ± 0.06 [c]	0.94 ± 0.08 [c]	1.20 ± 0.13 [b]	1.35 ± 0.04 [b]
Head-only	6.01 ± 0.14 [c]	6.00 ± 0.05 [b]	5.98 ± 0.11 [b]	6.00 ± 0.10 [ab]	1.14 ± 0.08 [a]	1.27 ± 0.11 [a]	1.35 ± 0.05 [a]	1.38 ± 0.02 [a]
Argon	6.11 ± 0.17 [bc]	6.04 ± 0.09 [b]	6.00 ± 0.08 [ab]	6.02± 0.11 [a]	0.94 ± 0.09 [b]	1.09 ± 0.15 [b]	1.29 ± 0.09 [a]	1.36 ± 0.02 [ab]
CO_2	6.30 ± 0.34 [ab]	6.21 ± 0.33 [a]	6.02 ± 0.09 [ab]	6.01 ± 0.09 [ab]	0.88 ± 0.06 [bc]	0.93 ± 0.08 [c]	1.17 ± 0.14 [b]	1.35 ± 0.03 [b]
Captive Bolt	6.29 ± 0.42 [ab]	6.23 ± 0.29 [a]	5.97 ± 0.10 [b]	5.96 ± 0.04 [b]	0.94 ± 0.13 [b]	0.99 ± 0.11 [c]	1.29 ± 0.10 [a]	1.36 ± 0.02 [b]

Table 3 *Course of pH and R-value with time in breast muscle related to different stunning methods.*

Data are given as group average ± standard deviation

stunned animals showed significantly higher values than the other stunning methods. At 2 hours, the argon stunned birds showed lower R-values than the head only stunned animals but higher than the CO_2 and captive bolt stunned animals. At 4 and 8 hours, the R-values of head-only and argon stunned birds were significantly higher than from the other three groups. After 8 hours, no differences were detected any more (results not shown). Up to 8 hours, the whole-body stunned animals showed lower R-values than the other stunning methods. Summarized it can be stated that breast muscles of head only stunned animals are close to rigor mortis already at 1 hour. Argon induced anoxia results in a slower rigor development but faster than captive bolt, CO_2 and whole-body electrical stunning.

Electrical stunning is reported to cause inhibition of glycolysis, compared to non-stunning, and hence lower R-values (Papinaho & Fletcher, 1995b; 1996). Lower R-values were also observed with high current electrical stunning when compared to argon stunning (Poole & Fletcher, 1998).

The R-value is closely related to the tissue ATP concentration. Table 4 shows the ATP concentration in breast muscle at 1 and 2 hours post mortem. The stunning method by time interactions were significant. Head-only stunning had a low muscle ATP concentration from the start of the measurements. The other four stunning methods resulted in higher initial ATP concentrations. Argon induced anoxia results in the fastest ATP degradation in the first few hours post mortem, while carbondioxide gave the slowest ATP degradation. From 4 hours on, no relevant differences between different stunning methods were detected. ATP degradation proceeds very fast in chicken breast muscle. Four hours after killing, approximately 25% of the amount present at 1 hour post mortem was present in the muscle tissue. Based on the observation that 1 hour after killing, approximately 50% of the initial ATP store was degraded (Schreurs & van der Heide, This thesis: Chapter 4), it can be calculated that after 4 hours, 10-15% of the initial ATP stock was still present in the breast muscle.

This is well below the 20 – 30%, gennerally accepted as the muscle ATP concentration at which rigor mortis sets in (DeFremery,1966).

Table 4 also shows the results of the determination of glycogen in the breast muscles. The interactions of stunning method by time were significant. Here again, head only stunning results in very low initial glycogen concentrations in the muscle. Whole-body electrical stunning showed the highest glycogen values. Argon stunning showed the second lowest glycogen stores in the muscle. After 4 hours, glycogen concentrations became very low and no more significant differences could be detected. The data on tissue lactate, as shown in table 4 are in close agreement with the ATP and glycogen data. Head only stunning shows by far the highest lactate concentrations in the muscle, whole body electrical and captive bolt stunning the lowest. All these data point to a retarded glycolysis in the breast meat of whole-body electrical and the CO_2 stunned animals. The glycolysis in the head-only electrical and to a lesser extent in the argon stunned animals was strongly accelerated, probably due to heavy convulsions after stunning. Birds showed an intermediate glycolytic rate after captive bolt stunning.

The results of the measurements of the myofibrillar fragmentation and of the sarcomere length are not shown in tables since no significant differences in stunning method main effects nor any significant interactions were detected. The myofibrillar fragmentation index is a measure of post mortem proteolytic degradation of the myofibrils into smaller fragments. During the time, the MFI slowly increased from 73 at 1 hour to approximately 100 at 8 hours. The MFI did not change anymore after this time up to 72 hours. Sarcomere length measurements did not show significant stunning method by time interactions nor were there any

	ATP		Glycogen		Lactate	
Time *post mortem*:	1 hour	2 hours	1 hour	2 hours	1 hour	2 hours
Stunning method	(N = 16)	(N = 16)	(N = 16)	(N = 16)	(N = 16)	(N = 16)
Whole body	5.4 ± 2.6 [a]	3.1 ± 1.9 [ab]	12.7 ± 8.0 [a]	6.0 ± 5.7 [ab]	0.49 ± 0.11 [cd]	0.59 ± 0.13 [c]
Head-only	1.5 ± 1.2 [b]	0.9 ± 0.9 [c]	0.37 ± 0.46 [c]	0.30 ± 0.62 [c]	0.79 ± 0.07 [a]	0.82 ± 0.11 [a]
Argon	4.6 ± 2.0 [a]	2.0 ± 1.7 [bc]	5.6 ± 6.0 [b]	2.5 ± 4.8 [bc]	0.59 ± 0.10 [b]	0.70 ± 0.11 [b]
CO_2	5.5 ± 2.0 [a]	4.4 ± 3.6 [a]	9.4 ± 6.9 [ab]	6.9 ± 7.0 [a]	0.47 ± 0.11 [d]	0.59 ± 0.09 [c]
Captive Bolt	4.4 ± 2.4 [a]	3.9 ± 2.9 [a]	6.7 ± 6.1 [b]	4.6 ± 5.7 [ab]	0.56 ± 0.11 [bc]	0.58 ± 0.18 [c]

Table 4 *Course of muscle ATP, glycogen and lactate concentration with time in breast muscle related to different stunning methods.*

Data are given as group average ± standard deviation

differences between stunning methods. Averaged over the stunning methods from 1.85 μm at 4 hours, the sarcomere length gradually and significantly increased to 1.93 μm at 24 hours.

Figure 1 shows the typical results of the electrophoresis of the muscle samples collected at different times post mortem. Statistical analysis of the densitometric optical densities of the titin and nebulin bands did not show any stunning method by time interactions nor were there any significant stunning method main effects. There was a gradual but significant decrease of the optical densities of both bands with time post mortem.

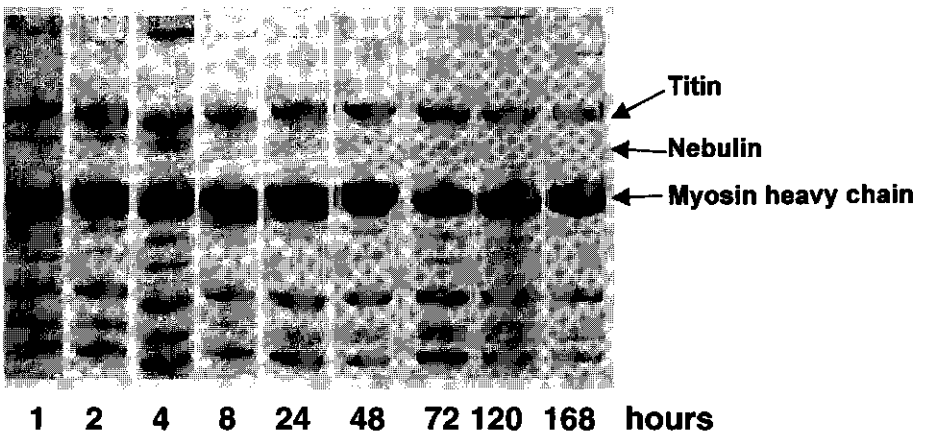

Figure 1 *Electropherogram of high molecular weight myofibrillar proteins at different times post mortem.*

Table 5 shows the results from the meat quality measurements. The water holding capacity (WHC) measurements did not show any significant stunning method by time interactions. Averaged over the stunning techniques, the samples showed a gradual significant increase in moisture loss from approximately 50 mg at 1 hour up to approximately 75 mg at 168 hours.

Stunning method	WHC (N = 144)	Shearforce (N = 144)
Whole body	59.6 ± 20.1^{bc}	18.0 ± 9.2^{b}
Head-only	71.5 ± 22.9^{a}	20.6 ± 11.0^{a}
Argon	61.9 ± 22.7^{b}	18.1 ± 9.8^{b}
CO_2	57.0 ± 20.7^{c}	18.5 ± 9.3^{b}
Captive Bolt	60.2 ± 19.5^{bc}	17.6 ± 8.1^{b}

Table 5 *Water holding capacity (WHC) and shearforce measurements of breast muscle related to different stunning methods.*
Data are given as group average ± standard deviation

Averaged over the different times, the head only electrically stunned birds showed the highest moisture loss while the CO_2 stunned birds showed the lowest moisture loss. The WHC is, as is meat color, closely related to initial pH drop. When the pH of the meat drops decreases very quickly early post mortem, meat is usually lighter and WHC is limited (Warris & Brown, 1987; Pearson & Young, 1989).

Shearforce measurements, as shown in table 5, of the Pectoralis muscle did not show any significant stunning method by time post mortem interactions. Averaged over the stunning methods there was a significant change from 29 N at 1 hour post mortem to 14 N at 24 hours post mortem. From 24 hours post mortem on, no shearforce changes were observed. Averaged over the different times post mortem there was a significant stunning method main effect. Breast muscles from birds that were head-only electrically stunned showed significantly higher shear values than the other four stunning types. It is clear that the acceleration of the initial glycolytic rate in the head-only stunned birds due to heavy convulsions had a detrimental effect on subsequent meat quality. The acceleration of glycolytic rate in the argon stunned birds had less negative side effects. Whether this is caused by differences in severity of the convulsions or that there is some other origin of these differences remains unclear in this study.

Mohan Raj *et al.* (1990) report a significant improvement of tenderness as measured by Volodkevitch shear force in argon stunned birds when compared with total body electrical stunning. In another study (Mohan Raj & Gregory, 1991), the same authors report no significant differences between argon induced anoxia stunned birds and broilers stunned with a whole-body electrical stunning device. Other comparisons of

gaseous stunning devices based on induction of anoxia by argon/CO_2 mixtures with different electrical stunning devices (Poole & Fletcher, 1998) show only early post mortem differences between treatments.

Heavy convulsions were mainly observed after head-only electrical stunning, argon induced anoxia, and captive bolt stunning. Only the first two methods induced a glycolytic acceleration probably due to these convulsions. The argon-induced anoxia however, results in a higher end pH, probably caused by rapid exhaustion of muscle glycogen stores due to heavy convulsions. It may be speculated that with this type of stunning, initially, during convulsions, blood circulation is still intact, thus causing less detrimental side effects on meat quality than head-only stunning. The head-only electrical stunning resulted in such a severe initial glycolytic rate increase that negative effects on meat quality were observed. This means that argon induced anoxia seems to be a method of stunning/killing broilers with certain advantages. Because of the glycolytic acceleration that is induced by the post mortem convulsions it may be possible to debone earlier than with the other stunning methods without the detrimental effects on meat quality that are usually associated with rapid deboning operations. CO_2 killing with initial stunning with a CO_2/O_2 mixture gives results comparable to normal practical whole-body electrical stunning.

Combined with the data on hemorrhage scores, the head-only electrical stunning method as used in this study resulted in the most negative effects on meat quality. The whole-body electrical stunning was slightly better because of the lack of detrimental metabolic effects, but this method gave worse hemorrhage scores. No detrimental effects on meat quality showed the CO_2 stunning method, but because of the slow initial glycolytic rate, there is a risk for cold-shortening when deboning operations are carried out too early. Captive bolt stunning showed neither a decreased meat quality nor high hemorrhage scores, nor a decreased initial glycolytic rate. Argon induced anoxia finally, showed low hemorrhage scores and an accellerated initial glycolysis, favorable with high speed deboning operations, but without any detrimental effects on meat quality.

Of course, the methods investigated in this study are merely a selection of all the possibilities of pre-slaughter stunning of broilers. Nevertheless, this study provides some indication of the impact of ante-mortem stunning operations on post mortem muscle metabolism and sub-

sequent meat quality. Performance of the different methods may be achieved by avoiding the detrimental effects of too severe a reaction of the birds' tissue to treatment. For head-only stunning, the application of a relaxation current as described by Hillebrand *et al.* (1996) may influence the severity of the convulsions and thus the negative effects on meat quality.

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Chapter 8:

General discussion

In the general introduction, under the heading: "Background and objectives of the project" a statement is made about the status of the field of research at the start of this project. It is stated that the knowledge obtained about underlying mechanisms of meat aging in chicken at that moment was fragmented, incomplete, and often contradictory. Systematic and detailed studies of the connection of muscular growth and postmortem metabolism in chicken muscle had never been conducted. Many studies reported about the influence of processing parameters on post mortem processes in chicken meat. Rarely however, these studies went beyond the onset of rigor mortis. If at all later events were studied, the measurements usually were related to the macro-effects of these processes, not with the underlying molecular and intracellular mechanisms. In chicken muscle tissue, figure 7 of chapter 1 of this thesis is the basis for the studies, here represented. As is already explained in chapter 1, the rigor development in the muscle determines the toughening of the muscle during the first hours post mortem. The speed of toughening, the slope of the first part of the curve, as well as the extent of toughening, the height of its maximum, is determined by factors related to energy metabolism. Energy-independent processes, such as proteolytic degradation of the structural myofibrillar proteins responsible for the lateral and longitudinal integrity of the muscular tissue, solely determine the post rigor tenderization of the muscle.

The present project had the objective to study the post mortem metabolism in more detail with some emphasis on the post rigor proteolytic events, in order to obtain a better understanding of the fundamental relationships between muscle physiology and meat quality parameters. Ideally, this would lead to the development of tools which would make it possible to predict and control poultry meat quality in a slaughterhouse environment. These considerations raised a number of questions and presumptions leading to several hypotheses, which were subsequently tested in a number of experiments as described in chapters 2 through 7.

First question to be answered was, whether the post mortem processes in chicken muscle were comparable to the processes observed in mammalian muscular tissue. As described in chapter 1, the preliminary literature study revealed a large amount of investigations conducted in mammalian species. Rigor development as well as energy metabolism was mainly studied in pig and beef. Post mortem proteolytic degradation of structural myofibrillar elements was almost exclusively studied in beef. This obviously, because meat quality problems in pork are mainly related to stress susceptibility and thus to energy metabolism. In beef, post mortem processes proceed relatively slowly, and thus meat quality problems are likely to be related to the fast processing speed of modern slaughter house practice and thus to both energy and protein metabolism. For chickens, with an extremely high energy metabolic rate, meat quality problems may be related to post mortem proteolytic events. However, the influence of the energy metabolism could not be ruled out a priori. In order to be able to study this proteolysis, methods had to be made available for the measurement of the proteolytic capacity of certain enzyme systems. On the basis of the literature research of chapter 1 on mammalian enzyme systems, it was concluded that the calpain/calpastatin system and the cathepsins probably were responsible for the post mortem proteolysis in chicken muscular tissue. However, it may be possible that in chickens, other enzyme systems may be (co-)involved in these processes. Although this is not very likely, with the present status of meat science, there is no way of knowing this for sure.

Chapter 2 describes the study of the chromatographic properties of the calpains in chicken muscle, as well as the development of a method for the measurement of the activity of these enzymes. It is clear that chicken muscle calpains are quite different from the mammalian isoenzymes. It was therefore necessary to adapt the methods described for mammalian tissue to the properties of the avian type of isoenzymes. The developed method is capable of measuring the proteolytic activity of the two isoenzymes separately under optimal circumstances. This would be the maximal proteolytic activity obtainable with the enzymes per given amount of tissue. But very little is known about the real *in situ* activity of these enzymes. Activity of the calpains is determined by several known, and probably some yet unknown, factors. It is therefore that the terms proteolytic capacity or proteolytic potential were introduced. The field experiment described in chapter 2 suggested that there were differences in proteolytic capacity of the muscle of chickens with different genetic backgrounds. Together with literature data this led to the presumption that the proteinases studied in this project, as they were involved in normal protein turnover in the living animal, would differ in chickens lines selected on the basis of juvenile growth rates and protein conversion efficiencies.

For the study in chapter 3, these presumptions were extended towards the fact that modern broiler chickens grow at the edge of 'what is metabolically possible'. This would mean that, based on the work of [1]Calzone *et al.* (1983) the maximum of protein synthetic capacity was reached and faster protein accretion could only be obtained by reducing the protein degradation side of the protein turnover equilibrium. The results of the study, described in chapter 3 indicated that body growth in modern broiler chickens is at least partly regulated by decreased protein breakdown. The faster growing animals showed a marked decrease in proteolytic capacity of their breast muscle tissue, while slow growing birds show high proteolytic capacities. It was concluded that the differences in metabolic parameters in different broiler chicken lines, selected for growth speed (GL-line) and favorable feed conversion ratio between 3 and 6 weeks of age (FC-line) could be used as natural sources of variation in the different metabolic enzyme activities.

From the literature study of chapter 1, it was concluded that there were virtually no data available on the basal, undisturbed, post mortem processes taking place in chicken breast muscle. In order to be able to compare the post mortem processes taking place in the breast muscle of different chicken lines, an experiment was set up to obtain data on the mechanisms responsible for rigor mortis development, proteolytic degradation and post mortem tenderness development under strictly controlled circumstances. The results indicated that, probably due to differences in hormonal regulation, the energy metabolism of the various selection lines differed considerably. From the results of the measurements, combined with data from the literature, it may be concluded that rigor mortis development proceeds within 6 hours after slaughter. This indicates that in normal poultry processing, it is necessary to include a waiting time of at least 6 hours, before deboning can be carried out, in order to avoid cold shortening problems. Quick cooling of the carcasses down to 12 °C and keeping them at this temperature for 6 hours would give the best results.

It was also concluded that the myofibrillar fragmentation index, based on differences in light scattering behavior of different suspensions of myofibrils, was to crude a measure to detect the probably small differences in proteolytic degradation of the cytoskeletal proteins in

[1] Calzone, F.J., R.G. Angerer and M.A. Gorovski, 1983. Regulation of protein synthesis in *Tetrahymena:* Quantitative estimates of the parameters determining the rate of protein synthesis in growing, starved and starved decilliated cells. J. Biol. Chem. 258: 6887-6898.

chicken muscle tissue. For measuring these small differences, more sophisticated methods had to be used. The development of these methods and the study of the kinetics of post mortem degradation of specific cytoskeletal proteins is described in chapter 6.

The results from this experiment indicate possible markers for monitoring the kinetics of the post mortem proteolytic degradation of the muscular ultrastructure. However, the techniques involved require a large effort and technical skill, and thus are probably only useful under experimental conditions . Nevertheless, the identified marker fragments establish good starting points for the development of new measuring techniques and devices which can be utilized in the slaughterline at the processing plant. The use of bio-sensors equipped with specific probes could possibly serve as in-line predictors of tenderness development. These probes could be applied to the meats' surface or in an invasive manner. Another possibility would be measurement of these markers in drip fluid. A whole new range of methods would become available to the poultry, and maybe also red meat, processing industry to monitor meat aging and to predict meat quality. It is therefore that this area seems very promising for future research.

Another finding of the study described in chapter 4 is the fact that the breast meat of very fast growing broilers seems to have not aged to completion within 48 hours. Although the differences with faster aging and slower growing lines are relatively small, these findings form an important indication to poultry breeders and the poultry processing industry. Extrapolating these facts to the future predicts that selection only for faster growth and higher slaughter yields will eventually lead to large meat aging and thus to meat quality problems. Especially consumer demands for maximal freshness of the meat, based on microbiological considerations, will inevitably lead to incompatibility with meat quality. Only solution for this problem is to dedicate more research towards the relationships between genetics, muscle growth and meat quality. When these relationships become more clear, breeding programs can be adjusted so that growth speed and yield, and eventually production costs, can be improved without impairing the intrinsic eating quality of the meat.

Chapter 5 is an extension of the work of chapter 4. The perception of meat quality by the consumer is a very complicated issue. Although there is some correlation between a parameter like sensory tenderness and objective measurements like shear force, there is more to this sensory tenderness than only forces related to mastication, as there is more to sensory juiciness than only the water holding capacity of the meat. The "mouth-feel" of meat depends on numerous different factors, which are, as a complication, not very well defined. What one consumer calls tenderness does not necessarily need to be the same "mouth-feel" as what some other customer would call tenderness. The same goes for practically every "sensory term". The experiment described in chapter 5 is an attempt to obtain insight in the relationships between the sensory perception of poultry meat quality and the objective measurement of what is sometimes called "instrumental tenderness". The conclusion can be drawn that, in order to be successful in developing objective instrumental measures for subjective sensory characteristics, it is important to clearly establish the different underlying physical and physicochemical processes related to sensory perception. As is shown by the experiment of chapter 5, generalized Procrustes analysis may be an important tool to identify the different "dimensions" of a sensory term, as is shown for the terms tenderness and juiciness. Moreover it is important to consider the fact that sensory perception of for instance tenderness is a dynamic process. To only record a one-point measure for this term does not do justice to the process of sensory perception. It may obscure true differences between studied groups or wrongly introduce differences that are not there. It is therefore of the utmost importance for meat, and in a generalized form food quality, that more effort is directed towards the study of

the sensory perception of meat quality. Not only studies to clearly identify the underlying physical and physicochemical processes of "mouth-feel" but also the dynamics of these processes have to be conducted. Introduction of so-called "time-intensity" methods, in which the dynamic course of some sensory aspect is studied, may prove very useful in meat research.

The final chapter in which experimental work is described is chapter 7, where the influence of different methods of ante mortem stunning on processes taking place in the muscle after slaughter and subsequent meat quality were studied. In general it can be concluded from this work that most differences found can be traced back to differences in peri mortem struggle of the animals. This struggle obviously has a large impact on post mortem energy metabolism and thus on rigor development. Clear differences in meat aging could not be established. The instruments however, used to study this, were of limited sensitivity, in other words, only capable of detecting large changes. Here, again the need for better and more sensitive measuring tools can not be over-emphasized.

The differences in hemorrhaging, in breast as well as in leg muscle, may partly be related to peri mortem struggle but more importantly, by the nature of muscle contractions during and after stunning. Heavy convulsion do not necessarily lead to more hemorrhages, provided that the muscular contractions evolve in a coordinated manner. This means that agonist and antagonist muscles do not contract simultaneously. The number of broken wing and keel bones may most probably also be reduced by this, provided the animals are allowed to freely flap their wings without hitting any stationary obstacles like steel bars in the slaughterline.

In this light, gaseous stunning methods, especially the argon induced anoxia described in chapter 7, may be very advantageous. However, animal welfare considerations have been deliberately excluded from this study. It is not always clear when the animals are rendered completely unconscious. The captive bolt stunning method is pretty accurate as is the whole body electrical stunning method normally used in practice. The head only stunning method also induces an epileptiform insult, so it is not very likely that the animals are conscious during the heavy convulsions following the stunning operation. Both gaseous stunning methods however, do allow for the suspicion that at least during the first phases of the stunning operation, the animals are aware of the fact that something unpleasant is happening. The moment of onset of unconsciousness is also a matter of discussion. It is not clear that the moment of loss of posture is a good indicator for this. Studies described in literature, utilizing evoked potentials, and measurements of electro-encephalograms have not given conclusive evidence for this. It is therefore necessary that studies are intensified to find a reliable indicator for the moment of onset of unconsciousness before new stunning methods are introduced. Some methods seem more humane than others but nothing is sure until good measures are established.

As a closing remark it an be stated that with the threat of a decreasing worldwide market for meat, it is important to uphold the present quality standards and improve them where possible. In order to be able to accomplish this, it is necessary to increase the effort, put into research. Product quality should be as important a consideration as are animal welfare, environmental and economical issues. In this light, fundamental biochemical and physiological studies are crucial to our understanding of something simple as meat quality.

Slotwoord

Dit werkje is tot stand gekomen door enkele bewuste keuzen én een enorme hoeveelheid toevalligheden. Het besluit om weer te gaan studeren is mij in eerste instantie ingegeven door Jan Mulder, destijds hoofdanalist van de afdeling nucleaire geneeskunde van het Lukas Ziekenhuis te Apeldoorn. Het besef dat ik een academische opleiding met goed gevolg zou kunnen afronden dank ik aan mijn oud collega en vriend Lex Moen. Mijn moeder maakte mij attent op de vacature van laboratorium assistent vleeskwaliteit op het Spelderholt, waar mij door Theo Uijttenboogaart als directe chef en Roel Mulder als afdelingshoofd alle vrijheid werd geboden om mij te ontplooien als onderzoeker. De laatste was het die mij attent maakte op de mogelijkheid om mijn HBO diploma door middel van een aanvullende studie te laten opwaarderen naar een volwaardig HLO (Ing) niveau. Wim de Wit ten slotte, gaf de eerste aanzet tot de opzet en uitvoering van het promotie onderzoek hetgeen nu heeft geresulteerd in dit boekje.

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Curriculum Vitae

Franciscus Johannes Gerardus Schreurs werd op 3 maart 1956 geboren te Vaassen waar hij tussen 1963 en 1968 de lagere school doorliep. Reeds op 7-jarige leeftijd wist hij wat hij later wilde gaan doen ..... op een laboratorium werken !! Van 1968 tot 1973 volgde hij derhalve HAVO aan het Veluws College te Apeldoorn. Na deze opleiding met goed gevolg te hebben afgesloten werd in 1973 begonnen met een medisch analisten opleiding aan de Laboratorium School te Deventer waar hij in 1975 afstudeerde in de microbiologische studierichting. Van 1975 tot 1976 werkte hij als analist op het Centraal Diergeneeskundig Instituut mee aan een onderzoekproject m.b.t. virale respiratoire aandoeningen bij mestkalveren. Vervolgens was hij 2½ jaar werkzaam, van 1976 tot 1979 als research analist bij de Gezondheidsdienst voor Dieren te Rozendaal, waar hij o.a. betrokken was bij onderzoek naar de resistentie tegen antibiotica van *Salmonella's* bij mestkalveren, en de ontwikkeling van een vaccin voor de opwekking van maternale immuniteit bij drachtige koeien tegen bepaalde *E. coli* stammen.

Vervolgens begaf hij zich op het pad van de humane geneeskunde, door van 1979 tot 1984 te gaan werken op de afdeling nucleaire geneeskunde van het Lukas Ziekenhuis te Apeldoorn. Hij was daar onder andere betrokken bij de kwaliteitsbewaking van de diagnostiek op het *in-vitro* laboratorium en bij de ontwikkeling van methoden voor de meting van de perfusie en filtratie efficiency van nieren m.b.v. radionucliden op het *in-vivo* laboratorium. Tijdens zijn werkzaamheden op dit ziekenhuis kwam hij tot de ontdekking dat zijn vooropleiding onvoldoende houvast bood om zich in de toekomst verder te kunnen ontwikkelen binnen het onderzoek. Daarom besloot hij opnieuw te gaan studeren. In zijn vrije tijd, gebruik makend van de studiefaciliteiten die zijn werkgever hem bood, doorliep hij de opleiding tot medisch nucleair werker. Het studeren sprak hem dusdanig aan dat onmiddellijk daarna een studie "Voeding en toxicologie" aan de Open Universiteit te Heerlen werd gestart.

In 1984 wisselde hij van werkgever en ging als productontwikkelaar werken bij de firma Eurodiagnostics b.v. te Apeldoorn waar hij betrokken was bij de ontwikkeling en productie van methoden voor de meting van hormonen en andere componenten in bloed m.b.v. radioactief gelabelde eiwitten. Na hier 2 jaar werkzaam te zijn geweest, wisselde hij voorlopig voor de laatste keer van baan, en wel naar het Centrum voor Onderzoek en Voorlichting voor de Pluimveehouderij (COVP-DLO) "Het Spelderholt" te Beekbergen. Hier was hij van 1986 tot 1992 werkzaam als laboratorium assistent, waarna hij zich vanaf 1992 als wetenschappelijk onderzoeker op het zelfstandig uitvoeren van wetenschappelijk onderzoek kon richten.

Ondertussen werd in 1990, in de avonduren, het HLO medisch biologisch ingenieurs diploma behaald met als afstudeerrichting levensmiddelen chemie. Toen hij in de loop van 1991 het aanbod kreeg om een stuk promotieonderzoek op te zetten hoefde hij niet lang na te denken. Van september 1991 tot september 1998 is hij hier, naast zijn overige werkzaamheden als onderzoeker, mee bezig geweest.