

COST- the acronym for European COoperation in the field of Scientific and Technical Research- is the oldest and widest European intergovernmental network for cooperation in research. Established by the Ministerial Conference in November 1971, COST is presently used by the scientific communities of 35 European countries to cooperate in common research projects supported by national funds. The funds provided by COST - less than 1% of the total value of the projects - support the COST cooperation networks (COST Actions) through which, with only around €20 million per year, more than 30.000 European scientists are involved in research having a total value which exceeds 2 billion per year. This is the financial worth of the European added value which COST achieves. A "bottom up approach" (the initiative of launching a COST Action comes from the European scientists themselves), "à la carte participation" (only countries interested in the Action participate), "equality of access" (participation is open also to the scientific communities of countries not belonging to the European Union) and "flexible structure" (easy implementation and light management of the research initiatives) are the main characteristics of COST. As precursor of advanced multidisciplinary research COST has a very important role for the realisation of the European Research Area (ERA) anticipating and complementing the activities of the Framework Programmes, constituting a "bridge" towards the scientific communities of emerging countries, increasing the mobility of researchers across Europe and fostering the establishment of "Networks of Excellence" in many key scientific domains such as: Physics, Chemistry, Telecommunications and Information Science, Nanotechnologies, Meteorology, Environment, Medicine and Health, Forests, Agriculture and Social Sciences. It covers basic and more applied research and also addresses issues of pre-normative nature or of societal importance.

PREFACE

European meat and fish producers are continuously challenged for efficiency of production, animal welfare and meat/fish quality. In meat/fish production muscle growth is the most important trait of the production economy and the muscle growth rate may influence the quality of the meat produced. Muscle fibres are formed during foetal development, and number and hypertrophic growth determines the growth rate of the animal to a large extend. The number of muscle fibre formed during foetal development is directly related to postnatal muscle growth. However, because studies on the number of muscle fibres are tedious and costly it is important that research in this area is complementary instead of duplicating. An initiative was consequently taken to initiate a COST Action entitled "The importance of prenatal events for postnatal muscle growth in relation to the quality of muscle based foods". The main purpose of the Action is to explain genetic and environ-mental variation in prenatal events (myogenesis and satellite cell behaviour) in an attempt to find new and alternative methods to be used in selection for optimising postnatal growth and meat/fish quality. Moreover the objectives are firstly to pass on the increased knowledge in this area to the scientific community, primary producers, and the derived food industry, and secondly to stimulate research, education, exchange of knowledge, technical experiences, and the mobility among scientists within the participating countries of this Action.

The present Action (COST Action 925) started in 2004, and the first two working group meetings were held in Oporto, Portugal and University of Thessaly, Greece, and results from these meetings were published in Archives of Animal Breeding, Volume 48, 2005 and in Archives of Animal Breeding, Volume 49, 2006, respectively.

The third work group meeting was held in Belek, Antalya, Turkey in collaboration with the Physiology Commission of the European Association for Animal Production, EAAP. The meeting was focused on the third milestone of the Action "*Environmental and genetic regulation of prenatal events and its importance for postnatal growth performance and meat quality*" and was carried out in serial sessions covering the two working groups:

- 1. **WG 1**: Environmental variation in prenatal events in relation to postnatal growth and meat/fish quality
- 2. WG 2: Genetic variation in prenatal events and its effect on postnatal growth and meat/fish quality

This special issue of Archives of Animal Breeding contains the contributions of the participants to the sessions organised as the 7. Satellite Symposium "Growth and Development" of the 57th Annual Meeting of the EAAP, serial working group meetings, and workshops. The papers and abstracts report on current scientific activities and results or review the status of knowledge in this research area.

We acknowledge and thank all the authors for their contribution to the sessions at the meeting and to this special issue. Furthermore, we appreciate COST for making this work possible. Finally, we thank the editor-in-Chief and the editorial board of Archives of Animal Breeding for the opportunity to publish the contributions of these working group meetings under COST Action 925.

Vice-chairman of COST 925
Marinus F.W. te Pas

Leader of WG 1 Neil Stickland Leader of WG 2 Klaus Wimmers Arch. Tierz. Dummerstorf 50 (2007) Special Issue, 05

The Royal Veterinary College, Royal College Street, London NW1 OUT, UK

JOANNE KARUNARATNE, STEPHANIE BAYOL, CLARE ASHTON and NEIL STICKLAND

Potential mechanisms for the nutritional control of muscle composition in developing pigs

(Potentielle Mechanismen von Fütterungseinflüssen auf die Muskelzusammensetzung beim Schwein)

Much of the variation within prenatal porcine litters has been ascribed to a variation in nutrition received by the developing fetuses. We have previously demonstrated that smaller fetuses develop a higher proportion of fat and connective tissue in their muscles than their larger littermates (KARUNARATNE et al., 2005). The aim of the present study is to identify the mechanisms which may explain this intralitter variation.

Twenty-three litters of porcine fetuses ranging from 36 to 86 days' gestation were used for this study. At slaughter the right *m.semintendinosus* was removed from the largest and smallest fetus from each litter and real time RT-PCR carried out in order to measure the transcript levels of specific genes. The left *m.semitendinosus* from the same fetuses was sectioned and used for the immunocytochemical localisation of IGFBP-5.

Paired t-tests showed that there was an upregulation in the expression of IGFBP-5, IGF-1 and PPAR γ , at least at later stages, in the smaller littermate compared to the largest. There was also a tendency for IGF-IR and myostatin to be upregulated in the smallest. Differences in levels of IGF-II and connective tissue growth factor (CTGF) were not demonstrated. The immunocytochemical results showed that IGFBP5 appeared to be located at higher levels in the extracellular matrix than in the muscle cells.

IGFPB5 has been shown to be upregulated with undernutrition in other studies. It has also been shown to inhibit muscle development and stimulate the growth of connective tissue. We suggest that IGFBP5, which has a known high affinity for extracellular matrix, is a key factor in the nutritional regulation of connective tissue content in muscles. PPAR γ is associated with lipid deposition and so this concurs with our previous results on fat content of muscles in littermate pairs. Myostatin is a negative regulator of muscle and was higher in the smaller muscled littermates. In conclusion, the expression of a number of genes which control muscle composition were found to be influenced by intralitter nutritional status.

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> Corresponding Author Prof. Dr. NEIL STICKLAND Department of Veterinary Basic Sciences, The Royal Veterinary College, Royal College Street, London NW1 0TU, UK

Arch. Tierz., Dummerstorf 50 (2007) Special Issue, 06-07

¹ICBAS – Institute of Biomedical Sciences Abel Salazar, University of Porto, Lg. Abel Salazar, 2, 4099-003 PORTO, Portugal.

² CIIMAR – Interdisciplinary Centre for Marine and Environmental Research, Rua dos Bragas, 289, 4050-123 Porto, Portugal.

³ Instituto Español de Oceanografia, Cabo Estay, Vigo, España.

PAULA SILVA^{1,2}, EDUARDO ROCHA^{1,2}, MERCEDES OLMEDO³, B. ÁLVAREZ-BLÁZQUEZ³, M.H. GALANTE¹ and LUISA M.P. VALENTE^{1, 2}

Temperature effects on muscle fibres number at hatch and first feeding in blackspot seabream (*Pagellus bogaraveo*)

(Temperatureinflüsse auf Muskelfaseranzahl beim Schlupf und erster Fütterung beim Graubarsch (*Pagellus bogaraveo*))

It has been shown that the thermal experience during the earliest phases of development can determine the larval and postlarval growth characteristics of teleosts (Clupea harengus, VIEIRA and JOHNSTON; 1992; Salmo salar, STICKLAND et al., 1988; Dicentrarchus labrax L., AYALA et al., 2000, 2001). In order to investigate the effects of the early temperature regime on prelarval stages of growth of Pagellus *bogaraveo*, this species was exposed during egg incubation until larvae mouth opening (vitelline phase) at two temperatures: natural temperature (\pm 14 °C) and 18.0 \pm 0.01 °C. Muscle growth was studied by morphometric and immunostaining techniques. The total number of fibres was counted in body cross sections, at both the postopercular and perianal levels. High incubation temperature accelerated embryonic (hatching) and prelarval (mouth opening) development. In newly hatched larvae, myotomes showed two muscular layers: a superficial monolayer of several fibres that extend hypaxially and epaxially away from the lateral line, and a deep layer composed of larger diameter fibres. At hatching, no significant influence of the incubation temperature was observed on muscle fibre number. At the end of the vitelline phase, three layers of muscle fibres were observed, with different antimyosin reactivities: a superficial monolayer, a presumptive slow red (present only as a small group of fibres adjacent to the lateral line nerve) and a fast-white layer. At mouth opening, the highest incubation temperature promoted an increased number of white fibres at the postopercular (N =199 (CV = 0.17)) vs lowest temperature (N = 146 (CV = 0.07)), being the white muscle fibre hyperplasia greater in the epaxial part of the larvae (P < 0.05). At hatching and mouth opening, the number of superficial monolayer fibres was similar for both tested temperatures. In conclusion, a slight increase of temperature during the vitelline phase of blackspot seabream promoted an epaxial hyperplastic growth of white fibres at the postopercular level, in the prelarval stage. It will be now very important to asses whether the initial fibre number gain can support a faster and/or greater posterior growth of fish under standard conditions.

Acknowledgments Supported by FCT (PhD Grant SFRH-BD-14068-2003).

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Corresponding Author LUÍSA M.P.VALENTE, CIIMAR – Interdisciplinary Centre for Marine and Environmental Research, Rua dos Bragas, 289, 4050-123 Porto, Portugal Arch. Tierz., Dummerstorf 50 (2007) Special Issue, 08-14

Institute of Animal Breeding and Genetics, Georg-August-University Goettingen, 37075 Goettingen, Germany

CARSTEN WERNER and MICHAEL WICKE

How is the embryonic development of the M. pectoralis superficialis in turkey embryos influenced by the incubation temperature? Theoretical background and hypothetical investigations

Abstract

MALTBY et al. (2004) presented a quite simple method to enhance the muscle fibre number of the M. semitendinosus in turkey poult by increasing the temperature between day 9 and 12 of incubation. Turkeys with a higher fibre number seem to have a lower hypertrophic growth to reach comparable meat yields after slaughter. It could be suggested that the reduced hypertrophy can decrease the incidence of deep pectoral myopathies or leg disorders and improve the well-being of the animals. However, it is not clear if the results by MALTBY et al. (2004) could be transferred to the M. pectoralis superficialis and what molecular mechanisms during embryonic myogenesis are influenced by the higher incubation temperature. Therefore in a short review morphological and molecular changes during the embryonic muscle development are presented. Morphological alterations include, for example, the recruitment of myogenic precursor cells from the dermomyotome, the outgrowth of the early muscle masses, the proliferation of the myoblasts or the differentiation of the cells to functional active fibres. In the second chapter the molecular changes in the myogenic cells during muscle development are presented focussing on the involvement of the myogenic regulatory factors (MRF) myf5, MyoD, MRF4 and Myogenin and the regulation of the mitotic cell cycle. The extra cellular signals that influence the myogenesis especially the IGF I-system or the *wnt* and Sonic Hedgehog signalling pathways are briefly presented. In the last section possible investigations are described to elucidate the thermal effects on the embryonic muscle development.

Key Words: turkey, myogenesis, incubation, muscle fibre number, cell cycle

Zusammenfassung

Titel der Arbeit: Wie wird die embryonale Entwicklung des M. pectoralis superficialis in Putenembryonen durch die Inkubationstemperatur beeinflusst? Theoretischer Hintergrund und hypothetische Untersuchungen

MALTBY et al. (2004) präsentierten eine recht einfache Methode, um die Muskelfaserzahl im M. semitendinosus von Putenküken zu erhöhen, indem die Temperatur zwischen Tag 9 und 12 der Bebrütung angehoben wurde. Puten mit einer größeren Muskelfaserzahl scheinen ein reduziertes hypertrophes Wachstum zu haben, um vergleichbare Fleischausbeuten nach der Schlachtung zu haben. Es ist anzunehmen, dass die geringere Hypertrophie das Auftreten von tiefen pectoralen Myopathien oder Beinveränderungen reduziert und das Wohlbefinden der Tiere verbessert. Es ist allerdings nicht klar, ob die Ergebnisse von MALTBY et al. (2004) auf den M. pectoralis superficialis übertragen werden können und welche molekularen Mechanismen während der embryonalen Myogenese durch die höhere Bebrütungstemperatur beeinflusst werden. Deswegen werden in einem kurzen Review morphologische und molekulare Veränderungen während der embryonalen Muskelentwicklung dargestellt. Morphologische Veränderungen beinhalten z.B. die Rekrutierung der myogenen Vorläuferzellen aus dem Dermomyotom, das Auswachsen der frühen Muskelmassen, die Proliferation der Myoblasten oder die Differenzierung der Zellen zu funktionell aktiven Muskelfasern. Im zweiten Abschnitt werden die molekularen Veränderungen in den myogenen Zellen während der Muskelentwicklung dargestellt, wobei auf die Beteiligung der myogenen Regulationsfaktoren (MRF) myf5, MyoD, MRF4 und Myogenin und die Regulation des Zellzyklus fokussiert wurde. Die extrazellulären Signale, die die Myogenese beeinflusssen, insbesondere das IGF I-System oder die wnt und Sonic Hedgehog Signalwege werden kurz besprochen. Im letzten Abschnitt werden mögliche Untersuchungen beschrieben, um die thermalen Effekte auf die Muskelentwicklung aufzuklären.

Schlüsselwörter: Pute, Myogenese, Bebrütung, Muskelfaserzahl, Zellzyklus

Introduction

Turkey strains have been intensively selected in the last decades for fast growth rates, improved feed efficiency and increased amount of muscling especially of the valuable parts of the carcass. This development resulted in changes of the muscle structure e.g.

by increasing fibre diameters. Consequences of this intensive hypertrophic growth are higher incidences of deep pectoral myopathies, leg disorders or vesicular alterations of the breast muscle surface (WILSON et al., 1990). An impact on the meat quality could be assumed although this feature is discussed very controversially (MALTBY et al., 2004). However, in order to reduce the incidence of the described pathological findings the reduction of the fibre size might be useful. But this alteration would negatively influence the breast and leg muscle yields. A better alternative would be the increase of the total muscle fibre number (MFN). This could be either realized by breeding assuming a heritability for the MFN in the turkey breast muscle of 0.12 to 0.49 (REHFELDT et al., 2000), or by changing the environment during myogenesis. Since the MFN is fixed in turkey around the time of hatch (STICKLAND, 1981) the environment could only be changed at specific periods during embryogenesis e.g. by varying the incubation temperature (MALTBY et al., 2004) or the lighting regime (ROZENBOIM et al., 2003). MALTBY et al. (2004) showed in turkey eggs that changing the temperature between day 9 and 12 of incubation from 37.5°C to 38.5°C increased the MFN in the M. semitendinosus of the turkey chicks 16 days post-hatch. ROZENBOIM et al. (2003) presented that intermittent photo stimulation of turkey eggs with green light during incubation increased the body and breast weight of the hens post-hatch. The latter authors did not determine if the green light influences the hyperplastic or -trophic growth of the turkey musculature but a positive effect on the MFN could be assumed, since the same group showed in an actual publication that in the pectoralis muscles of chicken the MFN could be influenced by green lighting (HALEVY et al., 2006).

The studies by ROZENBOIM et al. (2003) and MALTBY et al. (2004) did not clearly elucidate how the green lighting or the increased temperature influences the embryonic development resulting in a higher MFN. In the following chapters the embryonic myogenesis is described briefly focussing on the pathways that might be influenced by the described alterations of the incubation conditions.

Morphological changes during avian myogenesis

Chicken embryos are regularly staged according to HAMBURGER and HAMILTON (1951). The authors divide the embryogenesis into 45 stages. During the early development the avian embryo consists of the three germinal layers entoderm, mesoderm and ectoderm. The mesodermal germinal layer forms the somites. These "structures" are located in pairs on both sides of the notochord, the primitive vertebral column (CHRIST and ORDAHL, 1995). Somites are "epithelial balls" with a lumen containing mesenchymal cells and the amount of somite pairs corresponds to the vertebra number. In response to signals from the adjacent tissue of the somites the sclerotome is formed in the medio-ventral part and the dermomyotome in the dorsal part of the somites. The sclerotome is the origin of the axial skeleton and the ribs whereas the dermomyotome differentiates into the skeletal musculature and the dermis (CHRIST and BRAND-SABERI, 2002; HIRAO and AOYAMA, 2004; GEETHA-LOGANATHAN et al., 2005). In addition to the dorsoventral compartmentalization of the somites resulting in the formation of dermomyotome and sclerotome, mediolateral compartments are also formed in the somite especially in the dermomyotome. The medial part of the dermomyotome gives rise to the epaxial muscles and the dermis of the back and the lateral fraction forms the hypaxial musculature like the limb, thoracic and abdominal muscles (CHRIST and BRAND-SABERI, 2002). Epaxial muscles in chicken or turkey are, for example, the M. iliocostalis et longissimus a unique muscle oriented parallel to the vertebral column of the neck and breast (VOLLMERSHAUS, 1992).

Development of the hypaxial proceeds in certain steps. The lateral part of dermomyotome de-epithelializes and myogenic progenitor cells (myoblasts) migrate in to the somatopleura forming premuscle masses that continue to proliferate and subsequently differentiate. Depending on the muscles that will develop the myoblasts migrate either to the "muscle buds" giving rise to the thoracic and abdominal muscles, or at the limb level to the "limb buds" (CHRIST and BRAND-SABERI, 2002; GEETHA-LOGANATHAN et al., 2005). The formation of the limb buds occurs at stage 17 approximately 55 h after start of incubation in chicken and a little later in turkey embryos (HAMBURGER and HAMILTON, 1951). The premuscle masses consist of undifferentiated, proliferating precursor cells and differentiating myoblasts. Embryonic muscle growth is the result of a balance between proliferation and differentiation. During the proliferation of the myoblasts the myogenic precursor cells run through the cell cycle. The cell cycle usually consists of four different periods. The mitotic cell division (M-phase) and the DNA replication (S-Phase) are interrupted by two gap-phases – G1 between M and S-phase and G2 between S- and M-phase. A very important point in the cell cycle is the G1-S-phase transition. The myoblast could either progress to the S-phase, or exit the cell cycle to the GO-stage. The latter alteration induces the differentiation of the myoblasts characterized by fusion of adjacent precursor cells to myotubes and subsequent differentiation to mature myofibres (AMTHOR et al., 2002; CHRIST and BRAND-SABERI, 2002; OKSBJERG et al., 2004; HEYWOOD et al., 2005).

The process of fibre generation during avian myogenesis could be divided into two distinct phases. In the first phase at embryonic day (ED) 4 to ED 7 primary muscle fibres are formed. These cells are characterized by a larger size and the expression of specific myosin heavy chain (MyHC) isoforms. Between ED 8 to ED 15 secondary fibres are formed around the primary muscle cells using them as a scaffold (STOCKDALE and MILLER, 1987; WIGMORE et al., 1996; EDOM-VOVARD et al., 1999). Secondary muscle cells are smaller than the primaries and express different MyHC isoforms- mainly slow or fast fetal MyHC. During maturation of the secondary fibres the developmental myosin molecules are replaced by adult fast and slow MyHC isoforms (EDOM-VOVARD et al., 1999). It is interesting to note that the initial fibre formation occurs in the absence of innervation but further development depends on the formation of functional active motoneurons in the muscle tissue (FREDETTE and LANDMESSER, 1991; EDOM-VOVARD et al., 1999).

Molecular changes during avian myogenesis and regulation of these processes

Skeletal muscle development requires the coordinated expression of numerous proteins to determine mesodermal progenitor into myogenic cells, to increase the muscle cell number (proliferation) and to control the differentiation of these precursor cells into functional contractile muscles (OKSBJERG et al., 2004).

The formation of the dermomyotome in the somites requires signals originating from the adjacent ectodermal tissue traversing the extracellular matrix. The sclerotome depends mainly on the notochord and parts of the neural tube. Induction and

maintenance of the dermomyotome is mediated by wnt and Sonic hedgehog (SHH) proteins. The migrating cells in the dermomyotome are characterized by the expression of the transcription factors Pax3, Pax7 and Lbx 1. They populate the "muscle and limb buds" and express the transcription factors myf5 and MyoD – myogenic regulatory factors (MRF) (CHRIST and BRAND-SABERI, 2002; GEETHA-LOGANATHAN et al., 2005; HOLOWACZ et al., 2006). Prerequisites for the migration of the precursor cells are e.g. the activation of the tyrosine kinase receptor c-met by growth factors (e.g. HGF) secreted by cells of the "muscle or limb buds" (BLADT et al., 1995) and the expression of the cell adhesion protein N-cadherin (BIRCHMEIER et al., 1996; BRAND-SABERI et al., 1996). The epaxial and hypaxial outgrowth and the differentiation of the appropriate muscles are regulated differently. Development of the epaxial muscles depends on the neural tube and the notochord. The signalling molecule is SHH. For the hypaxial outgrowth the surface ectoderm is required (SCHMIDT et al., 2001; HIRAO and AOYAMA, 2004) but an influence of the axial structures (notochord, neural tube) was also presented (HUANG et al., 2003). The signalling proteins responsible for the formation of the hypaxial muscles are wnt proteins (Ectoderm) and SHH (Notochord, neural tube) (CHRIST and BRAND-SABERI, 2002; HOLOWACZ et al., 2006).

In the pre-muscle masses of the embryos the dividing myoblasts express MyoD and myf5. At the G1-S-phase transition is the key step during the cell cycle and the myoblasts have two options. They could either continue to the next DNA replication-phase (G1-S transition), or exit the cell cycle (G1-G0 transition). Myoblasts that exit the mitotic cell cycle down-regulate MyoD and myf5 and up-regulate the transcription factors MRF4 and Myogenin –other myogenic regulatory proteins (MRF) (LIU et al., 2005). All MRFs are under control of specific myogenic regulator proteins (e.g. (MEF2) (CHRIST and BRAND-SABERI, 2002).

The G1/S transition is controlled by D-type cyclins conjugated with the protein kinases CDK4 and 6 which act in mid-G1 and by cyclin E/CDK2 which operates in late-G1. One key substrate of these cyclin-dependent protein kinases is the retinoblastoma protein (Rb, and related proteins Rb2, p130, p107), a nuclear tumor suppressor protein. During G1-phase the kinases phosphorylate Rb. This results in the liberation of the pRb from specific transcription factors (e.g. E2f), proteins that initiate the expression of cell cycle progression genes (GALDERISI et al., 2003). An interesting feature is that the cyclin – dependent protein kinases sequester MyoD thereby keeping it inactive in the dividing myoblast (WEI and PATERSON, 2001). A key factor in the G1/S crossover is the regulation of the cyclin D expression in the myoblast. Increased expression of cyclin D could be initiated by signals through peptide growth factors (e.g. bFGF) or through cell-cell interaction (e.g. *wnt*) (WEI and PATERSON, 2001).

The G1-G0 crossover is characterized by the inactivation of the described cyclindependent protein kinases e.g. by serum withdrawal and reduced growth factor stimulation (e.g. FGF). This results in a reduced or missing phosphorylation of the Rb protein and a liberation and activation of the MyoD transcription factor which increases the expression of genes (e.g. Rb, Myogenin) that force and maintain cell cycle exit. MyoD binds Rb and prevents phosphorylation of the protein by the CDKs (WEI and PATERSON, 2001; OKSBJERG et al., 2004). The un-, respectively hypophoshorylated Rb binds E2F transcription factors thereby inhibiting the expression of cell cycle progression genes. On the other hand Rb inhibits the effects of the Id2 proteins. Id2 is a bHLH transcription factor with no DNA binding function that inhibits differentiation by binding to specific bHLH transcription factors (e.g. Myogenin). Inhibition of Id2 results in the exit from the cell cycle and differentiation of the cells (GALDERISI et al., 2003; COLOMBO and CABRELE, 2006; LASORELLA and IAVARONE, 2006). G1-G0 crossover is regulated by processes that, for example, inhibit the activity of the CDKs and reduce the cyclin concentration in the myoblast. The inhibition of the cyclin dependent protein kinases (CDK) is mediated by two classes of molecules that bind either to the kinase subunit like proteins of the Ink4 family (e.g. p16, p15, p18), or interact with the cyclin/CDK dimer like the Cip/kip family (e.g. p21, p27, p57) (GALDERISI et al., 2003). The cyclin concentration decreased in the myoblasts by increased degradation due to phosphorylation of the glucose metabolism (WEI and PATERSON, 2001) or by reduced synthesis e.g. after inhibition of the proliferation transcription factor E2F (GALDERISI et al., 2003).

Many of the intracellular alterations during myoblast development are regulated by growth factors. Unlike most growth factors e.g. the members of the TGF family which are believed to stimulate muscle proliferation and inhibit differentiation insulin-like growth factor I (IGF I) is able to stimulate both processes during embryonic myogenesis (TIFFIN et al., 2004; WINNER et al., 2006). This biphasic behaviour of the IGF makes the hormone one of the key regulators of embryonic myogenesis. The origin of the IGF protein is either the liver or other organs (endocrine), the adjacent cells (paracrine) or the same cell (autocrine). The initial effect of the hormone associated with the stimulation of myoblast proliferation is followed by cessation of this process and stimulation of differentiation (ADI et al., 2002; TIFFIN et al., 2004). IGF acts via a tyrosine kinase dependent receptor on two different signal transduction pathways - the MAPK/ERK1/2and the PI3K/ Akt pathway. The proliferative response of myoblasts is mediated by MAPK/ERK1/2, for example, by stimulating the expression of cyclin D (YANG et al., 2006). The exit from the cell cycle and the progess to myoblast differentiation is mediated by the PI3K/Akt pathway although interactions between this and the MAPK/ERK1/2 pathway could be suggested (VAN DEN VELDEN et al., 2006).

Possible influences of an increased incubation temperature on the embryonic myogenesis in turkey

Considering that the higher temperature between day 9 and 12 of incubation positively influences the fibre number not only of the M. semitendinosus – as shown by MALTBY et al. (2004) -, but also of the M. pectoralis superficialis an impact of the temperature on the cell cycle could be suggested. With regard to the complex molecular changes during the embryonic myogenesis the focus on specific pathways is necessary. The G1-S and the G1-G0 transition are important checkpoints that have to be considered since a higher fibre number is related with an increased mitotic activity of the myoblasts and/ or a delay of the G1-G0 transition. The latter is the prerequisite for the subsequent cell differentiation and the development of functional active muscle fibres. The increasing incubation temperature could influence the expression of proteins, enzyme activities or the stability of RNA and proteins. Important proteins are the cyclin-dependent protein kinases and there several inhibitors, the myogenic transcription factors MyoD, Myogenin and myf5, the Retinoblastoma protein or proteins of the involved signal transduction pathways (e.g. MAPK/ERK1/2, PI3K).

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Institut National de la Recherche Agronomique (INRA), Unité Mixte de Recherche sur les Systèmes d'Elevage, la Nutrition Animale et Humaine, 35590 Saint-Gilles, France

LOUIS LEFAUCHEUR and PATRICK ECOLAN

Changes in muscle proteome during development in the pig

(Veränderungen im Muskelproteom während der Entwicklung beim Schwein)

In the pig, the total number of myofibres (TNF) is definitely fixed at around 90 days of gestation (dg), whereas contractile and metabolic maturations mostly occur during the first three postnatal weeks. Myogenesis is a biphasic phenomenon with a primary generation of myotubes forming from 35 to 55 days of gestation (dg), followed by a second generation between 55 and 90 dg. Until recently, most studies dealing with myogenesis in the pig used a limited number of genes and/or proteins as markers. Our objective was to reassess pig skeletal muscle genesis and maturation using a proteomic approach. Samples were taken from the longissimus in 45, 50, 70, 90 and 113 (birth) day-old fetuses and in 8, 28 and 165 (100 kg BW) day-old pigs. Three different After extraction animals were analyzed per stage. in lysis buffer (urea/thiourea/CHAPS), 80 µg total muscle proteins were analyzed by twodimensional gel electrophoresis (2-DE) (BOULEY at al., 2004). Briefly, a nonlinear 3-11 pH gradient was used in the first dimension (88 kVh at 20°C). The second dimension was performed with 12.5% acrylamide gels using the Ettan DALT system. Gels were silver stained and scanned. The 2-DE reference gel was a mixture of all stages. Spot detection and quantification were performed using ImageMaster 2D Platinum software (GE Healthcare). Two-DE gels were normalized by dividing each spot volume by the total volume of all the matched spots within each 2-DE gel. About 870 spots could be matched between the gels. A principal component analysis (PCA) was conducted on centred and normalized data. The gels were clearly grouped according to the stage of development with a gradual progression from 45 dg to 165 postnatal days. To go further, an analysis of variance was carried out in order to select spots that were the most influenced by the stage of development (P<0.001, n=427 spots). These 427 spots were subjected to a second PCA followed by an ascending hierarchical clustering (AHC). Three groups of spots were obtained: 228 spots highly expressed during the fetal stages (group F), 88 spots highly expressed around birth (group B) and 89 spots highly expressed in late post-natal stages (group P). Then, spots from group F were further subjected to an analysis of variance to select those that were the most influenced by the stage of development during the fetal period (P<0.05, n=134 spots). Using a PCA followed by an AHC, these 134 spots could be clustered in 5 classes according to their precocity of development. Overall, 295 spots were selected and categorized in 7 classes. The next step will consist in the identification of the 295 spots using MALDI-TOF mass spectrometry.

Key Words: myogenesis, proteomic, electrophoresis, longissimus, pig

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Corresponding Author LOUIS LEFAUCHEUR, Institut National de la Recherche Agronomique (INRA), UMR SENAH, 35590 Saint-Gilles, France

E-Mail: Louis.Lefaucheur@rennes.inra.fr

Arch. Tierz., Dummerstorf 50 (2007) Special Issue, 17-21

Research Institute for the Biology of Farm Animals, Dummerstorf, Germany

MARCUS MAU, CLAUDIA KALBE, KARIN WOLLENHAUPT and CHARLOTTE REHFELDT

Isoflavones modify the growth response of porcine muscle satellite cell cultures to IGF-I and EGF

Abstract

Isoflavones, which are compounds of soy-based diets, have been reported to be specific inhibitors of protein tyrosine kinases that catalyze protein phosphorylation and play an important role in growth factor related signal transduction. Among others, the type 1 insulin-like growth factor receptor (IGF-1-R) and the epidermal growth factor receptor (EGF-R) act as protein tyrosine kinases and therefore could be negatively affected by isoflavones. This study was conducted to investigate, whether IGF-I and EGF stimulate porcine muscle cell growth in vitro and whether the responses are influenced by isoflavonic action. Both IGF-1-R and EGF-R were found to be expressed by RT-PCR and/or Western-Blot analysis in satellite cells from semimembranosus muscle of newborn piglets. The long-term effects (26 h) of 0.1, 1, 10 and 100 nM IGF-I or EGF, and of 10 nM IGF-I or EGF combined with 1, 10 and 100 µM genistein or daidzein on DNA synthesis were measured as 6 h-[3H]thymidine incorporation during exponential growth in serum-free basal medium. Both IGF-I and EGF increased DNAsynthesis rate with maximum responses of 234% and 367%, respectively, at 1 and 10 nM (p < 0.05), while a combined IGF-I/EGF (10 nM) treatment revealed additive effects (up to 478%). All concentrations of genistein combined with 10 nM of IGF-I or EGF (except 1µM genistein/IGF-I) reduced the stimulating effects of both growth factors in a dose-dependent manner (p < 0.05). In contrast, the combinations of daidzein with 10 nM IGF-I or EGF showed no effects (at 1, 10 µM) or even slightly increased (at 100 µM) the growth factor responses in DNA synthesis rate (p < 0.05). The results suggest that both IGF-I and EGF stimulate porcine muscle satellite cell growth. In contrast to daidzein, genistein substantially inhibits the growth response to IGF-I and EGF, which may result from interactions with the tyrosine kinase receptor molecules. These interactions and possible effects on cellular signal transduction remain to be investigated.

Key Words: genistein, daidzein, porcine satellite cells, IGF-I, EGF

Zusammenfassung

Titel der Arbeit: Isoflavone modifizieren das IGF-I- und EGF-abhängige Wachstum porciner Muskel-Satellitenzellkulturen

Isoflavone, die u.a. in Soja enthalten sind, hemmen spezifisch Proteintyrosinkinasen, welche die Phosphorylierung von Proteinen katalysieren und somit eine wichtige Rolle in der wachstumsfaktor-vermittelten Signaltransduktion spielen. Neben anderen Rezeptoren fungieren der insulin-ähnliche Wachstumsfaktor-Rezeptor Typ 1 (IGF-1R) sowie der epidermale Wachstumsfaktor-Rezeptor (EGF-R) als Proteintyrosinkinasen und können somit durch die Isoflavone negativ beeinflusst werden. Diese Studie diente dazu, herauszufinden, ob IGF-I und EGF das in vitro Wachstum porciner Skelettmuskelzellen stimulieren, und ob diese Stimulation durch Isoflavone beeinflusst werden kann. Sowohl der IGF-1R als auch der EGF-R werden in den Satellitenzellen, die aus dem M. semimembranosus neugeborener Ferkel isoliert wurden, exprimiert, was durch RT-PCR und Western-Blot gezeigt wurde. Die Langzeiteffekte (26 h) von 0.1, 1, 10 und 100 nM IGF-I oder EGF, sowie von 10 nM IGF-I oder EGF in Kombination mit 1, 10 und 100 µM Genistein oder Daidzein auf die DNA-Syntheserate wurden während der exponentiellen Wachstumsphase in serumfreiem Basalmedium über 6 h als [³H]-Thymidin-Einbau gemessen. Sowohl IGF-I als auch EGF erhöhten bei einer Konzentration von 1 und 10 nM die DNA-Syntheserate bis zu einem Maximum von 234% und 367% (p < 0.05), während die Kombination von 10 nM IGF-I mit 10 nM EGF additive Effekte zeigte (bis 478%). Alle Konzentrationen von Genistein in Kombination mit 10 nM IGF-I oder EGF (ausgenommen 1 µM Genistein/IGF-I) verringerten dosisabhängig die stimulierenden Effekte beider Wachstumsfaktoren (p < 0.05). Im Gegensatz dazu zeigten Kombinationen aus Daidzein und 10 nM IGF-I oder EGF keine Effekte (bei 1 und 10 µM), oder sie erhöhten (bei 100 µM) die wachstumsfaktor-vermittelte DNA-Synthese sogar signifikant (p < 0.05). Die Ergebnisse zeigen , dass sowohl IGF-I als auch EGF das Wachstum porciner Skelettmuskelzellen stimulieren. Im Gegensatz zu Daidzein inhibiert Genistein deutlich das IGF-I- und EGF-abhängige Wachstum der Zellen, was möglicherweise aus seiner Interaktion mit den Tyrosinkinaserezeptoren resultiert. Diese Wechselwirkungen und mögliche Effekte auf die Signaltransduktion müssen weiter untersucht werden.

Schlüsselwörter: Genistein, Daidzein, Schweinemuskelzellen, IGF-I, EGF

Introduction

Isoflavones, like genistein and daidzein, are potent effectors of porcine satellite cell growth (MAU et al., 2006). These plant-derived steroid-like dietary compounds display estrogenic activities in humans and animals, and thus are referred to as phytoestrogens (BARRET, 1996). The four major classes of phytoestrogens are the lignans, coumestanes, flavones and isoflavones (KURZER and XU, 1997). The isoflavones reach highest concentrations in legumes, especially in soybeans (0.2 - 1.6)mg/g dry weight; KURZER and XU, 1997), but can also be detected with lesser amounts in potatoes, fruits, vegetables and nuts (WHITTEN and PATISAUL, 2001). Because of being prominent food ingredients and due to their ability to interact with hormonal signalling, dietary-derived isoflavonic phytoestrogens are of great interest for cellular and developmental research. At the cellular level, isoflavonic phytoestrogens, like genistein and daidzein, are indicated to have inhibitory effects on protein tyrosine kinases (REN et al., 2001a). Especially genistein has been reported to directly interact with these enzymes (AKIYAMA et al., 1987) and in this way it could inhibit receptors of growth factors (e.g. IGF, insulin, EGF) by inhibition of tyrosine kinase phosphorylation. Both the type 1 IGF receptor (IGF-1R) and the EGF receptor (EGF-R) as targets for GF's and isoflavonic phytoestrogens have been shown to be expressed in porcine skeletal muscle (PENG et al., 1997; REN et al., 2001b). The insulin-like growth factor I (IGF-I) and the epidermal growth factor (EGF), are potent effectors of muscle satellite cell proliferation (ALLEN and RANKIN, 1990; BLACHOWSKI et al., 1993; HARPER and BUTTERY, 1995; ROE et al., 1995; DODSON et al., 1996; FLORINI et al., 1996; OKSBJERG et al., 2004). IGF-I and/or EGF were also shown to stimulate the proliferation of porcine embryonic myogenic cells (PAMPUSCH et al., 2003), satellite cell cultures (YI et al., 2001) and cell clones (DOUMIT et al., 1993).

At present, it is not known, to which extent the response of skeletal muscle cells to these growth factors would be modified by isoflavones. Therefore, the aim of this study was to investigate the effects of different dosages of growth factors (IGF-I, EGF) alone and in combination with genistein and daidzein on the *in vitro* growth of primary porcine muscle satellite cell cultures.

Material and methods

Tissue of *M. semimembranosus* was collected from newborn German Landrace piglets. Satellite cells were isolated, enriched and typified as described previously (KALBE et al., 2006). For the experiments cells were seeded in gelatine-coated 96-well microplates at about 5×10^3 cells per well. The cells were grown for 1 day in MEM α plus 10% fetal bovine serum (FBS) and 10% horse serum (HS). In each of 2 replicates, a total of 12 wells (GFs alone) or 10 wells (GFs combined with isoflavones) spread over two plates was used for each concentration of IGF-I or EGF (0.1; 1 ; 10 ; 100 nM), and of genistein (G) and daidzein (D) (0.1; 1; 10; 100 μ M) in serum-and phenol red-free basal medium with RPMI : MCDB 110 (4:1) supplemented according to DOUMIT et al. (1993). The cells were incubated with IGF-I and EGF alone or with 10 nM of either IGF-I or EGF in combination with G and D for 26 h. DNA synthesis was measured during the last 6 hours of incubation as [³H]thymidine incorporation (dpm/ μ g DNA) (REHFELDT and WALTHER, 1997). Data were subjected to analyses

of variance by using the GLM or mixed procedure of SAS with treatment and replicate as fixed factors and plate as random factor, if applicable.

Results

The expression of both the type 1 and type 2 IGF receptors and of the EGF-R was shown in the satellite cell culture by Western Blotting and/or real time RT-PCR (data not shown). Subsequently, changes in DNA synthesis rate and DNA amount in the cell culture in response to IGF-I and EGF were measured. DNA synthesis was increased by all GF concentrations used in response to 26 h of incubation (Fig. 1).



Fig. 1: DNA synthesis rate (LSMeans) in porcine satellite cell cultures after 26 h of incubation with IGF-I and EGF at concentrations ranging from 0.1 to 100 nM. Different letters indicate significant differences (p < 0.05).

For example, with 10 nM IGF-I DNA synthesis rate was doubled compared to the control, whereas 10 nM EGF even caused a 3.5-fold increase. In parts, the higher DNA synthesis rates were also reflected in slight increases in the amount of accumulated DNA in response to 1 and 10 nM IGF-I as well as to 0.1, 1 and 10 nM EGF, which is indicative of stimulated cell proliferation (data not shown).

In the following experiments 10 nM IGF-I were combined with 10 nM EGF or each of the growth factors was combined with different concentrations of isoflavones (Fig. 2).

The combination of 10 nM IGF-I with 10 nM EGF caused a 4.5-fold increase in DNA synthesis rate compared to the untreated control. DNA synthesis rate was also higher compared to IGF-I (146%) and EGF (117%) alone, which suggests that the effects of both growth factors are partially additive.

When IGF-I or EGF were combined with genistein or daidzein, the growth factor responses in DNA synthesis rate were modified significantly. With genistein the IGF-I- and EGF-dependent increase in DNA synthesis was substantially lowered with the greatest effects at the highest dosage. Only at a concentration of 1 μ M, genistein did not affect the IGF-I response. In contrast to genistein, daidzein did not attenuate the IGF-I- and EGF-dependent increases in DNA synthesis. Even an increase was seen at 100 μ M daidzein, but this might rather result from increased DNA repair activity instead of *de novo* DNA synthesis, which has been observed before (MAU et al., 2006). Lower DNA amounts (data not shown) were measured only in all combinations

with 100 μ M of genistein or daidzein, which were shown previously to cause cell death and thus to act toxic on porcine skeletal muscle cells (MAU et al., 2006).



Fig. 2: DNA synthesis rate (LSmeans) in porcine satellite cell cultures after 26 h of incubation with 10 nM IGF-I and EGF combined with genistein and daidzein at concentrations ranging from 1 to 100 μ M. $^{0}p < 0.05$ for difference to control

 $p^* < 0.05$ for difference to growth factor alone

Conclusions

From our findings we conclude that both IGF-I and EGF are efficient promoters of porcine muscle cell growth. The results after incubation with IGF-I support former results obtained with clonically derived porcine satellite cells by DOUMIT et al. (1993). However, in opposite, we were able to show also stimulating effects of EGF on porcine muscle cell cultures in serum-free basal medium. Furthermore, genistein depresses the growth factor-mediated stimulation in DNA synthesis rate in a dose-dependent manner, which may result from an inhibition of receptor tyrosine kinases (IGF-1-R, EGF-R). Daidzein shows almost no effects, but seems to promote the growth factor-dependent DNA synthesis rate at a concentration of 100 μ M. IGF-I and EGF may reduce, but are not able to prevent the toxic effects of genistein and daidzein at high concentrations (100 μ M), which have been observed previously. Conclusively, these results suggest that IGF-I and EGF at a certain extent may protect cells from adverse action of genistein.

Acknowledgements

This work was financially supported by the Deutsche Forschungsgemeinschaft (DFG; RE-978/11-1). The authors greatly acknowledge Angela Steinborn and Hilke Brandt for their skilled technical assistance.

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Corresponding Author

Dr. rer. nat. CHARLOTTE REHFELDT

Research Institute for the Biology of Farm Animals, Research Unit Muscle Biology & Growth Wilhelm-Stahl-Allee 2, 18196 Dummerstorf, Germany

Arch. Tierz., Dummerstorf 50 (2007) Special Issue, 22-23

¹Division of Nutritional Sciences, School of Biosciences, University of Nottingham, Sutton Bonington Campus, Loughborough, LE12 5RD, UK and ²Institute of Grassland and Environmental Research, Plas Gogerddan, Aberystwyth, SY23 3EB, UK

ZOE DANIEL¹, JOHN BRAMELD¹, NIGEL SCOLLAN² and PETER BUTTERY¹

Effect of maternal undernutrition on ruminant carcass and meat quality

(Auswirkung mütterlicher Unterernährung auf den Wiederkäuerschlachtkörper und die Fleischqualität)

We have previously shown that, in sheep, the majority of muscle differentiation and fibre formation takes place around d85 of gestation, with myoblast proliferation occurring before this time (FAHEY et al., 2005a). In a second study (FAHEY et al., 2005b), maternal nutrient restriction during the proliferation stage immediately before the period of major fibre formation (d30 to 70 gestation) resulted in a reduction in the numbers of fast fibres in 14 d lambs. Maternal undernutrition during (d55 to 95) and after (d85 to 115) major fibre formation did not alter the muscle fibre characteristics of 14 d lambs (FAHEY et al., 2005b).

Two sheep trials investigated whether such changes in muscle fibre characteristics persist into adulthood and what the consequences may be in terms of growth rates and carcass composition. Treatment groups were fed as for controls except that their nutrient supply was reduced to 50% of the recommended allowance from d 30 to 70 (Exp. 1) or d 30 to 85 (Exp. 2) of gestation, after which they were returned to the same level of nutrition as the control group. After weaning, twin lambs were individually housed and fed ad libitum to 24 or 17 wk of age for Exp 1 and 2 respectively. We specifically hypothesized that maternal nutrient restriction would result in a reduction in the numbers of muscle fibres in the resulting lambs and therefore lead to reduced lean growth but increased adiposity. There was, however, little or no effect of pre-natal dietary restriction during the time of muscle differentiation on the subsequent carcass quality of the adult lambs, suggesting an adaptation to the changes in muscle fibre composition seen previously at 2 wk. Allowing the lambs to grow to 24 wk (maternal restriction d30 to 70) showed some evidence of a small increase in the fat:lean ratio in the carcass of lambs subjected to maternal undernutrition, which had not been apparent when the animals were slaughtered at 17 wk (maternal restriction d30 to 85). Dietary restriction did not have significant effects upon the fibre type composition of the muscles. Presumably the animals had compensated during their growth. The question remains that had they received poor nutrition post-natally would they still have been able to compensate and would the failure to compensate influence the carcass composition?

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Corresponding Author Dr. ZOE DANIEL, Division of Nutritional Sciences, School of Bioscience, University of Nottingham, Sutton Bonington Campus, Loughborough, LE12 5RD, UK Arch. Tierz., Dummerstorf 50 (2007) Special Issue, 24

Department of Fisheries and Natural Sciences, Bodø University College, NO-8049 Bodø, Norway

C.A. JOHNSEN, M. BJØRNEVIK and C. SOLBERG

The effect of feeding regime on growth and muscle structure in Atlantic salmon (*Salmo salar* L.)

(Auswirkung eines Fütterungsregimes auf das Wachstum und die Muskelstruktur beim atlantischen Seelachs (*Salmo salar* L.))

The white muscle fibres in fish compose the major part of the skeletal muscle, and are the most valuable part of the fish from the consumer view. It is what we eat. The fish farmer is concerned about the fish growing as fast as possible, where as the consumer is focused on the sensorical quality. Important quality factors in fish are texture, colour, fillet gaping, taste and flavour. There are several indications that white muscle fibre size and recruitment have effect on texture, fillet gaping and colour in fish (JOHNSTON et al., 2000; BJØRNEVIK et al., 2004), and controlling the muscle fibre number and size may be a way to influence flesh quality in fish.

To examine the effect of restricted feeding on growth and muscle structure, farmed Atlantic salmon was fed two different feeding regimes from December 2004 to January 2006. One group was given restricted feeding, one meal every second day during winter and one meal a day during the rest of the year. The control group was fed one meal a day during winter, and 2-3 meals a day during the rest of the year. Both groups were fed to satiation. Fish were sampled for muscle structure and quality analyses in May 2005 and September 2005. The production time and total amount of feed was calculated for the whole experimental period. No differences in firmness and muscle structure between groups were found in May, but in September the restricted group had smaller mean white fibre diameter, larger fibre density and firmer texture.

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Arch. Tierz., Dummerstorf 50 (2007) Special Issue, 25-26

Nutrition Aquaculture & Genomic Research Unit, National Institute for Agronomic Research, Pôle d'Hydrobiologie, 64310 Saint Pée-sur-Nivelle, France.

HÉLÈNE ALAMI-DURANTE, NELLIE OLIVE and MICHÈLE ROUEL

Do European sea bass (*Dicentrarchus labrax*) juveniles coming from different temperatures have the same white muscle growth process?

(Wirken sich Herkünfte mit unterschiedlichen Temperaturen beim juvenilen europäischen Wolfsbarsch (*Dicentrarchus labrax*) auf den Wachstumsprozess weißer Muskulatur aus?)

European sea bass (*Dicentrarchus labrax*) is a Teleost fish of major interest for Mediterranean aquaculture. Its axial muscle growth occurred, as in other fish species reaching a large adult body size, both by hypertrophy and hyperplasia during larval and juvenile lives, and mainly by hypertrophy in adults (VEGGETTI et al., 1990). We recently showed that seawater temperature has a significant effect on the axial white muscle growth process (hyperplasia/hypertrophy) of European sea bass embryos and larvae(ALAMI-DURANTE et al., 2006). The objective of the present study was to determine if these temperature-linked changes in early axial white muscle growth process affect the subsequent white muscle growth process of juveniles.

To test this, *D. labrax* incubated and reared during larval life at constant temperatures of 13°C, 15°C or 20°C (ALAMI-DURANTE et al., 2006) were transferred at the end of larval life under ambient seawater temperature and natural light (Crete, 35°N). All juveniles were fed with a commercial diet for sea bass distributed *ad-libitum* by self-feeders. The juveniles coming from 13°C (L13), 15°C (L15) and 20°C (L20) were sampled between 29 and 35 days after the transfer, at 31-33mm total length. Muscle growth analyses were performed on transverse histological sections of juveniles stained with haematoxylin and orange G. Both the total cross-sectional area of a dorsal quadrant of white muscle and the individual outline of 200 white muscle fibres located in the deep part of this quadrant were measured by image analyses, in order to quantify total white muscle growth and white muscle growth process, respectively.

Results showed that, 29-35 days after the transfer to ambient seawater temperature, L13, L15 and L20 juveniles had a not significantly different body weight, a not significantly different total cross-sectional area of white muscle, and two distinct populations of white muscle fibres. Despite of these similarities, the white muscle growth process of the three batches of juveniles was not the same, as the juveniles had different proportions of small- and big- diameter white muscle fibres. The white muscle of L13 juveniles, which included the highest number of small-diameter white muscle fibres, grew more by hyperplasia than the white muscle of other juveniles. Incubation and larval rearing at low temperature improve thus European sea bass white muscle hyperplasia during the month following the transfer to ambient seawater temperature. Further work will be done in order to determine if these early-temperature linked differences in juvenile white muscle growth process persist at longer term.

Key Words: fish, skeletal muscle, hyperplasia, temperature

Acknowledgments

This work was part of the EU project "Muscle Ontogeny in Sea bass and Trout". We thank Prof. M. Kentouri and Ms. A. Sterioti from the Institute of Marine Biology of Crete, for fish rearing supervision and sample provision, respectively.

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> Corresponding Author Dr. HELENE ALAMI-DURANTE, Nutrition Aquaculture & Genomic Research Unit, INRA, Pôle d'Hydrobiologie, 64310 Saint Pée-sur-Nivelle, France.

E-mail: alami@st-pee.inra.fr

Arch. Tierz., Dummerstorf 50 (2007) Special Issue, 27-31

Animal Breeding and Genetics Centre, Animal Sciences Group of Wageningen University and Research Centre, Lelystad, The Netherlands

MARINUS F.W. TE PAS, INA HULSEGGE and MARCO H. POOL

Pathway analysis: Combining microarray data and physiological data to study myogenesis

Abstract

Microarray experiments investigate the changes in the expression of the transcriptome of a tissue during biological processes such as development of the tissue. Analysis usually produces a list of up and down regulated genes. While this in itself may highlight important biological processes taking place much information about relations between the genes may remain hidden in the huge amount of data. Databases can be searched via the internet might contain physiological information that can be invaluable for the understanding of the microarray results. However, the databases contain more information on humans and model animals such as mice and rats than other species. Therefore, we developed a set of PERL scripts enabling the automated search of the database for pathways using the list of expressed genes present on the microarray. A previously reported microarray experiment investigating prenatal myogenesis in pigs was used to search the KEGG database. Pathways returned by the KEGG database indicated that the gene expression patterns in several pathways suggest a single regulatory mechanism. Furthermore, pathways may be active in a specific myogenesis process such as proliferation of myoblasts or differentiation indicated by up regulated expression of most genes in that pathway. The results also indicate that pathways act together forming networks of pathways. This may give insight in higher level regulatory mechanisms taking place in the cell. We conclude that combining microarray and physiological data such as biochemical pathways in databases accessible via the internet is an important tool for gaining biological knowledge from microarray experiments.

Key Words: Bioinformatics, microarrays, Pathway analysis, KEGG Database, Myogenesis

Zusammenfassung

Titel der Arbeit: Biochemische Pfadanalyse: Kombination von microarray und physiologischen Daten zum Studium von Myogenese

Microarry Experimente untersuchen Änderungen in der Expression der Transcription eines Gewebes während der biologischen Prozesse zum Beispiel bei der Entwicklung dieser. Normalerweise erzeugt die Analyse eine Liste von auf und ab regulierten Genen. Bei diesen relativ großen Datenmengen können viele Informationen wichtiger biologischer Prozesse der Beziehungen zwischen den Genen unerkannt bleiben. Die über das Internet zu findenden Datenbanken enthalten wichtige physiologische Informationen, die für das Verständnis der microcarry Ergebnisse von unschätzbarem Wert sein können. Diese Datenbanken enthalten jedoch mehr Informationen über Menschen und Modelltiere wie z.B. Mäuse und Ratten als über andere Spezies. Daher entwickelten wir eine Reihe von PERL-Indizes für die automatische Datenbanksuche für biochemische Pfade, welche die Liste expressionierter Gene für microarry Experimente nutzen lassen. In einem zuvor beschriebenen microcarry Experiment, in welchem die pränatale Myogese bei Schweinen untersucht wurde, konnten diese Indizes für die Suche der KEGG Datenbank genutzt werden. Die durch die biochemischen Pfade mittels der KEGG aufgezeigten Wege zeigten einfache Regulationsmechanismen für das Muster der Genexpression. Außerdem können in spezifischen Myogeneseprozessen, wie der Zellteilung von Myoblasten oder der Differenzierung biochemische Pfade aktiv sein, und diese Pfade die Regulation der Genexpression der meisten Gene anzeigen. Die Ergebnisse zeigen auch, dass die biochemischen Pfade in einem Netzwerk fungieren. Dies gibt Einblick in das in der Zelle wirkende hohe Niveau der Regulationsmechanismen. Es wird geschlussfolgert, dass die Kombination von microarry und physiologischen Daten in den über das Internet zugänglichen Datenbanken ein wichtiges Werkzeug für biologische Erkenntnisse von microarry Experimenten bilden.

Schlüsselwörter: Bioinformation, Microarry, biochemische Pfadanalyse, KEGG Datenbank, Myogenese

Introduction

Mammalian myogenesis or muscle fibre formation is an exclusive prenatal process under strict genetic regulation (REHFELDT et al., 2004; STICKLAND et al., 2004; TE PAS and SOUMILLION, 2001). Livestock meat producing animals such as pigs are important model organisms because of a selection history of several decades for increased meat (muscle) growth (MERKS, 2000). Previously we reported on studies aiming to elucidate Transcriptome regulation during myogenesis in prenatal pigs using microarrays (TE PAS et al., 2005A, 2005B, 2006). We also reported on differential Transcriptome regulation in pig breeds differing in muscularity (CAGNAZZO et al., 2006) indicating the genetic bases underlying selection. Microarraying is a powerful technique to simultaneously analyse the changes in the mRNA expression of all genes in a cell type or tissue. Analysis of up or down regulation of groups of genes allowed to indicate processes taking place including not only proliferation and differentiation including the expression of muscle structural genes, but also energy metabolism.

Such microarray analyse however is limited to regulated individual genes and does not necessarily provide biological knowledge of processes taking place. Presently an overwhelming amount of information about physiological and biochemical processes in humans and model animals is available in databases on the internet. Similar physiological information from other species is more limited. This study aims to develop a methodology to combine the power of the microarray technique and the information available in the KEGG (Kyoto Encyclopaedia of Genes and Genomes) database containing information on a large number of physiological / biochemical pathways taking place in the cell (KANEHISA et al., 2006), called pathway analysis (PAPIN et al., 2004). The aim of this analysis is to gain more biological relevant information about the genetic regulation of myogenesis in pigs using physiological information from other species.

Materials and Methods

Development of PERL scripts to search the KEGG pathway database

The KEGG data base (http://www.genome.ad.jp/kegg/) contains general and speciesspecific information on biological pathways (KANEHISA et al., 2006). While searching the KEGG database for known pathways we found that genes were not found because they may be represented with several synonyms that were not all linked to the pathways in the KEGG data base. Therefore, we first linked the microarray data with a local MySQL installation of the Gene Ontology (GO) database (http://www.godatabase.org/cgi-bin/amigo/go.cgi) containing data of the monthly release of the GO database to collect all the synonyms of gene names (some of them obsolete) and added these to the file before searching the KEGG database. To automate the searching and retrieving of pathway data from the KEGG database a script (http://www.perl.com/) was written using the KEGG PERL API (KAWASHIMA et al., 2003). Direct links to each pathway for each gene were added to the file.

Microarray data

Microarray data have been previously reported (TE PAS et al., 2005A, 2005B, 2006).

Results and Discussion

Development of the pathway analysis tool

The amount of physiological information known and present in the databases varies between species. Physiological studies have been mostly conducted with human material and model (laboratory) species such as mice and rats, but less with livestock

animals. Comparative genomics suggests that data derived from these species can be extrapolated to other - related - species. Using such information the KEGG database contains both species-specific and general pathways called reference pathways. Especially for non-human and non-model-animal species it may be important that analyses can use this information. However, many software tools for pathways analysis such as Whole Pathway Scope (http://www.abcc.ncifcrf.gov/wps/wps_login. php?typ=download) and GOminer (http://discover.nci.nih.gov/gominer/) and others use species-specific ID's to search the pathway database resulting in a limited number of pathways retrieved for non-human and non-model animal-species including livestock. Therefore, we use gene names to search the KEGG and other pathway databases. Since many genes have multiple names often related to their history (e.g. the muscle regulatory gene MRF4 has been previously named myf-6 and herculin) and not all databases recognize all names, it is important to collect all known names of all genes. Therefore, we first searched the GO database and updated the gene list with all synonyms. The pathway database was searched with the updated gene list. If the gene was found in the database a link to all pathways where the gene was found was added to the file (Fig. 1).

It should be noted that physiological information for many genes is still lacking. Most genes with known pathway information were found in a single pathway. However, there are genes found in more than one pathway, and a limited number in over ten pathways. Presently it is unknown whether the lack of physiological information is causing this difference between genes, or that many genes are specific to a single pathway while others are more generally acting in multiple pathways. The latter genes may be also important to connect pathways into a network.

The pathway information was combined with the microarray information (Fig. 1). This study analyses a time series of microarrays expressing patterns of the genes related to different steps in the myogenesis process. The results showed that most genes within involved pathways had similar expression patterns during development suggesting a single regulatory mechanism. If we assume that up regulated expression means that the pathway is actively involved in regulation of the myogenesis process (e.g. proliferation of myoblasts or differentiation) we gain biological knowledge on how myogenesis is proceeding during the development of the embryo, and therefore about the mechanism of genetic regulation of myogenesis.

The information of pathways returned by the KEGG database search suggested that pathways connect into a network (Fig. 1). This higher level of analysis may provide insight into how biochemical reactions and physiological pathways interact inside and between the cells. Pathways are human made representations of biochemical processes, which may not fully be described by a single pathway. The overall is aim is enhancing physiological understanding of biology.

Myogenesis-related pathways – first results

The KEGG database search returned 88 pathways, 21 of which showed sufficient information (i.e. number of genes on the pathway with microarray information) for further analysis. The results indicated pathways specifically up regulated during proliferation of myoblasts. Others were specifically up regulated during differentiation. Primary and secondary waves of muscle fibre formation (REHFELDT et al., 2004; STICKLAND et al., 2004) differed in expression levels of pathways.

The results suggested two larger networks of pathways (Fig. 2). One network consisted of 14 pathways involved in the regulation of the expression of the actin cytoskeleton in the cells containing four independent links to processes initiating proliferation of cells, and one to processes inducing contraction of the cells. Another complex network of ten pathways may highlight the regulation of the complex pattern of glycolytic and oxidative energy metabolism observed during myogenesis (TE PAS et al., 2005A, 2005B, 2006) and the differences in energy metabolism expression between pig breeds during myogenesis (CAGNAZZO et al., 2006).



Fig. 1: Lay out of the pathway analysis method



Fig. 2: Networks of pathways involved in Regulation of Actin Cytoskeleton (A) and Regulation of energy metabolism (B). Pathways are indicated in boxes. Arrows denote interactions between pathways in the network.

Conclusion: New knowledge derived from the microarrays using pathways analysis The pathway analysis provided information about physiological processes taking place during myogenesis in pigs. Furthermore, higher level regulatory mechanisms of pathways acting together in networks are suggested that may provide insight in the network of biochemical reactions taking place in and between cells. Similar analyses in other experiments (e.g. *Salmonella enteritidis* infection in chicken) using the same tool confirm the usefulness of meta-analysis of large microarray datasets.

Acknowledgement

This research was supported by internal WUR funds (grant numbers 213.213.4000 and 4434.6057.00). Microarray data were from FP5 EU project PorDictor, contract number QLTR-1999-31363.

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Corresponding Author

MARINUS F.W. TE PAS, Wageningen University and Research Centre, Animal Sciences Group, Division of Animal Resource Development, ID-Lelystad, PO Box 62, 8200 AN Lelystad, The Netherlands Arch. Tierz., Dummerstorf 50 (2007) Special Issue, 32-33

Station de Recherches Avicoles, INRA, 37380 Nouzilly, France

CÉCILE BERRI, ESTELLE GODET, NADIM HAJ HATTAB and MICHEL J. DUCLOS

Effect of genotype and early nutrition on the number and activity of satellite cells in chicken Pectoralis major muscle

(Einfluss von Genotyp und früher Ernährung auf die Anzahl und die Aktivität von Satellitenzellen des Pectoralis major Muskels beim Huhn)

We conducted the present study to determine the implication of satellite cells during the early post-hatch growth, in the chicken *Pectoralis major* (P. major) muscle. In a first experiment, we compared chicks from two experimental lines divergently selected for high or low growth rate (HG and LG), which were either fed (F) as of hatch or 2 days later (DF for delayed feeding). We sampled the P. major muscle on days 2, 4, 7 post-hatch. We estimated the number of satellite cells on frozen muscle cross-section (10 μ m thick) using a monoclonal antibody against PAX7 (DSHB, Iowa, USA) which specifically label satellite cells and the total number of muscle nuclei by Hoechst 33258 staining. In a second experiment, we sampled the P. major muscle of 3 day-old LG and HG chicks injected with BrdU one hour before sacrifice (100 μ g BrdU/g). In a third experiment, we sampled the P. major muscle of BrdU injected HG chicks at hatch and on day 3 post-hatch. In this case, chicks were either fed as of hatch or unfed until 3 days. In these two last experiments, we estimated the number of satellite cells and nuclei as in the first experiment and the proliferative activity of cells by using a monoclonal antibody against BrdU (Biomeda).

Whatever the stage of development and the regimen, the HG chicks exhibited higher body and P. major muscle weight than the LG chicks. In HG chickens, delayed feeding (DF) induced a delay in growth and muscle development. In LG chickens, DF did not alter overall body growth, but decreased P. major muscle weight and yield at 2 and 4 days post-hatch.

The PAX7/total nucleus ratio decreased with age during the first week. This decrease occurred between 2 and 4 days in DF chicks but only after 4 days in F chicks. At 3 day post-hatch we detected a slightly higher PAX7/total nucleus ratio in LG than in HG birds. By contrast, the BrdU/total nucleus ratio or BrdU/PAX7 was much lower in LG than in HG chicks, suggesting a higher proliferative activity in the latter genotype. In the same way, feeding induced a significant increase in the BrdU/total nucleus and BrdU/PAX7 ratios between hatch and 3 days in fed birds suggesting increased cell proliferation. Feeding also induced a decrease in PAX7/total nucleus ratio between hatch and 3 days.

Assuming that PAX7 expression measures satellite cell number and BrdU incorporation cell proliferation, our data show that in the chick the variations in growth rate are related to variations in satellite cell proliferative activity rather than in satellite cell number. These results obtained on tissue section are in agreement with previous observations obtained on the same models by measuring mRNA levels on muscle extracts (BERRI et al., 2006).

Key Words: Muscle, Growth, satellite cell, proliferation

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Growth and differentiation of the chicken *Pectoralis major* muscle: Effect of genotype and early nutrition. Arch. Tierz., Dummerstorf **49** (2006) Special Issue, 31-32

Corresponding Author BERRI CECILE, INRA, Station de Recherches Avicoles, 37380 Nouzilly, France

E-Mail: berri@tours.inra.fr

Arch. Tierz., Dummerstorf 50 (2007) Special Issue, 34

National Institute for Agricultural Research, the joint Unit research for fish Physiology, Biodiversity and the environment, INRA Scribe, IFR140, Campus de beaulieu, 35042 Rennes, France

PIERRE-YVES RESCAN, JEROME MONTFORT and AURELIE LECAM

Dynamic gene expression in fish muscle during the compensatory growth induced by a food deprivation-refeeding sequence

(Dynamische Genexpression beim Fischmuskel während des kompensatorischen Wachstums nach Entzug und Wiederaufnahme der Fütterung)

Compensatory growth is a phase of rapid growth, greater than normal growth rates, that is triggered by adequate refeeding of animals following a period of weight loss caused by starvation. In this study, to know more on the system-wide integration of compensatory growth in muscle, we compared gene expression profiling in white muscle from one month fasted, 4, 7, 11 and 36 day-refed, trout using a trout microarray containing approximately 10000 cDNAs. Hierarchical clusterering analysis and SAM (significance analysis of microarrays) revealed the transient induction of a set of approximately 500 genes 4, 7 and 11 days post refeeding. This cluster was dominated by genes involved in transcription, ribosomal biogenesis, translation, folding of proteins, intracellular trafficking, ATP synthesis and cell proliferation. A cluster of 200 genes induced only from 7 days after refeeding onward was also identified that included a large set of genes encoding myofibrillar proteins. In contrast genes involved in protein catabolism and β oxidation were highly expressed in muscle of fasted animals and down regulated during muscle compensatory growth. Remarkably, some genes differentially expressed during the fasting-refeeding transition were themselves transcriptional regulators, belonging especially to the zincfinger protein family: they might be responsible in muscle of the gene-specific transcriptional adaptations induced by refeeding.

Acknowledgement This work was supported by INRA, le CIPA, l'IFOP and l'OFIMER.

> Corresponding Author PIERRE-YVES RESCAN Scribe-INRA, Campus de Beaulieu, 35042 Rennes, France

E-Mail: rescan@beaulieu.rennes.inra.fr

Arch. Tierz., Dummerstorf 50 (2007) Special Issue, 35-43

Institute of Genetics and Animal Breeding, Polish Academy of Sciences, Jastrzębiec, ul. Postępu 1, 05-552 Wólka Kosowska, Poland

J. WYSZYŃSKA-KOKO, M. PIERZCHAŁA, P. URBAŃSKI, and T. BLICHARSKI

Allele frequency of chosen candidate genes of a *MyoD* family and somatotropin axis in two groups of Polish Landrace and Polish Large White pigs of a high and low meatiness

Abstract

Body composition in adult animal is a result of a precisely regulated balance between the action of different systems of factors and hormones during body and tissue development. The central role in muscle tissue development is played by four genes of MyoD genes family: MYF3, MYF5, MYOG and MYF6. The factors and hormones of somatotropin axis are proved to be related with MyoD genes expression and are essential for body, muscle and adipose tissue development. In the group of these genes seven described mutations were chosen for the present study: MYF3 – two SNPs in exon 1, MYF5 – SNP in the promoter region, Myogenin – SNP in 3'flanking region, MYF6 – SNP in the promoter region. Novel mutations: GH –in exon 2 and GHR –in exon 10. Boars of two breeds: Polish Landrace and Polish Large White, divided into two groups – high and low average backfat thickness, were genotyped for chosen mutations. Statistically significant differences in the allele frequencies of $MYF3/BssSI_1$ polymorphism in Polish Large White and GHR/KspAI in Polish Landrace boars of the high and low fat content were stated.

Key Words: MyoD family genes, somatotropin axis, alleles frequency, Sus scrofa

Zusammenfassung

Titel der Arbeit: Allelfrequenzen ausgewählter Kandidatengene einer MyoD Familie und Somatotropinachse bei zwei Schweinegruppen der Polnischen Landrasse und Polnischen Large White mit hoher und niedriger Rückenspeckdicke

Die Körperzusammensetzung beim erwachsenen Tier ist das Ergebnis eines genau geregelten Gleichgewichtes zwischen dem Zusammenwirken von verschiedenen Systemen von Faktoren und Hormonen im Körper und der Gewebeentwicklung. Die Hauptrolle bei der Muskelgewebeentwicklung spielen vier Gene der *MyoD* Genfamilie: *MYF3*, *MYF5*, *MYOG* und *MYF6*. Faktoren und Hormone der Somatotropinachse wurden in ihrer Beziehung zur *MyoD* Genexpression untersucht, die für die Körper-, Muskel- und Fettgewebeentwicklung unabdingbar sind. Aus der Gruppe dieser Gene wurden sieben beschriebene Mutationen für die Studie ausgewählt: *MYF3* – zwei SNPs in exon 1, *MYF5* – SNP in der Promoterregion, *Myogenin* – SNP in der 3'd flanking Region, *MYF6* – SNP in der Promoterregion. Neuartige Veränderungen : *GH* –in exon 2 und *GHR* –in exon 10.

Eber aus zwei Rassen, Polnische Landrasse und Polnische Large White, wurden in zwei Gruppen geteilt (hohe und niedrige durchschnittliche Rückenspeckdicke) und nach ausgewählten Mutationen genotyped. Statistisch signifikante Unterschiede bei den Allellfrequenzen des *MYF3/BssSI_1* Polymorphismus beim Polnischen Large White und *GHR/KspAI* bei Polnischen Landrasseebern mit hoher und niedriger Rückenspeckdicke wurden festgestellt.

Schlüsselwörter: MyoD Genfamilie, Somatotropinachse, Allel-Frequenzen, Sus scrofa

Introduction

Body composition in adult animal is a result of a precisely regulated balance between the action of different systems of factors and hormones during body and tissue development. The central role in the processes of myogenesis is played by factors belonged to MyoD family, that regulate particular stages in myogenesis by activating expression of muscle specific genes. *MyoD* genes family consists of four genes coding for transcription factors: *MYF3* (TAPSCOTT et al., 1988), *MYF5* (BRAUN et al., 1989), *MYOG* (WRIGHT et al., 1989) and *MYF6* (BRAUN et al., 1990a). The products of *MYF5* and *MYF3* control the establishing the of the primary pool of

primary muscle cells, and their migration and proliferation during the development of muscle tissue (ARNOLD and WINTER, 1998). Experimental knock-out of one of these genes proved their capability to overlap the partial function of each other (BRAUN et al., 1992; RUDNICKI and JAENISCH, 1995). However, MYF5 is suggested to be responsible for migration of myoblasts (ARNOLD and WINTER, 1998), while MYF3 - proliferation of primary muscle cells (BUCKINGHAM, 1992). Downregulation of MYF5 and upregulation of MYF3 are correlated with an exit of myoblasts from cell cycle (BUCKINGHAM, 1992). The products of MYOG and MYF6 are known as crucial factors in myoblasts differentiation and maturation. Myogenin seems to be a crucial factor needed for differentiations of myoblasts. Knock-out experiments showed that mice lacking MYOG died perinataly and had no differentiated muscle fibers, although they had myoblasts in appriopriate number and location (HASTY et al., 1993; NABESHIMA et al., 1993). This gene seems to be irreplaceable in his functions although expression of transgene of 1.6 kb promoter of MYOG and coding sequence of MYF6 restores the function of myoblasts early fusion (ZHU and MILLER, 1997). The role of MYF6 gene is not fully known, but high MYF6 expression is correlated with the process of fusion of myotubes (ZHOU and BORNEMANN, 2001). It is also, together with low expression of other *MyoD* genes, proved to be a marker of maturation of muscle tissue and its low growth potential (TE PAS et al., 2000).

Growth of animal is largely under control of growth hormone (GH) and factors and receptors mediating its action called as somatotropin axis. The factors of this axis are also in relationship with *MyoD* genes family expression and directly are essential for body, muscle and indirectly adipose tissue development. There are several studies showing an association between polymorphism of the genes of somatotropin axis and carcass characteristics of pigs. FRANCO et al. (2005) showed associations of the point mutations in *GH*, *Pit1* and *GHRH* genes and traits related to growth rate and fat content in pig. Mutation in 3 intron of IGF2 gene is proved to cause major QTL effect on muscle growth in pigs (VAN LAERE et al., 2003). Igf1 and 2 upregulate *MYOG* gene expression and in this way influence the muscle specific genes expression (FLORINI et al., 1991). TE PAS and co-workers (2000) showed, that in the pigs line selected for high growth rate (so possibly having upregulated genes of somatotropin axis), *MYF3*, *MYF5* and *MYOG* are also upregulated in various groups of muscles, while in the pigs line selected for lean meat content (having high content of maturated muscle tissue but low growth rate), higher levels of Myf-6 were stated.

Point mutations in MyoD family genes were also investigated wether they had any relationship with carcass quality traits. URBAŃSKI et al. (2006) showed some interesting associations of *MYF3* point mutations and the loin traits and lean meat content, what seems to confirm the results of LEE et al. (2003) who mapped QTL for lean meat content in the region encompassing the *MYF3* locus. *MYF6* appeared to associate with right side carcass weight and growth rate, but this association was breed dependent (WYSZYŃSKA-KOKO et al., 2006).

On the base of the results of our previous study (URBAŃSKI et al. 2006, WYSZYŃSKA-KOKO et al. 2006) allele frequencies of several point mutations in *MYF3*, *MYF5*, *MYF6*, *MYOG*, *GH* and *GHR* genes were investigated in two groups of boars of extreme backfat thickness values.

Material and methods

Animals

700 Polish Landrace and Polish Large White breeding boars were used to choose among them two groups of animals of extreme values of backfat thickness. About 100 boars per breed were chosen. Average backfat thickness of group 1 of Polish Landrace boars was 6.7 mm, Sd=0.37; group 2 of Polish Landrace – 11.8 mm, Sd=0.84. Group 1 of Polish Large White - 6.6 mm, Sd=0,43; group 2 of Polish Large White – 11.9 mm, Sd=0.57. Measurement was done using ultrasound PIGLOG 105 (SFK Technology, Denmark) during performance-testing period in standarized conditions in Control Station, according to rutin living performance control protocol.

Blood collection and DNA extraction.

Blood samples were collected from all boars in standard procedure using collection tubes with EDTA. DNA was isolated from the whole blood according to method described by KAWASAKI (1990).

fragments		PCR product	primer sequences	references	PCR thermal profile
MYF3	exon 1 of MYF3 gene	330 bp	F 5'-ctgggatatggagctgctgt -3' R 5'-gcgttagtggtcttgcgttt-3'	URBAŃSKI et al. 2004a	1x 95°C-15' 32x 94°C-30'' 55°C-30'' 72°C-45'' 1x 72°C-7'
MYF5	promoter of <i>MYF5</i> gene	297 bp	F 5'-agtttagaggtgcagtcagtg -3' R 5'-gcacttcagtaatggagtgg-3'	URBAŃSKI et al. 2004b	1x 95°C-15' 32x 94°C-30'' 55°C-30'' 72°C-45'' 1x 72°C-7'
MYOG	3' flanking region of <i>MYOG</i> gene	353 bp	F 5'-tcaggaagaactggaggctg-3' R 5'-gtttcctggggtgttgc-3'	SOUMILLION et al. 1997*	1x 94°C-3' 38x 94°C-30'' 60°C-45'' 72°C-45'' 1x 72°C-5'
MYF6	promoter region of <i>MYF6</i> gene	322 bp	F 5'-aggaaagagatttacctgcttct-3' R 5'-atcgaatgcttcggtttg -3'	WYSZYŃSKA- KOKO, KURYŁ 2004	1x 95°C-15' 32x 94°C-40'' 52°C-40'' 72°C-50'' 1x 72°C-10'
GH	exon 2 of <i>GH</i> gene	bp	F 5'-gctcgatattgatgaccctgat-3' R 5'-agagatctgcttcccctgttaa-3'	JIANG et al, 1996*	1x 95°C-15' 28x 94°C-30'' 61°C-30'' 72°C-45'' 1x 72°C-7'
GHR	exon 10 of GHR gene	bp	F 5'- gctcgatattgatgaccctgat -3' R 5'- agagatctgcttcccctgttaa -3'	-	1x 95°C-3' 64°C-1' 72°C-1' 34x 95°C-1' 64°C-1' 72°C-45'' 1x 95°C-3' 64°C-1' 72°C-5'

Table 1

Amplified fragments, primer sequences and polymerase chain reaction thermal conditions

* references applying only to primer sequences

Polymerase chain reactions

Fragments of *MYF3*, *MYF5*, *MYF6*, *MYOG*, *GH* and *GHR* genes were amplified in polymerase chain reaction (PCR). Sequence of primers, the region amplified and temperature profile of the reaction are shown in Table 1. In case of *MyoD* genes, the reaction conditions of earlier studies were used. In case of *GH* and *GHR* genes, the primers were design using the Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) on the basis of sequence available in GenBank

database. For the fragments of *MYF3*, *MYF5* and *MYF6* genes the reaction mixes (12,5 μ l) comprised 30 ng genomic DNA (3 μ l), 1.5 mM MgCl₂ in PCR Buffer, 1xQ-Solution, 20 pmol of each primer, 0.25 mM of each dNTP and 0.3 U of HotStart Taq DNA Polymerase (Qiagen, Germany). For *MYOG*, *GH* and *GHR* genes fragments the reaction mix (12,5 μ l) comprised 30 ng genomic DNA (3 μ l), 1.5 mM MgCl₂ in PCR Buffer, 20 pmol of each primer, 0.25 mM of each dNTP and 0.3 U of AmpliTaq DNA Polymerase (Applied Biosystems, USA).

Sequencing

Sequencing was performed for the fragments of *GH* and *GHR* genes in case of randomly chosen samples of Polish Large White and Polish Landrace boars, using an ABI Prism 310 automatic sequencer and ABI Prism BigDye Terminator Cycle Sequencing Kit, according to the procedure described by the manufacturer.

Bioinformatic polymorphism searching

For detecting novel mutations in GH and GHR genes, bioinformatic tools were used. The obtained sequence of GH and GHR genes were compared with corresponding fragments of these genes using Sequencher 4.2 software (reg number 2304043, USA). Changes in nucleotide sequence were verified by PCR-RFLP analysis, for which analysis earlier restriction was performed using AnnHvb а (http://www.bioinformatics.org/annhyb/) V2.0 NEBcutter and (http://tools.neb.com/NEBcutter2/index.php) softwares.

Restriction analysis and electrophoresis

In case of MyoD genes, restriction enzymes and electrophoresis conditions were used on the basis of our earlier studies (URBAŃSKI et al. 2004, WYSZYŃSKA-KOKO et al. 2004). For *GH* and *GHR* the reaction mix of a total volume 10 µl contained 5 µl of the PCR product and 2,5 U of *DdeI* restriction enzyme for *GH* and *KspAI* for *GHR* fragment (New England Biolabs). The overnight digestion was followed by 3% agarose gel electrophoresis (Sigma).

Statistical analysis

Unbiased estimate of the P-value of a log-likelihood (G) was performed, based exact test for the estimations of differences in alleles frequency between groups of pigs (GOUDET et al., 1996). These calculation were made by using population genetics software package GENEPOP ver 3.4. (RAYMOND and ROUSSET, 1995).

Results

GH and GHR genes fragments sequence and polymorphism

To obtain *GH* and *GHR* genes fragments sequence, designed primers were amplified, purified and sequenced. The sequences were deposited in GenBank database under the accession numbers: the fragment of exon 2 of *GH* gene – DQ384866 and the fragment of exon 10 of *GHR* gene – DQ388035. In the next step the obtained sequences were compared *in silico* with known porcine *GH* and *GHR* – respectively – genes sequences, available in GenBank database. According to this procedure, two novel single nucleotide polymorphisms (SNPs) were found: transition G157A in *GH* gene, that appeared to change amino acid sequence from arginine to glutamine, and
transition A208G in *GHR* gene, not changing amino acid sequence. The novel SNPs existence was confirmed by restriction enzymes digestion. Allel A in *GH* gene fragment creates the restriction site for *Dde*I enzyme. Allel G in *GHR* gene fragment creates the restriction site for *KspA*I enzyme, together with reverse primer, changing one nucleotide in the obtained sequence (A \rightarrow C in the 18 position of primer).



Fig. 1: *Dde*I digestion of *GH* fragment. PCR product 426 bp is cut into 2 fragments 271 and 155 bp.



Fig. 2: *KspA*I digestion of the *GHR* fragment. The 230 bp fragment is cut into two fragments 211 and 19 bp.

Genotyping and alleles frequency

Breeding boars of Polish Landrace breed appeared to have slightly lower backfat thickness parameters (average backfat thickness: 9.4 mm, Sd=2.7) than Polish Large White boars (average backfat thickness: 9.5 mm, Sd=2.7). That was the reason of different border values of this trait for the two breeds analysed, needed to keep similar number of animals in both breeds. About 100 boars per breed were genotyped regarding chosen SNPs, using PCR-RFLP method. The polymorphisms were chosen basing on the results of our earlier studies: MYF3 gene - two SNPs in exon 1 both recognized by BssSI restriction enzyme; MYF5 gene - SNP in the promoter region, recognized by HinPI restriction enzyme and MYF6 gene - transition in promoter region, recognized by MspI restriction enzyme. Polymorphism MYOG/MspI was found and described by SOUMILLION et al. (1997). Polymorphisms in GH and GHR genes are novel SNPs found in the present study. The frequency of alleles in two groups of Polish Landrace and Polish Large White breeding boars of extreme backfat thickness values are shown in Table 2. In Polish Landrace breed only frequency of GHR alleles differed significantly (P≤0.05) between the two groups of boars. Polish Large White boars appeared to be monomorphic regarding to this SNP. In this breed the only statistically significant difference in alleles frequency between two groups of boars analysed (P≤0.05) was stated in case of MYF3/BssSI 1 polymorphic site. Differences in other polymorphic sites in both breeds appeared to statistically insignificant.

Table 2

Allele frequency in chosen polymorphic sites of two Polish Landrace and Polish Large White groups of boars differing in backfat thickness. By * the statistically significant differences ($P \le 0.05$) between allele frequency in I and II groups of boars were marked.

breed		Polish Landrace		Polish Large White	
locus	alleles	group		group	
		Ι	II	I	II
GH/DdeI	1	0.100	0.159	0.303	0.368
	2	0.900	0.841	0.697	0.632
GHR/KspAI	1	0.500*	0.660*	1.000	1.000
	2	0.500*	0.340*	0.000	0.000
MYF3/BssSI_1	1	0.750	0.744	0.792*	0.654*
	2	0.250	0.256	0.208*	0.346*
MYF3/BssSI_2	1	0.196	0.183	0.111	0.179
	2	0.804	0.817	0.889	0.821
MYF5/HinPI	1	1.000	0.956	0.947	0.925
	2	0.000	0.044	0.053	0.75
MYF6/MspI	1	0.375	0.382	0.280	0.283
	2	0.625	0.618	0.720	0.717
MVOCM	1	0.327	0.208	0.385	0.360
MYOG/Mspl	2	0.673	0.792	0.615	0.640

Discussion

The genes of *MyoD* family and somatotropin axis are studied as candidate genes for carcass quality and growth rate traits in pigs (PIERZCHAŁA et al., 2003; FRANCO et al., 2005; URBAŃSKI et al., 2006; WYSZYŃSKA-KOKO et al., 2006). Basing on our earlier studies, SNPs of *MyoD* family genes and two novel SNPs in *GH* and *GHR* genes were chosen for further studies. In the study of URBAŃSKI et al. (2006) sows of Polish Landrace and Polish Large White breed were divided into particular groups relating to carcass lean meat content and loin eye area. Extreme values for lean meat

content were 56% (first group <56%) and 63% (second group >63%), and for loin eye area – 49 cm² (first group <49 cm²) and 62 cm² (second group >62 cm²). The alleles frequency of the polymorphic site, here marked as *MYF3_BssSI_1*, in these two groups of both breeds remained unchanged. On the contrary, alleles frequency of the polymorphism here marked as *MYF3_BssSI_2*, differed highly significantly (P<0.001) between two groups of sows of both breeds, regarding lean meat content and loin eye area. Animals of GG genotype were much more frequent in the groups of higher meatiness values. The G566C SNP change amino acid sequence from arginine to proline in the region of transactivation domain of Myf-3 factor, thus being considered as a causative mutation. Proline existence caused by C allele changes the structure of α -helix in the region necessary for proper activation of the protein. The allele frequency of SNP in *MYF5* gene (URBAŃSKI et al. 2006), as well as SNPs in *MYF6* and *MYOG* (WYSZYŃSKA-KOKO, unpublished data), remained unchanged in these two groups.

Continuing the research on boars, easily available data of backfat thickness were used, as it is a constant element of predicting meatiness characteristics of boars used for breeding in Poland. It is known that lean meat content is highly related to fat content (and is dependent on it), as well as to growth potential (TE PAS et al., 2000). In case of *MyoD* family genes, the results similar to these, earlier obtained on sows, were expected. As it is shown in Table 2, no statistically significant difference in allele frequency of polymorphic site *MYF3_BssSI_2* was stated. It can be caused by several reasons. First, lean meat content and loin eye area, measured in the earlier study, can be formed independently on backfat deposition during body development. Second, the processes of meat and fat deposition are in part different in sows and boars, changed by sex hormones. Third, the mutations in *MYF3* gene are cause effects to small to be detected in the method shown in present study. To distinguish it, further more detailed analyses are necessary.

The difference in allele frequency in *GHR* gene in Polish Landrace boars seems to be interesting, as genes of somatotropin axis are more related to growth rate and fat content traits in pigs (TE PAS et al., 2000; PIERZCHAŁA et al., 2003; FRANCO et al., 2005). Because the mutation does not change amino acid sequence, it cannot be considered as causative one. Its relation to fat content in pigs should be confirmed in the other breeds.

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Corresponding Author JOANNA WYSZYŃSKA-KOKO, PhD Institute of Genetics and Animal Breeding, Polish Academy of Sciences, Jastrzębiec, ul. Postępu 1, 05-552 Wólka Kosowska, Poland

E-Mail: j.wyszynska-koko@ighz.pl

¹ Institute of Agricultural and Nutritional Sciences, Martin-Luther-University Halle-Wittenberg; A.-Kuckhoff-Str. 35, D-06108 Halle; Germany

² Institute of Biology; Martin-Luther-University Halle-Wittenberg; A.-Kuckhoff-Str. 35, D-06108 Halle; Germany * Present address: Research Institute for the Biology of Farm Animals (FBN) Dummerstorf,

Research Unit Muscle Biology and Growth, W.-Stahl-Allee 2, D-18196 Dummerstorf; Germany

STEFFEN MAAK^{1*}, KARSTEN NEUMANN² and HERMANN H. SWALVE¹

Polymorphic, putative regulatory elements at the *MYF5/MYF6* loci and expression analysis in neonatal piglets

Abstract

The myogenic factors (*MYF*) 5 and 6 are integral to the initiation and development of skeletal muscle and to the maintenance of its phenotype during post - natal growth. Thus, they are candidate genes for growth- and meat quality - related traits. We have recently identified and characterized several putative regulatory elements for the *MYF5* and *MYF6* loci covering 140 kb upstream of the *MYF5* transcription initiation site in several vertebrate species (mouse, pig, human, cow, dog, chicken and zebrafish). The organization of this locus is comparable between all investigated species with highly homologous distal enhancer elements. Single nucleotide polymorphism (SNP) analysis in pig revealed differences between commercial breeds and wild boar. In this study we investigated the identified polymorphisms on their relation to the hereditary disorder splay leg in piglet and on their influence on gene expression. The expression of *MYF5* in porcine skeletal muscle immediately after birth is neither affected by the identified SNPs nor are there differences in allele frequencies between splay leg and healthy piglets. A first analysis of the porcine 3' - UTR of both genes revealed several putative binding sites for microRNAs indicating further pathways for the temporal and spatial regulation of the expression of both loci.

Key Words: MYF5, MYF6, SNP, Expression, Pig, Splay leg

Zusammenfassung

Titel der Arbeit: Polymorphe, regulatorische Elemente in der MYF5/MYF6 – Genregion und deren Effekte auf die Genexpression bei neugeborenen Ferkeln

Die myogenen Faktoren (MYF) 5 und 6 sind unabdingbar für Differenzierung und Entwicklung der Skelettmuskulatur sowie zur Erhaltung ihres Phänotyps während des post - natalen Wachstums. Folglich sind beide Gene Kandidaten für Merkmale des Wachstums und der Fleischqualität. Wir haben in einer vorangegangenen Studie verschiedene, vermutlich regulatorisch wirksame DNA - Sequenzelemente für MYF5 und MYF6 im Bereich von etwa 140 kb stromaufwärts des MYF5 - Transkriptionsstarts bei verschiedenen Wirbeltieren identifiziert (Maus Schwein, Mensch, Rind, Hund, Huhn und Zebrafisch). Die Organisation der Loci ist in allen untersuchten Spezies ähnlich mit hochkonservierten distalen Enhancer - Elementen. In den Elementen wurden zahlreiche Polymorphismen (SNP) mit Allelfrequenz-unterschieden zwischen kommerziellen Rassen einerseits und Wildschweinen andererseits gefunden. In der vorliegenden Untersuchung stand die mögliche Beziehung zwischen den Polymorphismen und der Expression der Gene sowie zwischen SNP - Allelen und der Erbkrankheit Ausspreizen beim Saugferkel im Mittelpunkt. Die MYF5 - Genexpression im Skelettmuskel unmittelbar nach der Geburt stand in keinem eindeutigen Zusammenhang zu den identifizierten SNP. Ebenfalls wurden keine Beziehungen zwischen SNP - Allelen und dem Auftreten der Erkrankung gefunden. Eine erste Analyse des 3' - nicht - translatierten Bereiches (UTR) beider Gene zeigte die Existenz von mehreren Bindungsstellen für microRNAs. Dies deutet auf einen möglichen weiteren Regulationsmechanismus für die Expression beider Loci.

Schlüsselwörter: MYF5, MYF6, SNP, Expression, Schwein, Spreizer

Introduction

The myogenic factors *MYF5* and *MYF6* are members of the basic helix-loop-helix (bHLH) family of transcription factors and they are indispensable for initiation as well as for development of skeletal muscle (BRAUN et al., 1989, 1990). Because of their function, both genes have been investigated as candidates for growth related traits and meat quality in farm animals, respectively (TE PAS et al., 1999; LI et al., 2002). However, most of the described polymorphisms (SNP) are located in intronic regions

of the genes and thus, are considered as markers for functional mutations. Basing on investigations in mice (CARVAJAL et al., 2001) we have recently identified a set of conserved, putative regulatory sequences for the MYF5/MYF6 locus (E1 - E4; MAAK et al., 2006). They comprise stretches with a length between 200 and 500 bp and are located up to 140 kb upstream of the MYF5 initiation site. Several consensus motifs for binding of transcription factors are located within these conserved elements and make a role in the regulation of the MYF5 expression likely. Comparative sequence analysis in Pietrain, German Landrace and Wild boar revealed a total of 13 SNPs within the elements E1 - E4 but none in the enhancer elements with proven effects on embryogenesis in mice (H1, H2, M6EH; MAAK et al., 2006; FOMIN et al., 2004; BUCHBERGER et al., 2003). This may be due to different selection constraints on both sets of elements. Only recently, CHEN et al. (2007) identified several regulatory segments in the region +80 kb to -70 kb of MYF5 in zebrafish. They could demonstrate an effect of different segments on the MYF5 expression in different cell lineages during embryonic development and conclude a delicate orchestration of lineage specific gene expression by multiple upstream enhancers and repressors.

The aims of this study were (i) to investigate the relationships between the polymorphisms detected in the putative regulatory elements and the expression of MYF5 in M. bicep femoris of neonatal healthy and splay leg piglets, (ii) to relate SNPs in the promoters of MYF5 and MYF6 to the expression and (iii) to compare the 3' - UTRs of both genes across the species mentioned above in order to identify further regulatory motifs.

Materials and Methods

Detailed experimental procedures and primers for sequencing of the regulatory elements are described in MAAK et al. (2006).

Expression of *MYF5* and *MYF6* was analyzed with the QuantiTect SYBR Green PCR Kit (QIAGEN) on an ABI Prism 7000SDS (Applied Biosystems) essentially as described by the manufacturer.

The following primers were used:

Myf5f 5'-GCTGCTGAGGGAACAGGTGGA-3'

Myf5r 5'-CTGCTGTTCTTTCGGGACCAGAC-3'

Myf6f 5'-CGCCATCAACTACATCGAGAGGT-3'

Myf6r 5'-ATCACGAGCCCCCTGGAAT-3'.

All expression values were normalized for the individual expression of S18 rRNA which was amplified with the primers: S18f 5'-GACCATAAACGATGCCGACT-3' and S18r 5'-GGTGCCCTTCCGTCA-3'.

Sequence comparisons were done with DNASIS Max (MiraiBio, Alameda, USA) and the 3'-ends were analyzed with TargetScan 3.0 (http://www.targetscan.org) for microRNA - binding sites.

Results and Discussion

An overview about the observed allele frequencies at 13 polymorphic positions in the putative regulatory elements E1 - E4 is given in Table 1. There are no distinct differences in allele frequencies between six healthy and six splay leg piglets, respectively. Five new SNPs were detected in the *MYF6* promoter and one in the

promoter of *MYF5* (Table 1). The *MYF6* promoter polymorphisms are completely linked.

Table 1

	SNP	Allele frequency			
Element		Healthy (n=6)		Splay leg (n=6)	
		A1	A2	A1	A2
E1 (-138.4 kb)	A242C	0.25	0.75	0.17	0.83
	G536T	0.75	0.25	0.67	0.33
E2 (-124.7 kb)	G69T	0.50	0.50	0.50	0.50
	A211G	0.67	0.33	0.75	0.25
E3 (-119.5 kb)	C197T	0.50	0.50	0.50	0.50
	C430T	0.67	0.33	0.42	0.58
	A485G	0.70	0.30	0.50	0.50
E4 (-93.0 kb)	C158T	0.58	0.42	0.50	0.50
	C374T	0.83	0.17	0.83	0.17
	C411G	0.33	0.67	0.33	0.67
	A460C	0.33	0.67	0.13	0.87
	C467G	0.50	0.50	0.58	0.42
	C494T	0.25	0.75	0.25	0.75
MYF6 promoter	C255T	0.17	0.83	0.33	0.67
	InDel325T	0.83	0.17	0.67	0.33
	A373G	0.83	0.17	0.67	0.33
	A375G	0.83	0.17	0.67	0.33
	C429T	0.83	0.17	0.67	0.33
MYF5 promoter	C72T	0.67	0.33	0.60	0.40

Allele frequencies at SNPs in regulatory sequences for the MYF5/6 loci in healthy and splay leg piglets (Distance of the elements E1 -E4 relative to the transcription start of MYF5 is given in parentheses)

Elements E1 - E4 are located within a segment with potential impact on the expression of MYF5 in mice (CARVAJAL et al., 2001). Consequently we tested the relationship between SNPs in these elements and the expression of *MYF5* in M. biceps femoris of neonatal piglets. Furthermore, the effect of the respective promoter variants on the expression of *MYF5* and *MYF6* was tested, respectively. There was no significant effect of any of the polymorphisms on the expression of the respective genes (p = 0.08- 0.95). Considering the expression according to health status and sex there was again no significant effect on the *MYF5* expression but a significant interaction (p = 0.04) between sex and health status for the *MYF6* expression (Table 2). However, there is neither an indication for an involvement of the putative regulatory elements in the early post-natal expression of both genes in skeletal muscle nor for a relation between *MYF5/6* expression and congenital splay leg in piglets.

Table 2

Relative expression of MYF5 and MYF6 in M. biceps femoris depending on health status and sex

Health status	Sex	Ν	Relative gen	Relative gene expression		
			MYF5	MYF6		
Healthy	Male	3	0.85±0.14	1.07±0.12		
	Female	3	1.00 ± 0.08	0.65 ± 0.05		
Splay leg	Male	3	0.69±0.23	0.60±0.10		
	Female	3	0.88±0.19	1.01±0.29		

In order to identify further elements with potential impact on gene expression, we compared the 3' - UTR of both *MYF5* and *MYF6* between swine, cattle, human and

mouse. The alignment of the regions revealed several conserved blocks with a length of 10 - 20 bp. Analysis of the regions on potential binding sites for microRNAs (TargetScan v3.0) identified each one sequence for binding of a conserved microRNA (miR-20 - family for *MYF5* and miR-299-5p - family for *MYF6;* Figure). LEWIS et al. (2005) list *MYF5* among the predicted target genes with conserved binding site for miR-20 in human, rat mouse and dog. Our results extend this conservation on cattle and swine and points to a putative, common regulation of the expression via this microRNA. Furthermore, the 3'-UTR of *MYF5* contains another conserved binding site for a microRNA that does not belong to a conserved family (miR-511, Figure) in addition to numerous binding sites for species specific microRNAs in human and dog (data not shown).

	01	O	One company for DNU	Our second site for
	Stop	conserved site for hon- conserved miR-511	binding protein PUM1/2?	conserved site for conserved miR-20a/106b
Human	TGA —	——— AAGACAA —	— TGTAAAT ——	- CACTTTAT
Mouse	TGA —	****** _	******	****
Swine	TGA —	****** _	******	_ ****
Cattle	TGA	****** _	******	****
MYE6				
MYF6	Ston		Core sequence for RNA-	Conserved site for
			binding protein PUM1/2?	conserved miR-299-5p
Human	таа —		— тсталата —	
Mouse	таа —		- *******	******
Swine	таа —		******	**C****
Cattle	דאם —		******	******

Figure: Conserved motifs in the 3'-UTR of the *MYF5* and *MYF6* genes. The bars represent non-conserved sequences of different length between the conserved stretches

Beside binding sites for microRNAs, each one sequence motif in the 3'-UTRs of MYF5 and MYF6 could be identified as core sequence of a putative binding site for RNA-binding proteins of the PUF - family (XIE et al., 2005). According to the authors the 8-mer-motif "TGTANATA" ranks 16th among the highest conserved elements existing in 3'-UTRs of mammalian species. The role of the remaining stretches of conservation in the 3'-UTRs of the investigated genes remains unclear. In *MYF5*, there are five further elements of conservation in human mouse, swine, cattle and dog comprising two 11-mers, one 9-mer and two 8-mers (data not shown). The *MYF6*-3'UTR contains one 15-mer, two 12-mers and one 9-mer, however, their conservation includes the species mentioned above but the dog. Considering the discrepancy between the number of known microRNAs in human and swine (474 vs. 112; KIM et al., 2006), it is likely that several of the conserved motifs may comprise further binding sites for species-specific microRNAs.

The results on polymorphisms as well as the expression analysis of MYF5 and MYF6 in neonatal muscle make a direct involvement of these transcription factors in the etiology of congenital splay leg in piglets unlikely. However, our analysis is a further contribution to the definition of regulatory pathways of the developmental key factors *MYF5* and *MYF6*. A very complex array of upstream genomic elements orchestrate the

temporal and spatial expression of both factors (CARVAJAL et al., 2001; Chen et al., 2007) in mice and zebrafish, respectively. Comparative analyses indicate a similar mechanism in swine (MAAK et al., 2006). The increasing knowledge about the action of microRNAs in the process of development and growth of skeletal muscle (e.g. BOUTZ et al., 2007) defines an important direction of future research in meat producing animals.

Acknowledgement

We wish to thank Gerda Becke and Simone Jäsert for their expert assistance in sequencing and expression analysis.

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Corresponding Author STEFFEN MAAK Research Unit Muscle Biology and Growth, Research Centre for the Biology of Farm Animals, Wilhelm-Stahl-Allee 2, 18196 Dummerstorf / Germany

¹Research Institute for the Biology of Farm Animals (FBN), Research Unit Molecular Biology, 18196 Dummerstorf, Germany
 ²Research Institute for the Biology of Farm Animals (FBN), Research Group Functional Genomics,

18196 Dummerstorf, Germany

EDUARD MURÁNI¹, SIRILUCK PONSUKSILI² and KLAUS WIMMERS¹

Investigation of genetic and epigenetic mechanisms underlying stageand breed-specific differences in the transcription of *SPP1* gene during porcine myogenesis

Abstract

Comparative transcriptional profiling of seven key stages of myogenesis between Pietrain and Duroc breeds (EU-funded project PorDictor - QLK5-2000-01363) revealed association of the transcription of the Secreted phosphoprotein 1 gene (SPP1) with myogenesis and its consistent upregulation at all stages in Duroc compared to Pietrain embryos/fetuses. To identify regulatory DNA-variation that could be responsible for the breedassociated differences the 5'-upstream region (Acc.Nr. M84121) was comparatively sequenced. Two SNP were identified. In silico analysis of the 5'-upstream sequence pointed to SNP c.-13-11A>G located in the first intron as the functionally more relevant variable site. This SNP eliminates predicted CEBPB binding site in an evolutionary conserved region that was previously shown to harbor an enhancer element in the pig. Genotyping revealed that the G variant segregates in Duroc with a frequency of 57 % but is absent in Pietrain. A consistent negative effect of the G allele on transcription in vivo was shown by quantitative real-time PCR, but this effect did not reach statistical significance (p>0.05). In addition the G variant induces aberrant splicing of the first intron by generating a *de novo* and activation of one cryptic splice acceptor sites. DNA-methylation in the context of CpG dinucleotides is an established epigenetic mechanism involved in the regulation of myogenesis and a candidate mechanism controlling temporal changes in transcrition of SPP1 gene. The 5'-flanking region of the porcine SPP1 gene harbors a CpG island 2.5kb upstream from the transcription start. Our preliminary results indicate differential methylation of the CpG island in *musculus longissimus dorsi* during development.

Key Words: pig, expression analysis, mRNA splicing, single nucleotide polymorphism

Zusammenfassung

Titel der Arbeit: Untersuchung der genetischen und epigenetischen Mechanismen der Stadien- und Rassenspezifischen Transkription des SPP1 Gens während der Myogenese beim Schwein

Vergleichende Transcriptomanalyse von sieben Schlüsselstadien der Myogenese zwischen den Rassen Pietrain und Duroc (Eu-finanziertes Projekt PorDictor - QLK5-2000-01363) zeigte eine Beziehung zwischen der Transkription des Gens für Secreted phosphoprotein 1 (SPP1) und der Myogenese und seine konsistente Hochregulation an allen 7 Stadien bei Embryonen/Föten der Rasse Duroc im Vergleich mit denen der Rasse Pietrain. Um regulatorische DNA-Variation, welche für die Rassenunterschiede in der Expression von SPP1 Gen verantwortlich sein könnte zu identifizieren, wurde die gesamte 5'-upstream Region (Acc.Nr. M84121) vergleichend sequenziert. Zwei Punktmutationen wurden gefunden. In silico Analyse der Sequenz der 5'upstream Region wies auf die Punktmutation c.-13-11A>G im ersten Intron als die funktionell relevantere variable Stelle hin. Diese Punktmutation eliminiert eine CEBPB Bindungsstelle in einer evolutionär konservierten Region in der ein Enhancer-Element beim Schwein bereits nachgewiesen wurde. Genotypisierung ergab, dass die G-Variante in der Rasse Duroc mit einer Frequenz von 57 % segregiert, dagegen in der Rasse Pietrain vollständig fehlt. Die G-Variante zeigt einen konsistenten negativen Einfluss auf die Transkription, nachgewiesen mittels quantitativer Echtzeit PCR, allerdings konnte dieser Effekt nicht statistisch abgesichert werden (p>0.05). Überdies aktiviert die G-Variante alternatives Spleißen des ersten Introns durch Erzeugung einer de novo beziehungsweise Aktivierung einer kryptischen 3' Spleißstelle. DNA-Methylierung im Kontext von CpG-Dinukleoiden stellt einen etablierten Mechanismus der Regulation der Myogenese und einen potentiellen Mechanismus der Kontrolle der zeitlich-abhängigen Veränderungen in der Transkription des SPP1 Gens. Die 5'-flankierende Region des porcinen SPP1-Gens umfaßt eine CpG-Insel 2.5kb aufwärts vom Transkriptionsstart. Unsere ersten Ergebnisse deuten auf eine differentielle Methylierung der CpG-Insel im Musculus longissimus dorsi im Laufe der Entwicklung hin.

Schlüsselwörter: Schwein, Expressionsanalysen, mRNA Splicing, Punktmutation

Introduction

Secreted phosphoprotein 1 (SPP1 or Osteopontin, OPN) is a multifunctional matricellular protein mediating cell-adhesion, -migration and cellular signaling via integrin and CD44 receptors (SODEK et al., 2000; WAI et al., 2004). According to its multifunctionality and expression in various cell types SPP1 has been implicated in a number of physiological and pathological events, among others skeletal muscle regeneration (HIRATA et al., 2003). There is also mounting evidence for the involvement of SPP1 in myogenesis; SPP1 gene is a target of the muscle regulatory factors MYOD and MYF5 (ISHIBASHI et al., 2005) and was shown to be expressed in vitro in myoblasts and myotubes, with higher levels in the later (PEREIRA et al., 2006). Moreover SPP1 gene is downregulated in the BC3H1 cell line, which is myogenic but do not terminally differentiate, compared to the myogenic C2C12 cell line, which differentiate normally (SHARP et al., 2002). We recently showed upregulation of the SPP1 gene expression in prenatal musculus longissimus dorsi (m.l.d.) at 35 and 63-77 days post conception (dpc) in pigs, i.e. at the time points of the first and second myogenic wave respectively, and thus provided additional, in vivo, evidence in favor of the involvement of SPP1 in myogenesis (MURANI et al., submitted). In addition we showed consistent upregulation of the SPP1 gene in Duroc compared to Pietrain embryos (14 and 21dpc) and fetal m.l.d. (35, 49, 63, 77 and 91 dpc). The difference was highest at 14dpc (~20 fold), afterwards gradually decreasing until 49dpc (\sim 3.6 fold to \sim 1.3 fold) and remaining at similar magnitude until 91dpc. The aim of the present study is the identification of factors responsible for the observed breed-associated differences as well as factors regulating temporal changes in the expression of the porcine SPP1 gene. In order to meet these objectives we performed comparative and bisulfite sequencing of the 5'-upstream region of the porcine SPP1 gene and characterized a single nucleotide polymorphism (SNP) in the first intron.

Material and methods

Screening for DNA-variation

The 5'-upstream sequence of the porcine SPP1 gene (Acc. Nr. M841217) was amplified in six overlapping PCR fragments using each six individual DNA samples per breed. The PCR products were subsequently pooled within breed, purified using the NucleoSpin Extract II kit (MN, Düren, Germany) and directly sequenced on a MegaBace 750 capillary sequencer using Big Dye Terminator Cycle sequencing kit (Applied Biosystems, Darmstadt, Germany). Sequences of primers and PCR conditions used for comparative and bisulfite sequencing, genotyping, qPCR and RT-PCR are available from authors upon request. In silico analysis, including phylogenetic footprinting and transcription factor binding site (TFBS) analysis, was performed using online tools Mulan (http://mulan.dcode.org) TESS and (http://www.cbil.upenn.edu/cgi-bin/tess/tess) respectively. For genotyping single strand conformation polymorphism (SSCP) assays were established.

RNA isolation, cDNA synthesis and real-time quantitative PCR

Total RNA from fetal m.l.d. (91dpc) was isolated using TRI Reagent (Sigma, Taufkirchen, Germany). After DNaseI treatment (Roche, Mannheim, Germany) the RNA was cleaned up using the NucleoSpin RNA II Kit (MN). Template first strand

cDNA was synthesized using SuperScriptIII MMLV reverse transcriptase (Invitrogen, Karlsruhe, Germany) in a reaction containing 1 μ g RNA and 500 ng of oligo (dT)₁₁VN primer, according to the manufacturer's protocol. Expression of the SPP1 gene and the reference gene RPL32 were quantified by real-time quantitative PCR (qPCR) performed on a LightCycler 1.0 System using the LightCycler FastStart DNA Master SYBR^{plus} Green I (Roche Diagnostics, Mannheim, Germany). Expression profile of the porcine CCAAT/enhancer binding protein beta during porcine myogenesis was analysed on an iCycler iQ5 instrument (BioRad, München, Germany) using Absolute QPCR SYBR Green Mix (ABgene, Hamburg, Germany).

Splicing assay

RT-PCR was performed using primers amplifying a cDNA fragment spanning exons 1-6 and separated on 3% high-resolution agarose gel (ABgene). An aliquot of the RT-PCR was retained and cloned using the pGEM-T vector (Promega, Mannheim, Germany). Positive clones were screened by M13-PCR and those containing different splice variants sequenced using T7 and Sp6 primers.

Bisulfite sequencing

Genomic DNA from m.l.d. of 49-91 days old fetuses (n=4 per breed) and from adult muscle (n=4 from Pietrain only) was isolated by standard phenol-chlorophorm extraction. DNA from 21dpc embryos (n=1 per breed) and m.l.d. of 35 days old fetuses (n=4 per breed) was isolated using NucleoSpin RNA II Kit combined with NucleoSpin RNA/DNA buffer set (MN). One µg DNA was modified by sodium bisulfite according to GRUNAU et al. (2001) with minor modifications. A 422bp fragment of the CpG island, containing 19 CpG sites, was amplified in two nested PCR rounds and directly sequenced from the reverse side on an ABI 310 Automated sequencer. Peak heights were extracted from the chromatogram files using the PeakPicker software (GE et al., 2005). Methylation level of each CpG dinucleotide was calculated as the proportion of the G signal from the sum of A and G signals.

Statistical analysis

The effect of SPP1 genotype on its expression was analyzed using general linear model (PROC GLM; SAS V9.1, SAS Inst. Inc., Cary, NC), including fixed effects of genotype, stage and their interaction for across stage analysis and fixed effect of genotype for the analysis within stage 91dpc.

Results and Discussion

Identification and characterization of a SNP in the first intron of the porcine SPP1 gene

The emerging field of gene expression genetics greatly enhanced our knowledge about genetic factors responsible for individual variation in gene expression. As many as 25-50% of genes showing variation in their expression are affected by cis-acting regulatory DNA-variation in yeast and human (STAMATOYANNOPOULOS 2004; ROCKMAN and KRUGLYAK, 2006). We thus hypothesized, that the observed breed-associated differences in the expression of SPP1 might be caused by regulatory DNA-variation, which vary between Pietrain and Duroc. In human, regulatory variation affecting the expression of SPP1 gene has already been described (GIACOPELLI et al., 2004; HUMMELSHOJ et al., 2006). In order to identify such

variation between Pietrain and Duroc we comparatively sequenced the 5'-upstream region of the SPP1 gene including: 5'-flanking region and promoter, exon1, intron1, and exon2. Two SNP sites were identified: a C>T SNP in the 5'-flanking region (g.1804C>T) and a A>G SNP in the first intron (c.-13-11A>G). In silico analysis of the 5'-upstream region indicated, that the g.1804C>T SNP occurred in evolutionary poorly conserved sequence of a repetitive element (Mir, Fig. 1) and thus most likely possess no regulatory function. In addition genotyping of available embryos/fetuses for this SNP revealed that it is segregating in both breeds with frequencies of 83% in Pietrain and 49% in Duroc for the C allele. In contrast the c.-13-11A>G SNP is fixed in Pietrain for the ancestral A variant, segregating only in Duroc with a frequency of 57% for the mutant G variant. This SNP occurred in an evolutionary conserved region (Fig. 1) that was shown by reporter assay to harbor an enhancer element in the pig (ZHANG et al., 1992) and human (GIACOPELLI et al., 2003). Moreover in silico TFBS analysis revealed that the c.-13-11A>G SNP eliminates a putative CCAAT/enhancer binding protein beta (CEBPB) binding site. The orthologous site in the first intron of the human SPP1 gene has been shown to be a functional CEBPB binding site in vitro (GIACOPELLI et al., 2003). Because expression of the CEBPB

gene in prenatal muscle has not been reported previously, but is a prerequisite for a potential regulatory function of the CEBPB binding site at c.-13-11, we performed qPCR using embryos and fetal m.l.d. samples collected at seven key stages of myogenesis (14, 21, 35, 49, 63, 77 and 91 dpc).



Fig. 1: Evolutionary conservation and DNA-variation of the 5'-upstream region of the porcine SPP1 gene. At the top is the schematic representation of the sequence elements including exons represented as bars labeled by numbers in roman numerals and other elements labeled with their identity e.g. a CpG island. The arrowed line shows direction of the transcription. Beneath stacked-pairwise conservation profiles between porcine and corresponding bovine, canine, human and murine sequences generated using Mulan are shown. Bars above the conservation profiles indicate evolutionary conserved regions (>70% identity; >100bp). Numbered lines show position of the identified SNP: 1.g.1804C>T 2. c.-13-11A>G

This confirmed expression of the CEBPB gene at all seven stages (data not shown). To study the effect of the c.-13-11A>G SNP on the expression of SPP1 gene *in vivo* we genotyped available Duroc fetuses (12 per each of stages 49, 63, 77 and 91dpc) with expression data from a previous project (MURANI et al. submitted) and performed a separate qPCR analysis of its expression in fetal (91dpc) m.l.d. samples of additional seven individuals per each genotype. Both analyses consistently showed that the mutant G variant is associated with a decrease in the SPP1 gene expression (Figure 2). Although the difference is not significant at α =0.05 it is in line with the *in silico* evidence that the SNP disrupts a CEBPB-responsive enhancer element. However this result also indicates that the c.-13-11A>G SNP is unlikely causal for the differences in the expression of SPP1 gene the Duroc variant (here the G variant) is expected to be associated with higher expression.

The mutant G variant of the c.-13-11 SNP site generates a potential splice acceptor site (3'ss) only 10bp upstream of the naturally occurring intron1/exon2 junction. To prove whether this potential *de novo* 3'ss is used *in vivo* we performed RT-PCR using the same 91dpc fetal m.l.d. cDNA samples described in the preceding paragraph. As shown in Figure 3 carriers of the wild type A variant expressed only one, the naturally occurring, mRNA isoform. In contrast multiple isoforms were expressed by the carriers of the G variant.



Fig. 2: Effect of the c.-13-11A>G SNP on the expression of SPP1 gene. Left panel shows the results of the across stage analysis and the right panel the results of the analysis of additional 21 fetuses (91dpc).

Cloning and sequencing revealed two aberrantly spliced isoforms induced by the G allele: one with a 10bp longer 5'-leader sequence spliced at the *de novo* 3'ss generated by the G variant, and the other with a 36bp longer 5'-leader sequence spliced at a cryptic 3'ss located in the intron1 24bp upstream of the c.-13-11 SNP site. Mutations that affect hnRNA splicing account for up to 50% of disease-causing gene alterations in human and potentially represent the most frequent cause of hereditary disorders. Some 218 unique aberrant 3'ss activated by disease-causing mutations in 131 genes are presently known in human (VORECHOVSKY, 2006). However the relatively high frequency of the G variant argues against a major negative phenotypic effect, because in that case the G allele would be quickly eliminated given the high selection pressure on commercial pigs. Furthermore the aberrant splicing induced by the c.-13-11A>G SNP does not change primary structure of the SPP1 protein. In fact, the aberrant splicing may counteract the negative effect of the G variant on SPP1 gene transcription by enhancing translational efficiency. Aberrantly spliced mRNA isoforms of the human insulin gene with longer 5'-leader sequence, induced by a SNP in its first

intron, were reported to generate more proinsulin *in vitro* compared to the natural transcripts (KRÁLOVIČOVÁ et al., 2006). The dual effect of the c.-13-11A>G SNP on regulation of the SPP1 gene expression and its splicing is a unique feature. However, to confirm the regulatory effect of the c.-13-11A>G SNP and to separate it from a potential alteration of the transcript stability and translational efficiency of the aberrant splicing isoforms, *in vitro* models have to be established.



Fig. 3: Aberrant splicing of the SPP1 mRNA introduced by the c.-13-11A>G SNP

Methylation profile of the CpG island in the SPP1 gene 5'-flanking region during development of skeletal muscle

In silico analysis of the 5'-upstream sequence revealed that it harbors a ~300bp long CpG island at its distal end, ~2.5kb upstream from transcription start (Figure 1). Methylation of Cytosins that occurs exclusively in the context of CpG dinucleotides is an epigenetic mechanism that regulates, usually represses, gene transcription by chromatin condensation or interference with transcription factor binding. DNA-(de)methylation may be playing a role in imprinting muscle precursor cells that will then activate the myogenic program (PALLACIOS and PURI, 2006). Active demethylation of regulatory elements of MYOD and MYOG genes during myogenesis is associated with the onset of their expression (BRUNK et al., 1996; LUCARELLI et al., 2001). Moreover JOST et al. (2001) observed genome-wide demethylation in mouse myoblasts induced to differentiate. This was followed by partial remethylation of the genome after two days. We thus reasoned that dynamic changes in the methylation of the CpG in the distal 5'-flanking region of the SPP1 gene might be associated with the observed temporal changes in its expression during myogenesis.



Fig. 4: Methylation pattern of the CpG island in 5'-flanking region of the porcine SPP1 gene (left) and expression profile of the SPP1 gene (right) in Duroc and Pietrain embryos (14-21dpc) and fetal *musculus longissimus dorsi*

As shown in Figure 4, bisulfite sequencing revealed a nearly linear decrease in the average degree of methylation of the CpG island. The individual CpG sites showed either decreasing trend or little changes across stages (data not shown). It should be noted, that the level of methylation is overestimated here due to overscaled Guanine (or Cytosine, depending on direction of sequencing PCR) signals, a bias inherent to direct bisulfite sequencing (LEWIN et al., 2004). However, because the bias was the same for each stage the methylation pattern should be unbiased. The decreasing methylation degree of the CpG island shows no obvious relationship with the oscillating expression level of the SPP1 gene during myogenesis. The decrease in the methylation degree of the CpG island is a result of decreasing proportion of cells where the CpG island is present in its highly methylated state. The fact that the adult muscle shows the lowest methylation level indicates that myocytes are likely a cell type harboring the CpG island in its less methylated state. The identification of the different cell types harboring the CpG island in its differentially methylated states will help to understand the function of the CpG methylation in the regulation of the porcine SPP1 gene.

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Corresponding Author PD Dr. KLAUS WIMMERS Research Institute for the Biology of Farm Animals (FBN), Research Unit Molecular Biology, 18196 Dummerstorf, Germany

E-Mail: wimmers@fbn-dummerstorf.de

Department of Histology and Embryology, Veterinary Faculty, Kafkas University, Kars/TURKEY 36100

H. KOCAMIS

The Expression Profiles of Myostatin and Follistatin Genes in Quail (*Quoturnix quoturnix*) Embryonic Development

(Expressionsprofile des Myostatin- und Follistatingens in der Embryonalentwicklung der Wachtel)

Early embryonic development as well as skeletal muscle development are mainly regulated by major growth factors, such as IGFs and TGF-B family members. Myostatin, which is a member of TGF-B family, along with its binding protein follistatin have been demonstrated to inhibit muscle development during embryonic and postnatal development. In order to determine their differantial expression, total RNA was isolated from whole embryos on each of the embryonal days (E) 3 to 6 (n=6per day), from thoracic/abdominal half of the embryos at E 7 and E 8 (n= 6 per day), and from pectoralis muscle tisue at E 9 to E 16 (n= 6 per day). Myostatin and Follistatin cDNAs were synthesized by reverse-transcription polymerase chain reaction (RT-PCR). Myostatin mRNA was first detected on E 5, while follistatin mRNA was first detected on E3. Myostatin mRNA levels in pectoralis muscle declined from E 10 to E 12, and then sharply increased on E 13. Follistatin mRNA levels, with slight fluctations, remained at the E 3 level until E 13, on which day there was an approximately 11-fold increase. It is suggested that Myostatin and Follistatin play an important role in embryogenesis and skeletal muscle development of the quail embryos.

¹School of Biology, University of St Andrews, St Andrews, KY16 8LB, Scotland, UK.
²Department of Fisheries and Natural Sciences, Bodø University College, NO-8049 Bodø, Norway.

Ø. HAGEN^{1,2}, I. A. JOHNSTON¹ and C. SOLBERG²

Sexual dimorphism of fast muscle fibre recruitment in Atlantic halibut (*Hippoglossus hippoglossus* L.)

(Geschlechtsdimorphismus in der Rekrutierung von schnellen Muskelfasern beim Atlantikheilbutt)

Atlantic halibut (*Hippoglossus hippoglossus* L.) has for more than a decade been considered as a promising species for aquaculture, but are still to be considered as in the initiating stage. Despite its large growth potential (females of 300 kg are reported in the wild, males seldom reach 50 kg), Atlantic halibut displays a relatively poor growth in aquaculture. A well known and unwanted situation in aquaculture is the early maturation of males (NORBERG et al., 2001).

The first objective of the present study is to investigate muscle fibre recruitment in Atlantic halibut farmed commercially under ambient photoperiod condition in the grow-out phase. The second objective is to compare muscle fibre recruitment and the distribution of muscle fibre diameters in male and female halibut to see if the sexual dimorphism observed in size are reflected in muscle fibre distribution (CALVO, 1989). The third objective is to investigate the effects of precocious sexual maturation on muscle growth in males.

A group of commercially farmed Atlantic halibut reared under ambient conditions from Aga Marin's (Dønna, Norway) production were followed and sampled every third month during a 12 month production period (May 2004 – May 2005, n=100). The fish grew in average from 1.2 kg to 2.1 kg and all males matured, no maturation was observed amongst females. From the point of maturation females displayed a superior growth compared to males (P<0.01). Total fast fibre number were significant in all sampling points (P<0.001). For male and female of similar size (the August sampling) the distributions of fibre diameter were significantly different (P<0.05, nonparametric Kolmogorov-Smirnoff test): the peak probability density function of diameter was shifted towards larger sizes in males relative to females whereas females had a higher proportion of the smallest size class of fibres (P<0.001, GLM). The results illustrate a sexual dimorphism of muscle fibre recruitment patterns related to differences in body mass and highlight the adverse affects of sexual maturation in males on muscle growth.

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¹Department of Food Technology, University of Helsinki, P.O. Box 66 (Agnes Sjöbergin katu 2), FI-00014 Helsinki, Finland;
²MTT Agrifood Research Finland, Animal Production Research, Swine Research, Tervamäentie 179, FI-05840 Hyvinkää, Finland

¹M. RUUSUNEN, ²K. PARTANEN, and ¹E. PUOLANNE

Heart size and mean muscle fibre cross sectional area related to birth weight in pigs

(Beziehung zwischen Herzgröße, Muskelfaserquerschnitt und Geburtsgewicht beim Schwein)

It could be hypotetised that small heart size in relation to large muscle mass in the carcass and the large mean cross-sectional area of muscle fibres especially in light muscles may lower stress resistance. In this study the relationship between piglet birth weight and (i) the size of heart and (ii) the mean muscle fibre cross sectional area were investigated. Twenty pigs from 10 litters were slaughtered at the 165 days.

Muscle samples for analysing the mean fibre cross sectional area were collected from M. longissimus dorsi (LD), M. semimembranosus (SB), M. gluteus superficialis (GS), M. infraspinatus (IS) and M. masseter (M) about 30 minutes after slaughter. Samples were frozen in liquid nitrogen and stored at -80° C until analysed. The cross sectional area of muscle fibres were calculated from sections stained using the myosin ATPase method with an acid preincubation solution (pH 4.6). Stained sections were examined with an image analysis system using a computer program (KS300, Carl Zeiss Vision GmbH, Germany). The mean muscle fibre cross sectional area was calculated by dividing the whole analysed muscle area by the number of corresponding fibres. The day after slaughter the heart was weighed.

Birth weight of the piglet ranged from 0.9 to 2.2 kg and the weight of heart from 328 to 395 g. In the study, a positive correlation between the birth weight and the heart weight (r=0.597, p=0.005) was found. The live weight of the pigs ranged from 92.5 kg to 118.5 kg and the carcass weight from 70.8 to 92.5 kg but these did not correlate with birth weight.

The mean muscle fibre cross sectional area was larger in light muscles, LD, SB and GS as well as in dark IS muscle than in dark M muscle. Pigles with low birth weight had larger mean fibre cross-sectional area of muscle fibres in light muscles (r_{LD} =-0.521, p=0.018; r_{SB} =-0.434, p=0.056; r_{GS} =-0.496, p=0.026). The correlations were significant in LD and GS and marginally significant in SB. In dark IS and M no significant correlations were found. This indicates that during growth in pigs with low birth weight muscle fibre thickness grows more in light muscles than in dark muscles.

It was concluded that in pigs with low birth weight, the heart size is smaller and the cross sectional area of muscle fibres in light muscles is larger at slaughter age than in pigs with high birth weight.

Key Words: pig, birth weight, heart, cross sectional area of muscle fibre

¹Laboratory of Animal Nutrition and Animal Product Quality, Department of Animal Production, Ghent University, Proefhoevestraat 10, 9090 Melle, Belgium and ²Division of Gene Technology, Department of Biosystems, K.U. Leuven, Kasteelpark 30, 3001 Heverlee, Belgium

K. VAN DEN MAAGDENBERG $^{1\ast},$ E. CLAEYS 1, A. STINCKENS 2, N. BUYS 2 and S. DE SMET 1

The influence of the IGF-II genotype on muscle fibre type composition, cross sectional area and number of nuclei

(Einfluss des IGF-II Genotyps auf Muskelfasertypen, -querschnitte sowie Anzahl an Zellkernen)

The aim of this study was to investigate the effect of the QTN in the paternally imprinted IGF-II gene (Apat vs. Gpat allele; VAN LAERE et al., 2003) on muscle fibre characteristics. An equal number (n=12) of Apat and Gpat animals (almost equally distributed among castrates and females) was slaughtered at an average live weight of 110 ± 9 kg. Apat animals inherited the IGF-II mutation responsible for the higher muscle mass whereas Gpat animals carry the paternal wild type allele. Muscle fibre type distribution, cross sectional area (CSA), number of nuclei and PCNA positive (PCNA+) nuclei (Proliferating Cell Nuclear Antigen as a measure of proliferation according to HAWKE and GARRY, 2001) were determined in Longissimus muscle samples. Data were analysed with a univariate general linear model with IGF-II and gender as fixed factors, their interaction and live weight as covariate.

Birth weight and live weight at slaughter were not affected by the IGF-II genotype, but the carcass lean meat % was significantly higher (+5.3%) in Apat animals. Mean fibre CSA was significantly increased in Apat vs. Gpat animals (respectively 4932 and 3954 μ m²). No effect of the IGF-II genotype on the proportion of type I, IIA, IIX and IIB fibres was found. Also the number of nuclei and the number of PCNA+-nuclei were not influenced by the IGF-II genotype. However, Apat animals had a significantly higher ratio of primary to secondary fibres. No significant effect of gender was found, except for a higher carcass lean content and a higher number of PCNA+-nuclei next to oxidative fibres, but not the total number of PCNA+-nuclei.

The increased mean CSA clearly suggests an effect of the IGF-II mutation on muscle fibre hypertrophy postnatally, in line with increased postnatal IGF-II mRNA expression in Apat animals (VAN LAERE et al., 2003). The higher ratio of primary to secondary fibres also suggests some prenatal effect but further investigation is required.

Acknowledgement

This research is financially supported by the Institute for the Promotion of Innovation through Science and Technology, Brussels. The authors thank the COST program nr 925 for the STSM and S. Lescouhier and T. Luyten are acknowledged for their excellent technical assistance.

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Department of Physiological Sciences, Faculty of Veterinary Medicine, Warsaw Agricultural University, Nowoursynowska 159, 02-776 Warsaw, Poland

P. PAWLIKOWSKA and A. ORZECHOWSKI

Mitofusin 2 (Mfn2) – a switch to stop mitogenesis in insulin-dependent myogenesis *in vitro*

(Mitofusin 2 (Mfn2) – ein Schalter zum Beenden der Mitogenese bei der Insulin-abhängigen Myogenese in vitro)

The prerequisite for muscle differentiation is to withdraw cells from cell cycle. Being able to evoke two opposing effects insulin is both mitogen and differentiation factor because it stimulates cell proliferation and myotube formation in skeletal muscle myogenesis. Our previous results have shown that mitochondrial activity increased in response to insulin in differentiating muscle cells (PAWLIKOWSKA et al., 2006). Moreover, protein kinase kinase/extracellular-signal-regulated kinase (MAPKK/ERK - MEK) inhibitor PD98059 accelerated, whereas either the phosphatidylinositol 3-kinase (PI-3K) inhibitor LY294002 or blockade of mitochondrial respiration both abrogated insulin-mediated myogenesis.

Our present study points to the mitochondrial transmembrane protein called hyperplasia suppressor gene/mitofusin2 (HSG/Mfn2) which regulates both mitochondrial fusion (demonstrated by perinuclear mitochondria clustering) and insulin-dependent myogenesis *in vitro*. The molecular mechanism of this phenomenon is unknown, although immunoprecipitation studies indicate that during insulin-mediated myogenesis Ras protein (upstream activator of MAPK/ERK1/2 cascade) interacts with HSG/Mfn2 in muscle cells. Interaction of Ras with Mfn2 continued unless insulin was present and was blunted after PD98059 co-treatment.

It indicates that insulin-mediated myogenesis is augmented by inhibition of MEK, most likely by the lack of mitogenic signals opposing muscle differentiation. We suggest, that insulin stimulates Mfn2 protein expression which in turn binds to Ras and inhibits MEK-dependent signalling pathway. At the same time PI-3K-dependent signalling pathway is boosted, mitochondrial respiration increases, and the rate of myogenesis is accelerated.

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Comparative and Molecular Endocrinology Group, CCMAR, University of Algarve, Faro 8005-139, Portugal

N. SILVA, M.A. CAMPINHO and D.M. POWER

Comparative analysis of fTNT genes in Seabream (*Sparus auratus*) and (*Hippoglossus hippoglossus*)

(Vergleichende Analyse der fTNT Gene bei Goldbrasse (Sparus auratus) und Heilbutt (Hippoglossus hippoglossus))

The expression of fTnT genes in a flatfish which undergoes an overt TH driven metamorphosis and a roundfish which does not was studied throughout development and in adult tissue by means of Northen blot, semi-quantitative RT-PCR and in-situ hybridization. In both halibut (Hippoglossus hippoglossus) and sea bream (Sparus auratus) three alternative spliced forms of fTnT were identified which are generated by alternative exon splicing in the 5' region. In both halibut and sea bream the isoforms appear to be stage specific and correspond in sea bream to embryonic (efTnTsb), larval (LfTnTsb) and adult (afTnTsb) specific isoforms, while in halibut they correspond to embryonic/larval (efTnThh) and juvenile/adult (fTnThh-1 and -2) isoforms. In pre-metamorphic halibut larvae all three fTnThh isoforms are present although the most acidic form, efTnThh, is most abundant up until metamorphosis after which it is downregulated to almost undetectable levels. At metamorphosis and in subsequent stages fTnThh-2 is upregulated ~3-fold and becomes the most abundant isoform in halibut muscle. In contrast, fTnThh-1 expression is constant throughout development. Thyroxine treatment of pre-metamorphic halibut larvae leads to the precocious downregulation of efTnThh. In contrast, in the sea bream each isoform is characteristic of the principal developmental stages and the embryonic-acidic isoform (efTnTsb) is downregulated immediately after hatching and is replaced by a larval isoform, LfTnTsb. T3-treatment of sea bream juveniles had no effect on fTnT isoform expression. The results from this comparative analysis of fTnTs in two teleosts suggests molecular processing is common but that regulation of their expression during muscle ontogeny may be species-specific and adapted to their specific ecologies.

Department of Food Science, Faculty of Agricultural Sciences, Aarhus University, Blickers Allé 20, P.O. Box 50, DK-8830 Tjele, Denmark

PIA M. NISSEN and NIELS OKSBJERG

In vitro variation in primary satellite cell proliferation and differentiation within litters of pigs (In vitro Variation der Proliferation und Differenzierung von Satellitenzellen innerhalb von Würfen beim Schwein)

Within litters of pigs the fastest growing pig has a faster growth rate of the individual muscle fibres compared with the slowest growing pigs. Differences in growth rate of individual fibres partly reflect the rate of proliferation and differentiation of the satellite cells (SC). Thus, the aim of this study is to identify differences in proliferation and differentiation of SC within litters of pigs.

SC was isolated from semimembranosus from the lightest (LW), middle (MW) and heaviest (HW) weight female pig within 8 litters. Cells were seeded in 96-well plates and grown in proliferation medium until 80% confluence. The number of viable cells was estimated by addition of WST-1 and measuring the absorbance after 4 h. At 80% confluence medium was changed to differentiation medium, and differentiation was estimated by measuring the CPK-activity at 10 time-points. SC from MW and HW pigs grew at a faster rate and therefore reached confluence one day before cells from LW pigs. For differentiation, MW pigs showed an initial faster differentiation then LW and HW pigs, whereas at later time-points differentiation rate was only slower for LW than MW and HW pigs.

Thus, SC from LW pigs has both a slower proliferation and differentiation rate than SC from HW and MW pigs, when grown under the same *in vitro* conditions.

Corresponding Author PIA M. NISSEN, Department of Food Science, Faculty of Agricultural Sciences, Aarhus University, Blickers Allé 20, P.O. Box 50, DK-8830 Tjele, Denmark

E-Mail: piam.nissen@agrsci.dk

¹Department of Physiological Sciences, ²Department of Clinical Sciences, Faculty of Veterinary Medicine, Warsaw Agricultural University, 02-776 Warsaw, Poland.

A. BURDZIŃSKA¹, U. BARTOSZUK-BRUZZONE² and A. ORZECHOWSKI¹

Epigenesis does not affect myogenicity of muscle progenitor cells after extracorporeal propagation followed by autotransplantation

(Epigenesis beeinflusst nicht die Entwicklung von Vorläuferzellen zu Muskelzellen nach extrakorporaler Propagierung und Autotransplantation)

Irrespective to a high capacity for self-renewal, somatic stem cells have the ability to produce cells that differentiate to maintain tissue structure and function (LATHJA, 1979). Keeping this in mind cell transfer therapy is a dynamically developing technique in tissue engineering aiming to improve the regeneration process in damaged tissue organs (RAFII and LYDEN, 2003). Enormous hope connected with stem cells in therapy brings the hazard of genomic and non-genomic modifications that might evolve during "in vitro" cell engineering and passages. Studies on skeletal muscle-derived cells (MDCs) in the rat model of autotrasplantation gives unique opportunity to check if the risk of epigenetics affect myogenicity of transferred cells.

We decided to check if muscle-derived cells (MDCs) survive and develop into mature myotubes autologous transplantation. MDCs were recruited from specimens either by chemotactic migration or isolated by enzymic digestion. Next, they were selected for myogenicity, propagated and analyzed functionally. To follow the fate of deposited cells they were labeled *in vitro* by transfection with the molecular marker *lacZ* (β -galactosidase encoding gene). Fourteen days after autotransplantation serial cryosections were made perpendicularly to muscle fibers along the injection sites of labeled MDCs. The evaluation of cells survival after transfer was performed with Micro-Image software used to quantitate the average areas of spots of cells stained with X-Gal found in serial sections photographed digitally using UV-VIS-FLUO microscope.

Isolated, preplated muscle progenitor cells were positive for both desmin and Mcadherin. The cells were apparently functional when assessed by fusion index and myotube contractions. This was additionally confirmed by the elevated level of phospho-Ser473-Akt kinase found in MDCs after exposure to IGF-1 (100 ng/ml). In the sites of injection the auto-transplanted MDCs were observed to form clusters showing multinuclear appearance. Obtained results provide compelling evidence that epigenesis does not affect myogenicity of autologous MDCs transplants.

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University of Aarhus, Faculty of Agricultural Sciences, Dept. of Food Science, Research Centre Foulum, Blichers Allé 20, PO Box. 50, DK-8830 Tjele

NIELS OKSBJERG and PIA M. NISSEN

The use of primary muscle cell cultures as a tool to study nutritional regulation of myogenesis in pigs

(Die Anwendung der Primärmuskelzellen als ein Werkzeug für Studien über die ernährungsmässige Regulierung von Myogenese bei Schweinen)

Much of our knowledge on myogenesis stems from experiments on muscle cell cultures. However, a critical view on muscle cell cultures is necessary. This presentation addresses the possibility of using primary muscle cell cultures to study regulation of myogenesis and pinpoints advantages and disadvantages found as a result of a group discussion. Myoblasts and/or satellite cells can be isolated from foetal and postnatal muscles and grown in culture. When proliferating cells reach near confluence, a reduction in serum will withdraw cells from the cell cycle and induce differentiation into multinucleated primitive myotubes. We described/discussed 1) establishing porcine primary muscle cell cultures, 2) use of cell cultures as a bio-assay, 3) use of cell cultures to study effects of exogenous and intrinsic factors, 4) comparison of primary cells vs. cell lines, and finally 5) use of cell cultures to study effects of nutrition. The following items were pinpointed in the group discussion: i) cell cultures are easy to work with and cheap compared with in vivo experiments, ii) proliferation and differentiation can be studied, iii) functionality of genes can be studied, iv) should be regarded as complementary to the in vivo experiments, v) easier to generate a new hypothesis to test, vi) in vitro cultures avoid welfare issues, vii) good for multi-factorial nutrition experiments, viii) serum-free cell cultures may be needed to represent more physiological results.

Key Words: Proliferation, differentiation, energy substrate, endocrine, autocrine and paracrine regulation

Corresponding Author NIELS OKSBJERG University of Aarhus Faculty of Agricultural Sciences Dept. of Food Science Research Centre Foulum Blichers Allé 20, DK-8830 Tjele Dänemark

E-Mail: Niels.Oksbjerg@agrsci.dk

¹King's College London, UK
 ²Israel Oceanographic & Limnological Research, Israel
 ³University of Padua, Italy

A. ROWLERSON¹, B. FUNKENSTEIN², V. BALAS², T. SKOPAL² and G. RADAELLI³

Long-term culture of muscle explants from *Sparus aurata*

Abstract

We have developed an *in vitro* system for long-term culture of muscle explants from the gilthead seabream (*Sparus aurata*) fry. Changes taking place within the explants in culture were assessed by immunostaining for a variety of nuclear and cytoplasmic markers. The original fibres were damaged at the time of sampling, and subsequently degenerated, but the damage also provoked a proliferative response from myogenic precursor cells present in the explant, resulting in the formation of new muscle fibres, some achieving a mature 'fast-white' phenotype within 3 weeks.

Key Words explants, culture, myogenic precursor cells

Zusammenfassung

Titel der Arbeit: Langzeitkultivierung von Muskelexplantaten der Goldbrasse (Sparus aurata)

Ein in-vitro System zur Langzeitkultivierung von Muskelexplantaten gewonnen von Goldbrassen (Sparus aurata) wurde entwickelt. Um Veränderungen in den Explantaten in Kultur zu beobachten wurden Immunfärbungen einiger Zellkern- und Zytoplasmamarker vorgenommen. Bei Entnahme beschädigte Muskelfasern degenerierten, jedoch wird gleichzeitig die Proliferation myogener Vorläuferzellen im Explantat induziert, die zur Bildung neuer Muskelfasern führt. Von diesen weisen einige nach 3 Wochen den Phänotyp reifer, schneller weißer Fasern auf.

Schlüsselwörter: Explantate, Kulturen, myogene Vorläuferzellen

Introduction

So far there is no muscle cell line from fish equivalent to the widely-used and proliferation-competent mammalian *in vitro* model, C_2C_{12} , a mouse satellite cell line. Numerous laboratories have attempted primary culture of fish myoblasts, but the usual observation is that although myoblasts can be isolated and differentiate in culture, they do not proliferate (e.g. MATSCHAK and STICKLAND, 1995). Only a few investigators using rainbow trout find significant proliferation of myoblasts in culture (e.g. CASTILLO et al., 2004). This limits the usefulness of the primary culture system for investigating *in vitro* the physiological roles of growth factors like myostatin (MSTN) and insulin-like growth factors (IGFs) in fish.

Material and Methods

Full details can be found in FUNKENSTEIN et al., 2006. Explants obtained aseptically from *Sparus aurata* fry aged ca 5 months were pressed down firmly onto a substrate & cultured at 21 °C in L15 medium with antibiotics, glutamine and FCS. Cultured explants were observed daily, and the medium changed every 2 days. Explants were taken for analysis at intervals from 3 hours to 23 days. Muscle samples taken at day 0 were also used to make dissociated cell preparations. Explants/samples for histology & immunostaining were fixed in buffered cold 4% paraformaldehyde, dehydrated, wax-embedded and sectioned at 4 μ m for immunostaining.

Explants were evaluated using markers for: degeneration of mature fibres (TUNEL method), proliferation of cells, myogenic differentiation, and growth factors.

Antibodies used were: anti-PCNA (a nuclear marker of cellular proliferation), anti-Myf5 and anti-MyoD (nuclear markers of early myogenic determination), anti-DS (desmin, an early cytoplasmic marker of muscle differentiation), anti-MSTN, anti-IGF-I, anti-IGF-II, anti-IGF-IR (IGF receptor), anti-fast myosin (later marker for muscle differentiation), anti-PV (parvalbumin, a late marker for muscle fast fibre maturation). Results were compared with characteristics of dissociated cells in primary culture with muscle growth & regeneration *in vivo*.

Gene expression of IGF-I, IGF-II and MSTN was determined by RT-PCR using specific primers.

Results

Muscle in vivo:

All muscle fibres were PV+, and DS immunonegative. Only very rare nuclei were PCNA+, Myf5+ or MyoD+.

Dissociated myoblasts in culture:

Small mononucleate DS+ myoblasts were obtained from muscle samples by a standard protocol. Over the next few days these myoblasts fused, grew and laid down myofibrils, but although fibroblasts also present proliferated, myoblasts did not.

Explants at 3 hours:

Muscle fibres were all PV- (PV leaks out of damaged fibres) and there were very rare DS+ reactions on fibre peripheries. Some nuclei were PCNA+, a few Myf5+.

Explants at 2-4 days:

The large number of apoptotic muscle nuclei identified by TUNEL staining showed that the damaged original fibres died, but some live cells remained on the external surface of disintegrating mature fibres and proliferated as shown by an increase in PCNA+ nuclei. Some of the new mononucleate cells were myogenic, as shown by their DS+ cytoplasm, and Myf5+ nuclei. Some of these cells were weakly immunoreactive for IGF-I.

Explants from 5-23 days:

Proliferation of nuclei, and the proportion of newly differentiated myogenic cells (Myf5+) peaked at about 8d. The DS+ mononucleate cells aligned and fused to make multinucleate myotubes which were parvalbumin and fast myosin immunoreactive. From about 6 days IGF-II, IGF-IR and MSTN immunoreactivity gradually appeared in the multinucleate cells. By 23 days, many of the myotubes had increased in diameter, were packed with myofibrils, and were strongly PV-positive and immunoreactive for MSTN, IGF-I and IGF-I receptor.

Growth regulator gene expression:

Transcripts for IGF-I, IGF-II and MSTN were detected in the cultured explants, suggesting that the growth factors identified by immunostaining are being translated from their respective mRNAs.

Discussion

We have developed an *in vitro* system for long-term culture of muscle explants from *Sparus aurata* fry. Our results (described above, and in detail in FUNKENSTEIN et

al., 2006) show that although proliferation of myogenic cells was not obtained in dissociated primary cultures, proliferation from a myogenic cell population did occur in the explants, giving rise to new muscle fibres expressing growth regulators. The new fibres formed in the explants contained a 'fast' myosin and parvalbumin, resembling the regenerative process seen following muscle damage *in vivo*. The pattern of expression of IGF-I, IGF-II, IGF-IR and MSTN in the new cells of the explants was also similar to the events we have seen during myogenesis *in vivo* (RADAELLI et al., 2003a, 2003b; PERROT et al., 1999).

We therefore conclude that this culture system has potential for studying the interaction between MSTN and IGFs in regulating fish muscle growth, the kinds of muscle precursor cells present in fish muscle, and perhaps also for analysis of the ways in which strain differences and prior life history affect muscle growth performance.

Acknowledgements

Recent work on this project was supported by the Israel Academy of Sciences & Humanities (BF), and NATO (all authors).

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> Author affiliations: Dr. A. ROWLERSON Applied Biomedical Research, School of Biomedical & Health Sciences, King's College London, SE1 1UL, UK

Dr. B. FUNKENSTEIN, Ms. V. BALAS, Mr. T. SKOPAL Israel Oceanographic & Limnological Research, P.O. Box 8030, Tel Shikmona, HAIFA 31080, ISRAEL

Prof. G. RADAELLI Department of Experimental Veterinary Sciences, Faculty of Veterinary Medicine, University of Padua, Agripolis, 35020 LEGNARO (Pd), ITALY

Agroscope Liebefeld-Posieux, Swiss Federal Research Station for Animal Production and Dairy Products (ALP), 1725 Posieux, Switzerland

G. BEE, C. BIOLLEY, B. DOUGOUD, G. GUEX and W. HERZOG

Growth performance, carcass characteristics, and meat quality traits as affected by the birth weight and the applied feeding strategies during the growing and finishing period in pigs

(Einflüsse von Geburtsgewicht und Fütterung auf Wachstumsleistung, Schlachtkörperund Fleischqualitätsmerkmale beim Schwein)

Compared to heavier littermates, low-birth-weight pigs tend to exhibit slower growth, increased fat deposition, and impaired meat quality such as tenderness. Feeding strategies aiming to reduce large intra-litter variations in muscle growth will benefit production economy and improve quality uniformity of pork. Thus, the effects of 3 feeding regimes applied during the growing-finishing period on growth performance, carcass characteristics, and meat quality traits of the LM and dark (**STD**) portion of the semitendinosus were assessed in low- (**LW** = 1.12 kg) and high-birth-weight barrows (**HW** = 1.94 kg). Furthermore, in the LM proteolytic capacity was determined (30 min, 2 h, and 24 h postmortem) by casein zymography (μ - and m-calpain activity) and the extent of titin and talin proteolysis (30 min 24, 48, and 72 h postmortem) was monitored by SDS-PAGE and Western Blotting. From 21 litters the lightest and heaviest barrow were selected and randomly assigned to one of 3 dietary treatments; **AA**: ad libitum feed access from 27 to 102 kg BW, **RA**: restricted feeding from 27 to 63 kg and ad libitum feed access from 63 to 102 kg BW, and **RR**: restricted feeding from 27 to 102 kg BW.

Growth performance Regardless of the birth weight, AA- and RA-barrows grew faster (P < 0.01) than RR-barrows (0.77 vs. 0.68 kg/d). The ADFI differed (P < 0.01) among barrows of the 3 dietary treatment groups and was highest in AA- (2.12 kg), intermediate in RA- (2.00 kg), and lowest in RR-barrows (1.78 kg). In the 3 dietary treatment groups LW-barrows consumed more feed (204 vs. 193 kg; P < 0.01) and were less efficient (G/F: 369 vs. 382 g/kg; P = 0.02) than HW-barrows.

Carcass characteristics Carcasses of the RR-barrows were leaner (P = 0.01) and had less (P = 0.03) omental fat than AA- and RA-barrows (lean percentage: 55.2 vs. 56.9%; percentage omental fat: 1.5 vs. 1.8%). In accordance, percentage subcutaneous fat was lower (P = 0.05) in carcasses of RR- than RA-barrows (14.0 vs. 15.0%) with intermediate values in AA-barrows (14.5%). Regardless of the feeding strategy, LW-barrows were leaner (P < 0.01) than HW-barrows (55.0 vs. 56.6%).

Meat quality traits The LM of RA-barrows was lighter (L*: 52.7 vs. 50.6; P = 0.02) than the LM of AA- and RR-barrows. The b*-values (yellowness) were also higher in the LM of RA- than RR-barrows (3.4 vs. 2.7) with intermediate b*-values in the LM of AA-barrows (3.1). Regardless of the feeding strategy, the LM of HW-barrows was less (P = 0.05) yellow than of LW-barrows (2.8 vs. 3.3). The color of the STR was neither affected by the feeding strategy nor the birth weight. Compared to the LM of RA- and the STR of AA-barrows, both muscles tended to be less ($P \le 0.08$) tender (LM: 3.8 vs. 4.2 kg; STD: 4.7 vs. 4.0 kg) in RR-barrows as indicated by the higher shear force values.

Postmortem proteolysis In line with the results for shear force, μ -calpain activity determined at 30 min and 24 h postmortem in the LM of barrows with ad libitum feed access in the finishing period (RA- and AA-barrows) was higher (P < 0.01) than in restrictedly fed barrows. Regardless of the feeding strategy, intact titin tended (P = 0.09) to be less degraded at 48 and 72 h postmortem in LW- than HW-barrows. The extent of talin proteolysis was neither affected (P > 0.68) by the feeding strategy nor the birth weight.

Conclusions The present findings revealed that regardless of the applied feeding strategy low birth weight was mainly associated with impaired carcass quality whereas only small effects were observed for meat quality traits. Furthermore, compensatory growth positively affected meat tenderness probably by increasing the proteolytic capacity at the time of slaughter.

Key Words: Birth weight, compensatory growth, meat quality

¹ Department of Meat and Carcass Quality, IRTA-Monells. Finca Camps i Armet, E-17121 Monells, Girona. Spain; ² Department of Animal Nutrition and Management, Swedish University of Agricultural Sciences, PO Box 7024, 750 07 Uppsala, Sweden;

³Department of Food Science, Swedish University of Agricultural Sciences, PO Box 7051, 750 07 Uppsala, Sweden

NÚRIA PANELLA-RIERA⁽¹⁾, MARIA NEIL⁽²⁾ and JANA PICKOVA⁽³⁾

Evaluation of the influence of dietary fat content and fatty acid composition in four diets based on different fat sources on loins (*M. Longissimus dorsi*) of newborn piglets

Abstract

The aim of this study was to explore if a change in dietary fatty acid content in sow feed had impact on the loin (*M. Longissimus dorsi*) fatty acid (FA) composition of newborn piglets. Four cereal based diets were used: a conventional low fat (3%) sow diet (LF diet) and three high fat (6%) diets: Saturated diet (HFSat diet), Omega-6 diet (40 % high fat oats, HFn-6 diet) and Omega-3 diet (20 % high fat oats and Linseed oil, HFn-3 diet). Loins from newborn piglets (four per treatment) coming from 39 selected litters (mean parity=3.9) were used to analyse FA composition in triacylglyceroles (TG) and phospholipids (PL) fractions by Gas Cromatography (GC). Statistical analysis was performed using proc GLM (SAS v.8.02). Both TG and PL fractions presented significant changes among treatments. In TG fraction, HFn-3 group showed a higher C18:3 *n-3* content (p<0.001) than the other groups, and the same results were found for C20:5 *n-3* (p<0.001), C22:5 n-3 (p<0.001) and C22:6 *n-3*. Similarly, in PL fraction, HFn-3 diet showed a higher content of n-3 (p<0.001) than the other diets. The results indicated than loins from newborn piglets were affected by sow diet.

Key Words: Fat sources, linseed oil, newborn piglets, sow diet

Zusammenfassung

Titel der Arbeit: Untersuchung des Einflusses von Fettgehalt und -zusammensetzung von vier Diäten mit unterschiedlichen Fettquellen auf den *M. longissimus dorsi* beim neugeborenen Schwein

Das Ziel der Untersuchungen war festzustellen, ob Beziehungen zwischen dem Fettsäuregehalt in Sauenfutter und der Fettsäurezusammensetzung (FA) im *M. longissimus dorsi* bei neugeborenen Ferkeln bestehen. Vier verschiedene Diäten wurden untersucht: Eine konventionelle fettarme Sauendiät (3%, LF Diät) und drei fettreiche Diäten (6%), eine gesättigte Fettsäurediät (HFSat Diät), eine Omega-6 Diät (40% fettreicher Hafer, HFn-6 Diät) und eine Omega-3 Diät (20% fettreicher Hafer und Leinkuchenöl, HFn-3 Diät). Die Lendenmuskeln (*M. Longissimus dorsi*) von neugeborenen Ferkeln (vier pro Behandlung), stammten aus 39 ausgesuchten Würfen. Die Untersuchung der Fettsäurezusammensetzung erfolgte mittels Gaschromatophie in Triacylglycerol (TG) - und Phospholipid (PL) Fraktionen. Für die statistische Auswertung wurde das Programm GLM (SAS v.8.02) verwendet. Beide, die TG Fraktion und die PL Fraktion, zeigten signifikante Unterschiede zwischen den Behandlungen. Bei der TG Fraktion zeigte die HFn-3 Versuchsgruppe einen höheren C18:3 *n-3* Gehalt (p<0,001) als die anderen Gruppen. Dieselben Ergebnisse wurden für C20:5 *n-3* (p<0,001), C22:5 n-3 (p<0,001) and C22:6 *n-3* gefunden. Ähnlich war es bei der PL Fraktion, die HFn-3 Diät zeigte einen höheren Gehalt von n-3 (p<0.001) und ein niedrigeres *n-6/n-3* Verhältnis (p<0.01) als die anderen Diäten. Die Ergebnisse zeigten, dass eine Sauendiät die Lendenmuskeln (*M. longissimus dors)i* von neugeborenen Ferkeln beeinflusst.

Schlüsselwörter: Fettzusammensetzung, Leinölkuchen, neugeborene Ferkel, Sauendiät

Introduction

Litter size, piglet birth weight and survival have been important issues for the farming industry during a long time. Still mortality around 10% or more for piglets after birth has been accepted and is very common. This development in pig farming has been ascribed the selection for lean growth resulting in less physiologically mature status of piglets. The Swedish lean pig is no exception, generally suffering the same losses of piglets. So far sow diets have been composed with regard to protein, vitamins,

minerals and energy demands, which have been more generally known as important, while the needs for essentially fatty acids in animal nutrition have not been fully recognized. Indeed, according to the recent BSAS standards (WHITTEMORE et al., 2003), the minimum required level of essential fatty acids is unclear, and 1.0-2.5% linoleic acid in diets for all pigs is suggested as a guideline. Today, the most commonly used fat sources in pig diets are cereals, which contain mostly monounsaturated fatty acids and *n*-6 polyunsaturated fatty acids. Attempts have not been made to design the composition of lipids to fulfil the physiological needs for polyunsaturates including a well balanced ratio of *n*-6/*n*-3 fatty acids (INNIS, 1999). The most obvious goal has been a cheap and not easily oxidised lipid source resulting in high ratio *n*-6/*n*-3 fatty acids, often exceeding 10:1.

Studies on piglet performance as an effect of sow fatty acid nutrition have been performed by ROOKE et al. (2001 a, b). The sources of fatty acids investigated in these experiments were mainly of fish origin. It is well established that the nervous system of newborns has a large demand for n-3 polyunsaturated fatty acids. The retina and brain of mammals is in general very rich in docosahexaenoic fatty acid, 22:6n-3 (DHA). The need of DHA from external sources in piglet retina and brain has been demonstrated by LESKANICH and NOBLE (1999).

The aim of this study was to explore if a change in dietary fatty acid content from low fat, high fat-saturated, and two high fat-unsaturated diets (n-6 rich and n-3 rich, respectively) had impact on the tissue composition of newborn piglets.

Materials and methods

Animals, diets and experimental design

The study was performed on 39 litters. Sows were blocked with a randomised uneven (block) design, according to date of weaning of previous litter and parity/age group, to one of four experimental dietary treatments. The diet was given for the whole period of pregnancy, starting before fertilization until weaning. There were no gilts included, and mean parity of sows was 3.9 (range 2-8). During service and pregnancy period sows were housed in large pens (52 m²) on deep straw bedding, with a maximum of 16 sows per group. One week before expected farrowing sows were moved to individual farrowing pens (8.5 m²), without crates, with partly drained (2.5 m²), and partly heated floors. Sows had free access to water.

The diets were fed to purebred Swedish Yorkshire sows at the Swedish University of Agricultural Sciences, Funbo-Lövsta experimental station. They consisted on four cereal based diets (with soy as protein enhancement): Low Fat diet: conventional low fat (3 %) sow diet (LF diet) and three high fat (6%) diets:

- High fat saturated Diet (HFSat diet)
- High fat *n*-6 Diet: 40 % of high-fat oats was included (*HFn*-6 diet)
- High fat *n*-3 Diet: 20 % of high-fat oats and linseed oil as a source of *n*-3 fatty acids (*HFn*-3 diet)

Sampling and lipid analysis

Immediately following birth, one piglet per litter, preferably the third born was stunned by CO2, bled to death and dissected. The longissimus muscle was cut out with the subcutaneous fat layer included. Loins from newborn piglets (20 animals, 5 per treatment) were collected and stored in -80 °C until analyses.

Lipids in loins were extracted according to HARA and RADIN (1978) to analyze fat content and FA composition in the main lipid fractions, triacylglycerols (TG) and phospholipids (PL) by using the thin-layer chromatography (TLC) methodology, which was performed in the solvent system hexane:diethylether:acetic acid (85:15:1, v/v/v). Methylation of the PL and TG muscle lipids was performed with dry methanol and BF3 addition, described by APPELQVIST (1968) and resulting fatty acid methyl esters (FAME) were stored in hexane at -80 °C until further analysis. The analysis of FAME was performed by using gas chromatography (GC).

Statistical Analysis

All samples were analysed in duplicate. A least square analysis was performed using General Linear Model (GLM) procedure program of SAS statistical package (SAS, 1999). The effect of diet was included in the model as fixed effect.

Results and discussion

Diets and animals

FA composition of the four diets used in the experiment is shown in Table 1. Fat content was 3 % by weight in the control feed (LF diet) compared to 6 % in the high fat feeds (HFSat, HFn-6 and HFn-3). The highest level of 18:3n-3 was found in the linseed oil added feed (HFn-3 feed), and it was nearly 6 times higher in comparison to saturated and HFn-6 feeds. Accordingly, HFn-3 feed has the highest level of PUFA n-3 and therefore, the lowest value of n-6/n-3 ratio (1.95). The highest level of SAFA was found in HFSat feed (43 %), and the lowest one in HFn-3 feed (17 %). The PUFA content in control feed was comparable, by percentage (53 % vs. 51 %) to the PUFA content in the HFn-3 feed. No differences were found between HFn-6 and HFn-3 feed, which included linseed oil (Alternativ Förädling AB, Glanshammar, Sweden), this was added by hand at feeding time, as a top-dress at a rate of 1% by weight.

Table 1

Fatty acid composition (% of identified) of the four diets used in the experiment

	LF	HFSat	HFn-6	HFn-3
Fat content, %	3	6	6	6
12:0	0.22	1.88	0.26	0.44
14:0	0.30	1.38	0.27	0.30
16:0	17.1	34.5	14.7	13.0
16:1	0.21	0.22	0.19	0.13
18:0	2.44	4.69	3.42	2.56
18:1 n-9	23.7	27.3	40.1	29.9
18:1 n-7	1.17	0.81	1.57	0.82
18:2 n-6	48.2	24.9	34.2	33.9
18:3 n-3	4.37	2.34	3.13	17.35
20:0	0.23	0.27	0.32	0.22
20:1 n-9	0.74	0.34	0.76	0.73
22:0	0.17	0.13	0.16	0.06
SAFA	20.6	43.0	19.2	16.7
MUFA	26.0	28.7	42.7	31.8
PUFA	52.6	27.26	37.30	51.2
PUFA n-6	48.2	24.92	34.17	33.9
PUFA n-3	4.37	2.34	3.13	17.4
n-6/n-3	11.0	10.7	10.9	1.95

Abbreviatons: LF: conventional low fat (3%); HFSat: high saturated FA (6%); HFn-6: High n-6 FA (6%); HFn-3diet: High n-3 FA (6%).
The sow data are presented in Table 2. Mean litter size across treatments was 12.6 (incl. stillborn piglets). No significant differences were found in litter sizes; however, sows fed the two unsaturated high fat diets (HFO and HFL) had the largest litters, 11.7 and 13.6 respectively.

		LF	HFSat	HFn-6	HFn-3
No of sows		10	10	9	10
Sow parity		4.0	4.2	4.2	3.4
Sow LW (kg)					
	at service	204	208	207	200
	after farrowing	260	259	254	244
Sow backfat (mm)					
	at service after farrowing	16.1 20.1	16.3 20.7	14.8 18.2	15.4 20.5

Table 2 Size, parity, live weight (LW) and backfat thickness of the sows included in the trial

Abbreviatons: See Table 1.

Piglet loin fatty acid content

Results of total fat content show no differences (P>0.05) among diets $(4.05 \pm 0.72 \%)$ although three of them were designed as high fat diets, containing twice the total amount of fatty acid. This suggests that the extra fat if stored it would probably be as an extra fat tissue was not found in muscle tissue as intramuscular fat.

Piglet loin fatty acid composition

TG fraction had higher saturated (SAFA) and monounsaturated (MUFA) fatty acid content whereas the long-chain PUFA of interest (arachidonic acid, AA; eicosapentaenoic acid, EPA; docosapentaenoic acid, DPA and DHA) were more prevalent in the PL fraction than in the TG. However, α -linolenic acid (18:3 n-3), which was the one used to enrich HFn-3 diet, was higher in TG fraction. Despite these differences in fatty acid content, similar diet effects were observed in both fractions regarding to FA composition on piglet loin muscle: they showed significant changes among treatments, specially the linseed group (HFn-3 diet), which is a source of α -linolenic acid, (Table 3 and Table 4).

HFSat diet did not modify FA composition in piglet loins in comparison with LF diet. However, some differences could be found when studying HFn-3 and HFn-6 diets.

In this study, n-6 fatty acids were composed of 18:2n-6, 18:3n-6, 20:3n-6; 20:4n-6, 22:4n-6 and 22:5n-6, and the n-3 fatty acids were composed of 18:3n-3, 20:3n-3, 20:5n-3, 22:5n-3 and 22:6n-3. With respect to total n-6 PUFA content, no differences were found among diets. However, linoleic acid (18:2n-6) content was higher when HFn-6 and HFn-3 diets were used in the TG (P<0.01) and the PL (P<0.1) fractions. On the other hand, arachidonic acid (20:4n-6) content was lower in HFn-6 and HFn-3 diets in comparison to control one in the TG fraction, whereas in PL no differences were found.

Both fractions (TG and PL) showed differences (P<0.01) in n-3 fatty acid content among diets: α -linolenic acid content was greater (P ≤ 0.001) in HFn-3 diet group than in other treatments, and same results were found in longer n-3 fatty acids, such as EPA

(20:5n-3; P \leq 0.001), DPA (22:5n-3; P \leq 0.001) and DHA (22:6n-3; P \leq 0.01). Therefore, the n-3 content in newborn piglets' loins was higher in HFn-3 diet (P \leq 0.001) and, consequently, n-6/n-3 ratio was lower (P \leq 0.01) in comparison with other diets. Same results were found in growing pigs by KOUBA et al (2003) when using a high linseed diet (6%).

Fatty acid	LF diet	HFSat diet	HFn-6 diet	HFn-3 diet	S.E.	p-value
Fat Content	4.05	3.99	3.30	4.32	0.51	ns
14:0	4.63	4.71	4.14	4.63	0.34	ns
14:1	0.12	0.11	0.05	0.16	0.04	ns
16:0	33.4	35.4	34.6	31.8	1.05	ns
16:1tr	3.80	3.80	3.69	3.90	0.17	ns
16:1 n-7	8.21	7.97	7.79	8.35	0.68	ns
17:0	1.12	0.96	1.01	0.99	0.07	ns
18:0	6.15	5.38	5.54	4.86	0.46	ns
18:1 n-9	23.4	22.0	20.2	21.9	1.77	ns
18:1 n-7	6.29	6.01	7.98	5.68	1.11	ns
18:2 n-6	5.23°	5.68 ^{bc}	7.15 ^{ab}	8.03 ^a	0.51	**
18:3 n-6	0.57	0.54	0.57	0.66	0.04	ns
18:3 <i>n-3</i>	0.13 ^b	0.09 ^b	0.12 ^b	0.61 ^a	0.03	***
20:0	0.18	0.15	0.16	0.14	0.03	ns
20:1	0.40	0.37	0.39	0.35	0.04	ns
20:3	0.26	0.26	0.24	0.22	0.04	ns
20:3 n-6	0.44	0.46	0.55	0.63	0.06	ns
20:4 <i>n-6</i>	1.11	1.45	1.26	1.31	0.26	ns
20:3 <i>n-3</i>	0.11 ^a	0.09 ^a	0.02 ^b	0.13 ^a	0.02	*
20:5 <i>n</i> -3	0.02 ^b	0.02 ^b	0.00 ^b	0.09 ^a	0.01	***
22:4 n-6	1.10	1.13	1.20	1.03	0.15	ns
24:0	0.27	0.17	0.26	0.18	0.20	ns
22:5 n-6	0.57	0.89	0.57	0.63	0.26	ns
24:1	0.08	0.07	0.03	0.07	0.03	ns
22:5 n-3	0.37 ^b	0.43 ^b	0.36 ^b	0.90 ^a	0.06	***
22:6 n-3	0.33 ^b	0.45 ^b	0.34 ^b	0.80 ^a	0.07	**
% ID FA	95.5	95.7	96.6	95.8	0.33	ns
SAFA	45.8	46.8	45.7	42.6	1.14	ns
MUFA	42.4	40.4	40.1	40.6	1.07	ns
PUFA	9.98	11.23	12.2	14.8	1.26	†
PUFA <i>n-3</i>	0.96 ^b	1.08 ^b	0.85 ^b	2.54 ^a	0.14	***
PUFA n-6	9.02	10.2	11.3	12.3	1.13	ns
n-6/n-3	9.46 ^b	9.68 ^b	13.6 ^a	4.83 °	0.70	***

Table 3 Fatty acid composition (% of identified) of loin triacylglycerols

Abbreviatons: ns: non significant; *. p<0.05; **. p<0.01; ***. p<0.001

a.b.c. least squares means within a row with different superscript differ (p<0.05)

LF diet: conventional low fat (3%); HFSat diet: high saturated FA (6%); HFn-6 diet: High n-6 FA (6%); n-3diet: High n-3 FA (6%).

IDFA: Identified Fatty Acid; **SAFA**: Saturated Fatty Acid; **MUFA**: Monounsaturated Fatty Acids; **PUFA**: Polyunsaturated Fatty Acids; *n-3* Fatty Acids; *n-6*: *n-6* Fatty Acids.

Non-essential fatty acids are readily obtained by the foetus either by synthesis from appropriate precursors or by absorption via the placenta, although the placental transfer of fatty acids in swine has been reported to be quite limited (RAMSAY et al, 1991). In this study we have observed an elevation in n-3 PUFA in pig loins born to sows fed HFn-3 diet, which was enriched with linseed oil.

Fatty acid	LF diet	HFSat diet	HFn-6 diet	HFn-3 diet	S.E.	p-value	
14:0	0.26	0.25	0.26	0.38	0.08	ns	
16:0	16.0	16.8	16.3	16.2	0.93	ns	
16:1tr	0.38	0.34	0.34	0.43	0.09	ns	
16:1 n-7	1.90	1.75	1.82	1.79	0.10	ns	
17:0	1.47	1.22	1.32	1.38	0.11	ns	
18:0	13.7	13.6	13.8	14.2	0.73	ns	
Unknown	2.85	2.42	2.48	2.25	0.61	ns	
18:1 n-9	16.0	17.0	16.8	17.2	0.61	ns	
18:1 n-7	7.38	7.94	7.28	7.31	0.37	ns	
Unknown	2.12	1.42	1.80	1.68	0.29	ns	
18:1 n-5	1.75	1.58	1.49	1.36	0.14	ns	
18:2 n-6	10.8	11.3	13.2	13.3	0.73	Ť	
18:3 n-6	0.39	0.30	0.31	0.36	0.05	ns	
18:3 <i>n-3</i>	0.01 ^b	0.02 ^b	0.04 ^b	0.36 ^a	0.03	***	
20:3	0.57	0.48	0.37	0.33	0.07	ns	
20:3 n-6	1.18	1.03	1.19	1.31	0.07	ns	
20:4 <i>n-6</i>	16.9 ^a	16.4 ^{ab}	15.2 ^b	13.4 °	0.52	**	
20:5 <i>n-3</i>	0.29 ^b	0.28 ^b	0.23 ^b	0.88 ^a	0.06	***	
22:4 <i>n-6</i>	1.77 ^a	1.73 ^a	1.76 ^a	1.15 ^b	0.07	***	
24:0	0.23	0.19	0.16	0.00	0.17	ns	
22:5 n-6	1.73 ^a	1.84 ^a	1.69 ^a	1.04 ^b	0.18	*	
22:5 n-3	0.84 ^b	0.80^{b}	0.85 ^b	1.60 ^a	0.07	***	
22:6 n-3	1.05 ^b	1.07 ^b	1.06 ^b	1.61 ^a	0.08	***	
% ID FA	97.8	96.9	98.4	96.2	0.97	ns	
SAFA	31.7	32.1	31.8	32.1	0.67	ns	
MUFA	27.47	28.6	27.8	28.2	0.67	ns	
PUFA	35.0	34.8	35.6	35.1	0.71	ns	
PUFA <i>n-3</i>	2.20 ^b	2.17 ^b	2.18 ^b	4.45 ^a	0.17	***	
PUFA n-6	32.8	32.6	33.4	30.6	0.77	ns	
n-6/n-3	15.0^{a}	16.0^{a}	15 5 ^a	6 90 ^b	1 47	**	

Table 4					
Fatty acid	composition	(% of identified)	of loin	phosphol	lipids

Abbreviatons: see Tab. 3; †. p<0.1.

Conclusion

To conclude, diet seems to be of importance for the piglet production of sows, giving higher litter size in litters where diets were rich in polyunsaturated fatty acids. From our data in the present study, an effect of diet could be recognised on the piglet loin FA composition: the inclusion of linseed oil (18:3n-3) in the sow diet increased n-3 fatty acid content in newborn piglets' tissues. Therefore, it may alter immune function and incidence of inflammatory disease in these piglets.

Sow diets is of great importance to the newborns. The FA composition of the diet is well reflected in the tissue of piglets, here the muscle. It is known that n-3 PUFA have implications on the nervous development in mammals. We can also recognise that the dominant fatty acid in linseed oil, 18:3 n-3 gave a higher DHA level in piglet tissue, indicating a higher desaturation of n-3 fatty acids. This effect was found both in the TG and the PL fractions. This study indicates that the difference of DHA proportion and the low n-6/n-3 ratio of the investigated muscle in the linseed group can be

ascribed to the sow diets. DHA is known to contribute to the development of the nervous functions, which is also recognised by a behaviour study performed on the same piglet groups (GUNNARSSON et al. 2003).

Acknowledgements

This work was supported by the COST ACTION 925 and the Swedish MISTRA funding to FOOD 21.

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Corresponding Author NÚRIA PANELLA-RIERA Department of Meat and Carcass Quality, IRTA-Monells. Finca Camps i Armet, E-17121 Monells, Girona. Spain. Arch. Tierz., Dummerstorf 50 (2007) Special Issue, 79

¹ CCMAR Centre of Marine Sciences, University of Algarve, Portugal.
 ² Laboratory of Aquaculture & Artemia Reference Center, University of Ghent, Belgium.

J. DIAS¹, R. RUEDA-JASSO², L. CONCEIÇÃO¹ and M.T. DINIS¹

Effect of dietary non-protein energy levels on muscle lipids and oxidative status in Senegal Sole (*Solea senegalensis***) juveniles** (Einfluss des Nicht-Protein Energiegehalts der Diät auf Muskelfette und oxidativen Status bei der Senegal-Seezunge (*Solea senegalensis*))

A study was undertaken to determine the effect of dietary non-protein energy levels on muscle and liver fatty acid content and oxidative status in Senegal sole (Solea senegalensis) juveniles. Four isoproteic (crude protein: 52% DM) diets were formulated to contain one of two lipid levels (11 and 21% DM). Furthermore, within each dietary lipid level the digestible carbohydrate content was varied by the incorporation of extruded or crude peas meal. Triplicate groups of 50 sole (IBW: 23.6 ± 1.2 g) were grown in recirculated seawater (temperature: 20°C; salinity: 35 ppt) over 67 days. At the end of the study, liver and muscle samples were withdrawn for analyses of fat content, fatty acid profile, TBARS and activity of selected antioxidant enzymes. During the experimental period the mean fish weight about doubled in all treatments. Dietary treatments affected tissue lipid content, with highest values being generally observed in fish fed high digestible carbohydrate levels. The fatty acid composition of muscle samples were little affected by dietary treatments. The low lipid content of muscle and its highly conservative fatty acid profile suggest that muscle may not be a preferential lipid storage site in Senegal sole. The TBARS values were higher in fish fed diets with high lipid level. The activities of antioxidant enzymes catalase (CAT) and superoxide dismutase (SOD) were higher in livers of fish fed diets with the high lipid level. CAT and SOD activities and TBARS values were also related to the dietary starch type, given that the higher oxidation rates were observed in fish fed the diets containing digestible carbohydrates. This study suggests that lipid and carbohydrate energy sources interact in the definition of the fish oxidative status, and that Senegal sole diets should contain a low lipid level and raw starch, in order to produce fish less prone to oxidation.

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Department of Physiological Sciences, Faculty of Veterinary Medicine, Warsaw Agricultural University, 02-776 Warsaw, Poland.

P. PAWLIKOWSKA, B. PAJAK and A. ORZECHOWSKI

Metabolic programming establishes resistance of soleus muscle to glucocorticoid-induced muscle cachexia

(Metabolische Programmierung bedingt Resistenz des *M. soleus* gegen Glukokortikoid-induzierte Kachexie)

Metabolic programming leads to molecular changes which determine the contribution of the particular fiber type to skeletal muscle. Thus, muscle development is related to changes in morphological, biochemical and mechanical criteria. Soleus muscle (SM) represents oxidative muscles featured by the extreme resistance to catabolic action of glucocorticoids. In contrast to glycolytic-oxidative muscle (gastrocnemius muscle, GM), the somatic index of SM increased in dexamethasone-treated (DEX) growing rats (ŁOKOCIEJEWSKA et al., 2006). In turn, somatic index of GM increased by cotreatment with sodium ascorbate (ASC) or ascorbic acid phosphate (ASC-P) indicating that antioxidants might directly counteract catabolic action of glucocorticoids or that they indirectly improve GM sensitivity to insulin.

The aim of this study was to establish the relationship between the expression of selected biochemical indices of insulin action in muscles (serine-threonine kinase Akt, phospho-Ser473-Akt, subunit I and IV of mitochondrial cytochrome c oxidase) and the activity of selected enzymes (lactate dehydrogenase - LDH, phosphofructokinase – PFK, isocitrate dehydrogenase – ICDH) in SM isolated from rats treated with DEX, ASC or ASC-P alone or combined together.

No changes were observed in the expression of Akt, phospho-Ser473-Akt, subunit I and IV of mitochondrial cytochrome c oxidase in soleus muscle. The activity of LDH was not affected, either. Interestingly, PFK activity in SM was significantly elevated in experimentally treated animals (P<0.001) and the highest was observed after ASC-P. In turn, ICDH activity peaked in SM after dexamethasone treatment (P<0.05) and dropped during the co-treatment with ascorbate (DEX/ASC).

Results of immunoblotting indicate, that dexamethasone does not affect insulin sensitivity in SM. However, the rise in the activity of the step-limiting enzyme of carbohydrate catabolism (PFK) suggests that SM started to utilize glucose both during DEX-induced hyperglycemia and after ASC- or ASC-P-induced sensitization to insulin. Taken together, these results pointed to the metabolic programming as a crucial factor that determines the resistance of SM to glucocorticoid-dependent muscle cachexia.

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Sodium ascorbate (ASC) and ascorbic acid phosphate (ASC-P) differently modulate glucocorticoiddependent metabolic effects in growing rats. Arch. Tierz., Dummerstorf **49** (2006), 41-51 Arch. Tierz., Dummerstorf 50 (2007) Special Issue, 81

The Royal Veterinary College, Royal College Street, London NW1 0TU, UK.

IBRAHIM ALBOKHADAIM and NEIL STICKLAND

Environmental Influences on Early Muscle Development and Consequences for Posthatch Muscle Growth and Activity in Atlantic salmon

(Umwelteinflüsse auf die frühe Muskelentwicklung und ihre Auswirkungen auf Muskelwachstum und Aktivität nach dem Schlupf beim Atlantischen Seelachs)

Larval muscle development in Atlantic salmon is known to be affected by temperature. The aim of this study was to identify the critical time window for this influence and to study the consequences for posthatch muscle growth and activity.

Two groups of salmon eggs were incubated at either 10° C or 5° C from fertilization until hatching. A third group was incubated at 10° C until the eyed stage and then at 5° C to hatch. From hatch onwards all three groups were reared at 5° C. Fish from all groups were sampled at the eyed stage and at 6 weeks and 21 weeks after first feeding. Transverse sections at the level of the vent were used for determining muscle cellularity and for immuno-cytochemistry.

At 6 weeks after first feeding fish reared at 10° C from fertilization until hatching were heavier than the other two groups. At 21 weeks after first feeding fish reared at 5° C from fertilization until hatching were heavier than the 10° C fish. At the eyed stage and at 6 weeks after first feeding the number of white muscle fibres was not different between the three different groups. However, at 6 weeks there were more muscle fibres of smaller diameter in white muscle for the fish initially reared at 5° C, and more larger diameter muscle fibres for the fish initially reared at 10° C. MyoD and myogenin expression were studied at both 6 weeks and 21 weeks. More MyoD and myogenin were found in fish reared at 5° C throughout than the other two groups. The activities of fish were measured using an activity monitor at 3 weeks, 6 weeks and 21 weeks after first feeding. Fish initially reared at 5° C were significantly more active than the other two groups.

The results indicate that incubation temperature up to the eyed stage in salmon may be critical for longer term muscle growth. There is also clear evidence of larval programming of fish activity, which is probably related to muscle growth and phenotype.

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¹Teagasc, Pig Production Development Unit, Moorepark Research Centre, Fermoy, Co.Cork, Ireland.
 ²Teagasc, Ashtown Food Research Centre, Ashtown, Dublin 15, Ireland.
 ³The Royal Veterinary College, Royal College Street, London NW1 0TU, UK

PEADAR G. LAWLOR¹, P. BRENDAN LYNCH¹, M. KAREN O'CONNELL¹, LOUISE M^CNAMARA¹³, PAULA REID² and NEIL C. STICKLAND³

The influence of over feeding sows during gestation on reproductive performance and pig growth to slaughter

Abstract

Increasing feed levels for sows in early to mid gestation has been found to increase muscle fibre number in the semitendinosus muscle of progeny thereby increasing their growth rate and improving food efficiency. Sows (n = 238) blocked at mating on parity and weight were assigned at random to the following gestation feed allowances (1) 30 MJ DE/day throughout gestation, (2) as for 1 but with 60 MJ DE/day from day 25 to 50 of gestation, (3) as for 1 but with 60 MJ DE/day from day 50 to 80 of gestation, (4) as for 1 but with 60 MJ DE/day from day 25 to 80 of gestation and (5) as for 1 but with 45 MJ DE/day from day 80 to 112 of gestation. At weaning, three pigs (heavy, medium and light birth weight) were selected from each of 80 litters and penned individually. Feed intake and pig weight was recorded up to slaughter at c.157 days of age. Treatment had little effect on pig weight (P > 0.05), daily gain (P > 0.05) or feed conversion efficiency (P > 0.05). Carcass back-fat thickness measured at 6cm from the edge of the split back between the 3rd and 4th last rib was 10.6, 9.9, 9.2, 11.0 and 10.6 (s.e.d. 0.39 mm; P < 0.05) for Treatments 1 through 5 respectively. Birth weight was 1.81, 1.51 and 1.15 (s.e.d. 0.025kg; P < 0.001), slaughter weight was 95.5, 96.0 and 88.0 (s.e.d. 2.09kg; P < 0.01) and carcass weight was 72.2, 72.8 and 66.2 (s.e.d. 1.61kg; P < 0.01) for heavy, medium and light birth weight pigs, respectively. Females and males had similar birth weight, slaughter weight and carcass weight. Kill out yield was 768 and 744 (s.e.d 2.0g/kg; P < 0.01) and lean meat yield was 597 and 584 (s.e.d. 3.4g/kg; P < 0.001) for females and males, respectively. In conclusion, increasing gestation feeding allowance had little effect on pig performance to slaughter but did reduce carcass fatness. Light birth weight pigs had a lighter live-weight at slaughter and lighter carcass weight than medium and heavy birth weight pigs which were similar in this regard.

Key Words: sow, gestation feeding, lactation, muscle fibre, growth, carcass, reproduction

Zusammenfassung

Titel der Arbeit: Einfluss der Überfütterung von Sauen während der Trächtigkeit auf die Reproduktionsund Mastleistungen

Bei einem höheren Fütterungsniveau von Sauen während der frühen und mittleren Trächtigkeit wurde eine Erhöhung der Muskelfaseranzahl im *M. semitendinosus* ihrer Nachkommen, verbunden mit einer erhöhten Wachstumsrate und einer verbesserter Futtereffizienz, gefunden. 238 Kreuzungssauen, die künstlich besamt wurden, verabreichte man in verschiedenen Trächtigkeitsstadien fünf unterschiedliche Futterrationen: (1) 30 MJ DE/Tag während der Trächtigkeit, (2) wie bei 1 aber mit 60 MJ DE /Tag vom 25. bis zum 50. Trächtigkeitstag, (3) wie bei 1 aber mit 60 MJ DE / Tag vom 50. bis zum 80. Trächtigkeitstag, (4) wie bei 1 aber mit 60 MJ DE / Tag vom 25. bis zum 80. Trächtigkeitstag, (5) wie bei 1 aber mit 45 MJ DE / Tag vom 80. bis zum 112. Trächtigkeitstag.

Nach dem Abferkeln wurden drei Ferkel (mit schwerem, mittlerem und leichtem Geburtsgewicht) aus jedem der 80 Würfe selektiert und einzeln aufgestallt. Futterverbrauch und Körpergewicht wurden ermittelt bis zur Schlachtung am etwa 157. Lebenstag. Die Versuche hatten einen geringen Einfluss auf das Körpergewicht (P > 0,05), tägliche Zunahme (P > 0,05) bzw. Steigerung der Futtereffizienz (P>0,05). Die Rückenspeckdickenmessungen am Schlachtkörper zwischen der 3. und 4. letzten Rippe zeigten 10,6 mm, 9,9 mm, 9,2 mm, 11,0 mm and 10,6 mm (s.e.d. 0,39mm; P < 0,05) für die Gruppen 1 bis 5. Für Schweine mit einem hohen, mittleren und niedrigen Geburtsgewicht betrug das Geburtsgewicht 1,81 kg, 1,51 kg and 1,15kg (s.e.d. 0,025kg; P < 0,001) und das Schlachtgewicht 72,2 kg, 72,8 kg and 66,2 kg (s.e.d. 1,61kg; P < 0,01). Weibliche und männliche Tiere hatten ähnliches Geburtsgewicht, Schlachtgewicht und Schlachtkörpergewicht. Die Schlachtkörpergewichtszunahme betrug 768 g and 744 g (s.e.d 2,0g/kg; P < 0,01) und die Magerfleischzunahme 597 g and 584 g (s.e.d. 3,4g/kg; P < 0,001) für weibliche und männliche Tiere. Zusammenfassend ist zu sagen, dass eine Erhöhung des Fütterungsniveaus während der Trächtigkeit einen geringen Einfluss auf die Leistungen der Schweine bis zur Schlachtung hatte, es führte nicht zu einer Reduzierung des Fettgehaltes des Schlachtkörpers. Schweine mit einem geringen Geburtsgewicht hatten ein geringeres Schlachtgewicht und geringeres Schlachtkörpergewicht als Schweine mit einem mittleren bis hohem Geburtsgewicht, die sich bei diesem Versuch nicht deutlich unterschieden.

Schlüsselwörter: Sau, Fütterung in der Trächtigkeit, Laktation, Muskelfasern, Wachstum, Schlachtkörper, Reproduktion

Introduction

Primary muscle fibres are believed to be genetically fixed and are thought to be unaffected by conditions in utero (DWYER and STICKLAND, 1991). However secondary muscle fibres which form between day 54 and 90 of gestation are vulnerable to conditions in utero which can be influenced by environmental factors including nutrition (STICKLAND, 1996). Increasing nutrition during mid gestation for sows increases the ratio of secondary to primary muscle fibres in progeny and this has benefits in terms of increased growth rate and improved feed conversion efficiency in the latter stages of pig growth (DWYER et al., 1994).

This is thought to have particular implications with modern hyperprolific sows where intra uterine growth retardation occurs in some foetuses due to the detrimental effects on placental development caused by uterine crowding in the early post-implantation period. This effectively limits the nutrient availability to the embryo during myogenesis which can result in reduced numbers of muscle fibres in piglets at birth, low birth weight and increased intra-litter variability in birth weight (FOXCROFT et al., 2006; WU et al., 2006). Increased maternal feed intake during early to mid gestation may help to alleviate the negative impact of uterine crowding in these highly prolific sows. Potential improvements in this regard and subsequently in carcass and meat quality are thought to be greatest in lighter litter-mates since they normally have lower numbers of muscle fibres compared with heavy litter-mates (REHFELDT and KUHN, 2006).

In spite of the positive effect on the foetus, excessive feed intake during gestation may reduce lactation feed intake of sows and thus increase weight loss in this critical period. Excessive lactation weight loss reduces the subsequent reproductive performance of sows (THAKER and BILKEI, 2005). For this reason the practice of increasing gestation feed intake to increase uniformity and growth of the litter may have a negative impact on the subsequent reproductive performance.

The objective was to examine the effect of 5 gestation feeding regimens on intra-litter variation in piglet weight at birth, growth of light, medium and heavy birth weight progeny to slaughter and carcass quality. The effect of increasing gestation feed allowance on lactation feed intake of sows, sow weight change and reproductive performance was also investigated.

Materials and Methods

Sows and treatments

Multiparous Landrace x Large White sows (n = 238) were artificially inseminated using the pooled semen from 7 boars Hylean Large White boars (Hermitage AI, Co. Kilkenny, Ireland). The sows were balanced on parity at mating and assigned to the following gestation feeding allowances: (1) 30 MJ Digestible energy (DE)/day throughout gestation, (2) as for 1 but with 60 MJ DE/day from day 25 to 50 of gestation, (3) as for 1 but with 60 MJ DE/day from day 50 to 80 of gestation, (4) as for 1 but with 60 MJ DE/day from day 25 to 80 of gestation and (5) as for 1 but with 45

MJ DE/day from day 80 to 110 of gestation. Between day 110 and farrowing all sows were fed 25 MJ DE/day of lactation diet (Table 1). Once farrowed sows were liquid fed (3.4:1 water to feed DM) a lactation diet by a computerised feeding system (Big Dutchman, Vechta, Germany). A lactation feed curve increasing from 25 MJ DE/kg at farrowing to 95 MJ DE/kg at weaning (28 ± 0.9 days) was used and sows were fed twice daily. The ingredient composition and chemical analysis of the diets fed are shown in Table 1.

Table 1

Ingredient composition and nutrient content of experimental diets (g/kg)

Diet type	Gestation	Lactation	Starter ⁵	Link ⁵	Weaner	Finisher
Steam flaked wheat						
Wheat		423.9			455.4	404
Barley	892.9	350			225	364.1
Soya 50	75	160			180	200
Full fat soya					100	
Soya oil	10	40			10	10
Mineral and vitamins ¹	1.5	1.5			3.0	1.0
Lysine HCl ²	0.5	2.0			4.0	3.0
DL-Methionine ²		0.7			2.0	0.8
L-Threonine ²		0.8			1.5	1.0
Di-calcium phosphate	5	5			5.0	
Limestone flour	11	12			11	13
Salt	4	4			3	3
Pulmotil ³				+		
Phytase 5000 iu/g ⁴	0.1	0.1			0.1	0.1
Analysed chemical composition	(g/kg)					
Dry matter	871	873	870	870	872	870
Crude Protein	132	158	200	200	196	178
Fat	31	56	90	75	43	27
Crude fibre	45	35	25	30	36	37
Ash	44	46	60	60	50	44
Lysine	6.2	9.1	16.0	15.0	13.1	11.1
Digestible energy (MJ/kg)	13.0	14.2	16.3	15.4	14.1	13.7

¹Provided per kilogram of complete diet:

Gestation and lactation diets: Cu, 15 mg; Fe, 70 mg; Mn, 62 mg; Zn, 80 mg; I, 0.6 mg; Se, 0.2 mg; vitamin A, 10000 IU; vitamin D₃, 1000 IU; vitamin E, 100 IU; vitamin K, 2 mg; vitamin B₁₂, 15 μ g; riboflavin, 5 mg; nicotinic acid, 12 mg; pantothenic acid, 10 mg; choline chloride, 500 mg; Biotin, 200 μ g; Folic acid, 5 mg; vitamin B₁, 2 mg and vitamin B₆, 3 mg.

Weaner diet: Cu, 155 mg; Fe, 90 mg; Mn, 47 mg; Zn, 120 mg; I, 0.6 mg; Se, 0.3 mg; vitamin A, 6000 IU; vitamin D₃, 1000 IU; vitamin E, 100 IU; vitamin K, 4 mg; vitamin B₁₂, 15 μ g; riboflavin, 2 mg; nicotinic acid, 12 mg; pantothenic acid, 10 mg; choline chloride, 250 mg; vitamin B₁, 2 mg; vitamin B₆, 3 mg; and endox, 60 mg.

Finisher diet: Cu, 15 mg; Fe, 24 mg; Mn, 31 mg; Zn, 80 mg; I, 0.3 mg; Se, 0.2 mg; vitamin A, 2000 IU; vitamin D₃, 500 IU; vitamin E, 40 IU; vitamin K, 4 mg; vitamin B₁₂, 15 μ g; riboflavin, 2 mg; nicotinic acid, 12 mg; pantothenic acid, 10 mg; vitamin B₁, 2 mg and vitamin B₆, 3 mg.

² Synthetic amino acids

³Link diet contained 200mg Tilmicosin per kg of feed provided from Pulmotil G100 (Eli Lilly and Company Limited, Basingstoke, Hampshire, England).

⁴ Sow, weaner and finisher diets contained 500 FTU phytase per kg finished feed from Natuphos 5000 (BASF, Ludwigshafen, Germany). ⁵ Commercial diets for which the ingredient composition was not disclosed.

The sows were weighed and back-fat depth recorded at day 0, 25 50, 80 and 110 of gestation, at weaning, at service and at day 110 of their subsequent gestation. Back-fat depth was measured at the P2 position using an ultrasound scanner (Lean meater, Renco Corporation, Minneapolis, Minnesota, USA). Sow feed intake was recorded during gestation, lactation and between weaning and oestrus (5 ± 2.1 days after weaning). Actual lactation weight loss (%) was calculated as ((weaning weight – weight at day 110) / weight at day 110) x 100. Estimated lactation weight loss (%) was calculated as (weaning weight – (weight day 110 – (total born x 2.28)) / (weight day 110 – (total born x 2.28)) x 100. The value of 2.28 kg is an estimate of increased

weight in the gravid uterus and in mammary tissue attributed to each pig in a litter (NRC, 1998).

After each farrowing, number of pigs born alive and number of pigs born dead was recorded. Each pig was individually weighed and tagged for identification purposes at birth. Litter size was standardised at farrowing to c. 10 pigs per litter across treatments by cross fostering. Creep feed (starter diet) was fed from day 12 to weaning to all litters. Pre-weaning deaths were recorded and at weaning individual pig weights and number weaned per litter was recorded.

Progeny

Three pigs selected on birth weight (one light, one medium and one heavy) and sex were taken from each of 80 litters (N = 16 sows per treatment) at weaning. In total 240 pigs (120 male and 120 female) were then individually penned and followed through to slaughter at c.158 days of age. Intake and growth rate of these pigs was recorded to slaughter. Each pig was fed 2 kg starter diet, 5 kg link diet followed by weaner diet as dry pellets to day 49 post-weaning. A dry pelleted finisher diet was fed during the period from day 49 post-weaning to slaughter at day 130 post-weaning. Pigs were fed 3 times daily in the first week and *ad-libitum* thereafter, with care being taken to avoid feed wastage and spoilage. Intakes were recorded weekly and pigs were weighed at days 28, 49, 90 and 130 post-weaning.

Housing

Dry sows were penned individually in basket stalls (2.4m x 0.6m; O'Donovan Engineering, Coachford, Ireland) up to day 110 of gestation. Temperature was maintained at 20 to 22 $^{\circ}$ C.

From day 110 of gestation until weaning, sows were accommodated in farrowing rooms with 10 pens per room. NPD type farrowing crates (O'Donovan Engineering) were used. Water was available *ad-libitum* from one nipple drinker per pen and supplemental water was provided by lever valve where necessary. Temperature was maintained at 22 °C except around farrowing when temperature was increased to 24 °C for 48 hours.

Pigs were penned individually from weaning to day 49 post-weaning in fully slatted (Faroex, Manitoba, Canada) pens 1.2 m x 0.9 m. Between day 49 and day 130 post-weaning pigs were penned in fully slatted pens (concrete slats; 75mm solid and 20mm slots) 1.81m x 1.18m. Water was available *ad-libitum* from one drinking bowl (BALP, La Buvette, Charleville Nord, France) per pen. Each pen had a single stainless steel feeder 30 cm wide (O'Donovan Engineering). Temperature was maintained at 28 °C in the first week and reduced by 2 °C per week to 22 °C in the fourth week after weaning. Air temperature was maintained at 20 to 22 °C between day 49 and day 130 post-weaning.

Slaughter

Pigs were slaughtered at when they reached 158 days of age. They were transported 107 km to the abattoir and killed by bleeding after CO_2 stunning. Backfat thickness was measured at 6 cm from the edge of the split back at the level of the 3rd and 4th last rib using a Hennessy grading Probe (Hennessy and Chong, Auckland, New Zealand). Lean content was estimated according to the following formula (Department of Agriculture and Food, 2001):

Estimated lean meat content (g/kg) = 603 - 8.47x + 1.47y

where x = fat depth (mm); y = muscle depth (mm)

Carcass weight was estimated by multiplying the weight of the hot eviscerated carcass, (minus tongue, bristles, genital organs, kidneys, flare fat and diaphragm) 45 minutes after slaughter by 0.98. Kill-out proportion (g/kg) was calculated as carcass weight/slaughter weight.

Statistics

The experiment was a completely randomised design. Sow and litter data were analysed using the general linear models procedure of SAS (SAS, 1996) with effects for treatment, parity grouping and the interaction effect of treatment x parity grouping. There were 5 parity groupings; 1, 2, 3, 4 and 5+. The litter was the experimental unit.

The performance of individually housed pigs between weaning and slaughter was subjected to analysis of variance using GENSTAT (Genstat, 1993) for a split plot design. The experiment was conducted in two time periods or batches. In the model batches were included as blocks, gestation feeding treatment was the main plot, weight category was the sub-plot and gender was included as the sub-sub-plot. The individual pig was the experimental unit.

Results

Daily and total lactation energy intake of sows was less for treatment 4 than any of the other treatments (P < 0.05; Table 2). However, actual and estimated lactation weight loss of sows was not affected by treatment (P > 0.05). There was a tendency towards a treatment effect for subsequent farrowing rate (P = 0.10) where treatment 1 was lowest and treatment 5 highest. There was a numerical reduction in subsequent born alive of 0.5 pigs with treatment 4 compared with the control (Treatment 1), however this difference was not significant (P > 0.05).

Table 2

Effect of treatment on lactation feed intake, sow weight loss, litter size and farrowing rate

	/	0 ,			0		
	1	2	3	4	5	se	Significance
Mean lactation energy intake (MJ/day)	76.2 ^a	75.2 ^a	75.3 ^a	67.2 ^b	73.9 ^a	2.16	*
Total lactation energy intake (MJ)	2135 ^a	2110 ^a	2120 ^a	1852 ^b	2058^{a}	62.5	*
Actual lactation weight loss (%)	19.8	20.6	18.7	18.7	19.8	0.94	
Estimated lactation weight loss (%)	9.9	11.7	8.0	9.2	9.6	1.29	
Subsequent farrowing rate (%)	66	79	84	84	93	6.2	P = 0.10
Subsequent born alive (No.)	11.0	10.7	11.1	10.5	11.4	0.53	
Subsequent born dead (No.)	1.77	1.56	1.61	1.48	1.28	0.334	
1 obso							

^{1 abc,} Within rows, means without a common superscript differ significantly (P < 0.05).

Mean daily lactation energy intake was higher for parities 2, 3 and 4 than parity 1 (P < 0.05; Table 3). Actual weight loss during lactation was higher for parity groups 1, 2, and 3 than for parity group 5 to 8 (P < 0.001); however estimated lactation weight loss was not affected by parity (P > 0.05). Parity group 5 to 8 had the lowest subsequent farrowing rate (P < 0.001) and born alive (P < 0.01).

Treatment had no effect on born alive (P > 0.05; Table 4), mean birth weight (P > 0.05), coefficient of variation of birth weight (CV; P > 0.05), weaning weight (P > 0.05), CV of weaning weight (P > 0.05) or average daily gain (ADG) from birth to weaning (P > 0.05). However, the total number of piglets born dead was influenced

Table 3	
Effect of parity group on lactation feed intake, sow weight loss, litter size and farrowing rate	

	1	2	3	4	5 to 8	se	Significance
Mean lactation energy intake (MJ/day)	66.7 ^b	76.4 ^a	75.9 ^a	76.4 ^a	72.0 ^{ab}	2.17	*
Total lactation energy intake (MJ)	1973	2117	2101	2001	2026	62.5	
Actual lactation weight loss (%)	22.4 ^c	20.4^{bc}	20.4^{bc}	18.3 ^{ab}	16.1 ^a	0.94	***
Estimated lactation weight loss (%)	11.5	10.0	9.0	10.5	7.4	1.29	
Subsequent farrowing rate (%)	85 ^a	86 ^a	89 ^a	88^{a}	57 ^b	6.2	***
Subsequent born alive (No.)	12.0 ^a	11.8 ^a	11.0 ^a	10.9^{ab}	9.4 ^b	0.53	**
Subsequent born dead (No.)	1.54	1.35	2.21	1.44	1.16	0.334	

^{1 abc.} Within rows, means without a common superscript differ significantly (P < 0.05).

Table 4

Effect of treatment on litter size, piglet growth and within litter variation in pig weight

	1	2	3	4	5	se	Significance
Born Alive (No.)	11.46	9.80	10.23	9.40	11.05	0.510	
Total Born Dead (No.)	0.97^{b}	1.02 ^b	1.91 ^a	1.70^{ab}	1.30 ^{ab}	0.275	*
Birth weight (kg)	1.52	1.54	1.59	1.65	1.49	0.040	
CV Birth Weight (%)	19.0	16.4	16.6	17.9	18.9	1.41	
Weaning Weight (kg)	7.4	7.9	7.9	7.7	7.6	0.15	
CV Weaning Weight (%)	18.9	18.8	16.7	19.4	17.6	1.16	
ADG birth to weaning (g/d.)	211	228	225	217	217	5.0	

^{1 ab}, Within rows, means without a common superscript differ significantly (P < 0.05).

There was no parity group effect on total number of piglets born dead (P > 0.05; Table 5) mean birth weight (P > 0.05), CV of birth weight (P > 0.05), weaning weight (P > 0.05), CV of weaning weight (P > 0.05) or average daily gain (ADG) from birth to weaning (P > 0.05). The total number of piglets born alive tended to increase up to parity 3 and then decreased for subsequent parities (P < 0.10).

Table 5 Effect of parity group on litter size, piglet growth and within litter variation in pig weight

	1	2	3	4	5 to 8	se	Significance
Born Alive (No.)	10.33	10.98	11.19	9.82	9.64	0.510	P = 0.10
Total Born Dead (No.)	1.26	1.13	1.81	1.42	1.47	0.275	
Birth weight (kg)	1.52	1.60	1.55	1.59	1.52	0.040	
CV Birth Weight (%)	18.0	17.6	17.9	16.8	18.4	1.41	
Weaning Weight (kg)	8.0	7.9	7.6	7.6	7.6	0.15	
CV Weaning Weight (%)	16.4	17.6	20.5	18.2	18.6	1.16	
ADG birth to weaning (g/d.)	229	226	213	214	216	5.0	

Gestation feeding regimen had little effect on pig weight (P > 0.05), daily gain (P > 0.05) or feed conversion efficiency (P > 0.05; Table 6). Carcass back-fat thickness was lower for Treatment 3 than Treatments 1, 4 and 5 (P < 0.05). Treatment 3 also tended to have the highest lean meat yield (P = 0.11).

Birth weight was 1.81, 1.51 and 1.15 (s.e.d. 0.025kg; P < 0.001), slaughter weight was 95.5, 96.0 and 88.0 (s.e.d. 2.09kg; P < 0.01) and carcass weight was 72.2, 72.8 and 66.2 (s.e.d. 1.61kg; P < 0.01) for heavy, medium and light birth weight pigs, respectively (Table 7). Females and males had similar birth weight, slaughter weight and carcass weight. Females had higher kill out yield (P < 0.01) and lean meat yield (P < 0.001) than males.

Table 6	
Effect of gestation feeding regimen on pig weight and carcass quality	
Gestation feeding regimen	

12345s.e.d.SignificanceBirth weight (kg)1.531.441.481.481.520.087Weaning weight (kg)7.68.18.37.77.60.58Weight Day 48 (kg)30.128.629.428.729.11.70Weight Day 90 (kg)62.955.558.358.957.03.94Weight Day 131 (kg)97.389.892.295.191.34.37Carcass weight (kg)73.667.768.872.469.63.38Kill aut (adap)7567527547607574.7								
Birth weight (kg) 1.53 1.44 1.48 1.48 1.52 0.087 Weaning weight (kg) 7.6 8.1 8.3 7.7 7.6 0.58 Weight Day 48 (kg) 30.1 28.6 29.4 28.7 29.1 1.70 Weight Day 90 (kg) 62.9 55.5 58.3 58.9 57.0 3.94 Weight Day 131 (kg) 97.3 89.8 92.2 95.1 91.3 4.37 Carcass weight (kg) 73.6 67.7 68.8 72.4 69.6 3.38 Kill out (adap) 756 752 754 760 757 4.7		1	2	3	4	5	s.e.d.	Significance
Weaning weight (kg)7.68.18.37.77.60.58Weight Day 48 (kg)30.128.629.428.729.11.70Weight Day 90 (kg)62.955.558.358.957.03.94Weight Day 131 (kg)97.389.892.295.191.34.37Carcass weight (kg)73.667.768.872.469.63.38Kill out (adap)7567527547607574.7	Birth weight (kg)	1.53	1.44	1.48	1.48	1.52	0.087	
Weight Day 48 (kg)30.128.629.428.729.11.70Weight Day 90 (kg)62.955.558.358.957.03.94Weight Day 131 (kg)97.389.892.295.191.34.37Carcass weight (kg)73.667.768.872.469.63.38Kill out (alax)7567527547607574.7	Weaning weight (kg)	7.6	8.1	8.3	7.7	7.6	0.58	
Weight Day 90 (kg) 62.9 55.5 58.3 58.9 57.0 3.94 Weight Day 131 (kg) 97.3 89.8 92.2 95.1 91.3 4.37 Carcass weight (kg) 73.6 67.7 68.8 72.4 69.6 3.38 Kill out (alap) 756 752 754 760 757 4.7	Weight Day 48 (kg)	30.1	28.6	29.4	28.7	29.1	1.70	
Weight Day 131 (kg) 97.3 89.8 92.2 95.1 91.3 4.37 Carcass weight (kg) 73.6 67.7 68.8 72.4 69.6 3.38 Kill out (a/a) 756 752 754 760 757 4.7	Weight Day 90 (kg)	62.9	55.5	58.3	58.9	57.0	3.94	
Carcass weight (kg) 73.6 67.7 68.8 72.4 69.6 3.38 Kill out (a/a) 756 752 754 760 757 4.7	Weight Day 131 (kg)	97.3	89.8	92.2	95.1	91.3	4.37	
Will put (a/ba) 756 752 754 760 757 47	Carcass weight (kg)	73.6	67.7	68.8	72.4	69.6	3.38	
Kin out (g/kg) 750 755 754 760 757 4.7	Kill out (g/kg)	756	753	754	760	757	4.7	
Fat (mm) 10.6^{a} 9.9^{ab} 9.2^{b} 11.0^{a} 10.6^{a} 0.39 *	Fat (mm)	10.6^{a}	9.9^{ab}	9.2 ^b	11.0^{a}	10.6^{a}	0.39	*
Muscle depth (mm) 51.3 50.2 49.9 50.0 51.8 1.02	Muscle depth (mm)	51.3	50.2	49.9	50.0	51.8	1.02	
Lean meat yield (g/kg) 589 593 598 583 589 4.1 P = 0.11	Lean meat yield (g/kg)	589	593	598	583	589	4.1	P = 0.11

Table 7

Effect of birth weight category and gender on pig weight and carcass quality

	Weight Category					Gender			
	High	Medium	Low	s.e.d.	Р	Female	Male	s.e.d.	Р
Birth weight (kg)	1.81 ^a	1.51 ^b	1.14 ^c	0.025	***	1.48	1.5	0.028	
Weaning weight (kg)	8.9 ^a	8.2 ^b	6.5 ^c	0.17	***	7.8	7.9	0.21	
Weight Day 48 (kg)	31.1 ^a	29.9 ^a	26.6 ^b	0.66	***	29.5	28.9	0.63	
Weight Day 90 (kg)	60.9 ^a	60.1 ^a	54.6 ^b	1.53	**	59.4	57.7	1.2	
Weight Day 131 (kg)	95.5 ^a	96.0 ^a	88.0^{b}	2.09	**	91.9	94.4	1.68	
Carcass weight (kg)	72.2 ^a	72.8 ^a	66.2 ^b	1.61	**	70.3	70.5	1.27	
Kill out (g/kg)	757	756	755	2.8	NS	768	744	2.0	**
Fat (mm)	10.1	10.5	10.2	0.35	NS	9.7	10.9	0.32	**
Muscle depth (mm)	51.0^{ab}	51.5 ^a	49.4 ^b	1.19	*	51.8	49.5	0.74	**
Lean meat yield (g/kg)	593	590	589	3.8	NS	597	584	3.4	***

Discussion

Minimising weight loss during lactation is critical when attempting to achieve an early return to oestrus after weaning (TANTASUPARUK et al., 2001; EISSEN et al., 2003; THAKER and BILKEI, 2005) and a high litter size at the subsequent farrowing (THAKER and BILKEI, 2005; EISSEN et al., 2003). Increasing the gestation feed allowance in the present study reduced lactation appetite in sows, however, this did not increase lactation weight loss and therefore subsequent reproductive performance was not affected by treatment. THAKER and BILKEI (2005) suggest that weight loss during lactation should not be greater 10% for multiparous sows if early return to oestrus, high farrowing rate and a high subsequent litter size are to be achieved. The estimated lactation weight loss in the present study was very close to this target for all treatments and this may explain why subsequent reproductive performance was not negatively affected due to treatment. Farrowing rate actually tended to increase for all

treatments where gestation feed allowance was increased with the greatest increase found for treatment 5. This finding was unexpected as lactation weight loss was similar for all treatments. In this regard it is important to point out that farrowing rate for treatment 1 (control) was exceptionally low at 66%.

Increased gestation feeding as practised here was found to have little effect on piglet birth weight. This has been the experience in a number of other similar studies (DWYER et al., 1994; NISSEN et al., 2003; HEYER et al., 2004; BEE, 2004; KING et al., 2006). The present study also found that intra-litter variation in piglet birth weight and weaning weight was not changed due to maternal feeding treatment. Others have also found this to be the case for birth (HEYER et al., 2004; KING et al., 2006) and weaning weight (KING et al., 2006). Overfeeding between day 50 and 80 of gestation caused an increase in the number of piglets born dead per litter. This has not been previously reported in studies of this kind. The number of piglets born dead almost doubled on this treatment compared to the control.

REHFELDT and KUHN (2006) in their review concluded that maternal feeding above requirements seems not to be very effective in improving foetal growth but that the small littermates could benefit from the additional nutrient supply. The present study followed small, medium and light littermates to slaughter but found little response from additional gestation feed. There was no birth weight by gestation feeding treatment interaction or simple effect of gestation feeding on pig weight, growth or feed conversion efficiency between weaning and slaughter. This is contrary to the results of DWYER et al. (1994) but supports the results from many other studies where these variables were not positively affected by increased gestation feeding (NISSEN et al., 2003; LAWLOR and LYNCH, 2004; HEYER et al., 2004; BEE, 2004). Some reports actually found a reduction in the growth rate of progeny as a result of increasing the gestation feed allowance (HEYER et al., 2004; BEE, 2004).

Increasing sow feed allowance between day 50 and 80 of gestation did reduce carcass fatness in progeny and tended to increase lean meat yield. Other studies have found little effect of gestation feeding treatment on carcass fatness (HEYER et al., 2004) or lean meat yield (NISSEN et al., 2003; HEYER et al., 2004; BEE, 2004). On the contrary BEE (2004) found that increased energy supply during gestation actually increased carcass fatness.

Pig weight at all stages from day 48 post-weaning to slaughter and carcass weight was less for light than either medium or heavy birth weight pigs. The advantage of a heavy weight at birth for increased weaning weight and increased post weaning performance was previously reported (LAWLOR et al., 2002). This advantage is most likely due to an increased number of fibres in the muscle of pigs that are heavier at birth-weight compared to their lighter littermates (NISSEN et al., 2004; REHFELDT and KUHN, 2006) and because lighter pigs are less mature at birth (lower fat and protein and less water in the whole body) than their heavy littermates (REHFELDT and KUHN, 2006). The latter study also found that lean meat yield and loin muscle area at slaughter were also lower for light birth weight pigs than their heavier littermates. This difference was thought to be because light birth weight pigs had lower fibre numbers and a higher proportion of large fibres. In the present study muscle depth was the only carcass quality measure that differed for the three birth

weight groups and then only the medium birth weight group was found to have a higher muscle depth than the light birth weight pigs. Histological analysis still remains to be done on samples generated from these pigs but one could speculate that fibre number might be similar for heavy and medium birth weight pigs and lower for the light birth weight pigs.

In conclusion, increasing feed intake during gestation was found to reduce appetite of sows during lactation but did not increase weight loss during this period. Born dead was found to increase where sow feed intake was increased between day 50 and 80 of gestation. Birth weight, weaning weight and the intra-litter variation for these two variables were not affected by gestation feeding. Post-weaning weights up to and including slaughter and carcass weight were not affected by increased gestation feeding. However, carcass fatness increased where maternal feed level was increased between day 50 and 80 of gestation and there was tendency for lean meat yield to increase on this treatment also. All post-weaning weights and carcass weight were similar for heavy and medium birth weight pigs but lower for light birth weight pigs.

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Corresponding Author PEADAR G. LAWLOR Teagasc, Pig Production Development Unit, Moorepark Research Centre, Fermoy, Co.Cork, Ireland

E-Mail: peadar.lawlor@teagasc.ie

Arch. Tierz., Dummerstorf 50 (2007) Special Issue, 92-96

Departament de Fisiologia, Facultat de Biología, Universitat de Barcelona, Ave. Diagonal, 645, Barcelona 08028, Spain.

D. GARCÍA DE LA SERRANA, N. MONTSERRAT, M. CODINA, J. SÁNCHEZ-GURMACHES, A. IBARZ, M. BELTRÁN, I. NAVARRO and J. GUTIÉRREZ

Looking for markers of muscle growth and flesh quality in fish

Abstract

Substantial progress in aquaculture has been made in the last ten years. Research on flesh quality and especially on muscle growth regulation is, however, still insufficient. In vitro and in vivo studies were performed in order to evaluate AKT, AKTP, MAPK, MAPKP, and c-Met as indicators of growth and muscle quality and to determine the best conditions in terms of feeding and water circulation for sustainable and efficient fish aquaculture. Expression analyses on the level of protein and/or mRNA in myocyte cultures promote the in vitro studies as an useful tool to identify the molecules that may indicate the stage of the muscle development and presence of satellite cells, in an attempt to determine those conditions that result in better muscle growth and flesh quality.

Key Words: rainbow trout (Oncorhynchus mykiss), gilthead sea bream (Sparus aurata), expression analysis, myocyte culture, myogenesis

Zusammenfassung

Titel der Arbeit: Suche nach Markern von Muskelwachstum und Fleischqualität beim Fisch

In den vergangenen Jahren sind große Fortschritte in der Aquakultur zu verzeichnen. Erkenntnisse zur Qualität von Fisch und Regulation des Muskelwachstum sind aber noch unzureichend. In in vitro und in vivo Studien wurden AKT, AKTP, MAPK, MAPKP und c-Met als Indikatoren für Muskelwachstum und –qualität untersucht und Einflüsse von Fütterungs- und Haltungsbedingungen auf das Muskelwachstum analysiert. Expressionsanalysen auf der Ebene der Proteine und/oder mRNA weisen Myozyten-Kultivierung als hilfreich für die Identifizierung von Markern der Myogenese zur Bestimmung von Haltungsbedingungen für optimiertes Muskelwachstum und Fischqualität aus.

<u>Schlüsselwörter:</u> Regenbogenforelle (*Oncorhynchus mykiss*), Goldbrasse (*Sparus aurata*), Expressionsanalysen, Myozyten-Kultivierung, Myogenese

Introduction

A great deal of progress in aquaculture has been made in the last ten years and knowledge of growth protocols, nutrition requirements, diet formulation etc., for many fish species has increased significantly. The substitution of vegetable products for fish meal and fish oil has also been studied, and we are now much closer to finding the key to producing fish food with less dependency on fish compounds. Research on flesh quality and especially on muscle growth regulation is, however, still insufficient. Fish myogenesis is controlled by molecules similar to those described in other vertebrates; MRFs (MyoD, myogenin, mrf4, myf5), MEFs, and myostatin are described in several species (JOHNSTON, 2006). The continuous growth of many cultured species offers an interesting model for the study of myogenesis regulation.

Our objectives are to study the molecules in fish muscle that may be indicators of growth and muscle quality and that will be useful for the selection of species families and to determine the best conditions for sustainable and efficient fish aquaculture.

Material and methods

In vitro studies were done with rainbow trout (*Oncorhynchus mykiss*) and gilthead sea bream (*Sparus aurata*) juveniles from Truchas del Segre and Cupimar, respectively, maintained in the fish facilities of the Faculty of Biology (University of Barcelona). Trout and gilthead sea bream myocytes were isolated following CASTILLO et al. (2002), and MONTSERRAT et al. (2007). Myocyte culture was used to investigate the appearance of regulatory molecules throughout the *in vitro* development. The molecules studied were AKT, AKTP, MAPK, MAPKP, and c-Met. Western blot analyses were performed as described (CASTILLO et al., 2006). PCNA was studied by microscope observation of muscle cells stained with the antibody, and MyoD2 was analyzed by real time PCR based on the sequence described by TAN and DU (2002). Flow cytometry and a specific antibody were used to analyze the presence of the

marker CD34.

In vivo studies. Trout juveniles were fed a control diet with fish meal or a diet containing plant protein instead of fish meal for 3 months. Trout juveniles were maintained in regular tanks or in tanks with water circulation to provoke forced swimming activity for 3 weeks. Dorsal muscle was taken and kept frozen until the preparation of samples for Western blot analysis.

Results and Discussion

In vitro studies

In fish myocytes we found the presence of MAPK and AKT at different stages of the in vitro development (Figure 1). The MAPK and its phosphorylated form present the highest levels at early stages, in myogenic precursor cells and myocytes. Later its expression decreased, and its response to IGFs was much lower. Considering the role of MAPK (CASTILLO et al., 2004) in proliferation, it makes sense to find these molecules highly expressed at the beginning of the culture and reduced once differentiated.

On the other hand, AKT and AKTP levels were present during the different stages of myocyte development, and they seemed to be even more active in the differentiated myotubes. These results agree with the AKT pathway's involvement in differentiation and in several metabolic processes (CASTILLO et al., 2006), which are maximized at the myotube stage.

Rainbow trout and gilthead sea bream are different in terms of phylogeny, and biological cycle, and the cell culture is done at different salinity and temperature (MONTSERRAT et al., 2007). Furthermore, the time required to reach different muscle stages is longer in sea bream than in trout. However, it is interesting to note that in both species MAPK and AKT activation follow a similar pattern, suggesting a good evolutionary conservation of these pathways and their regulative role in muscle growth.



Figure 1.

STIMULATION of MAPK and AKT PHOSPHORYLATION by IGF-I (100 nM) in gilthead sea bream muscle cells at different stages of development. Cells were incubated with peptides for 30 minutes, lysed, and 30 µg of protein were loaded in each lane and subjected to a 10% SDS-PAGE under reducing conditions. Results of three independent experiments are shown as a percentage of stimulation over basal and normalized for the content of total MAPK or Akt. Different letters indicate significant differences (P<0.05).

c-Met is the receptor of the hepatic growth factor and is considered a good marker of the satellite cells (WOZNIAK et al., 2003). Our studies on rainbow trout and gilthead sea bream showed that in a myocyte culture, c-Met presents the highest expression during the first and especially the middle stages of the culture. However once the cells reached the myocyte stage the levels decreased rapidly, and in the stage of myotube c-met was almost non detectable (Figure 2). Myocytes exposed to IGF-I (10-100nM) for 24 h presented higher levels of c-Met than control cells.



Cell development stage

Figure 2.

c-Met EXPRESSION IN RAINBOW TROUT MYOCYTE CULTURE at different stages of cell development. Cells were lysed and 40µg of protein were loaded in each lane and were subjected to 12% SDS-PAGE under reducing conditions. Primary antibody against c-Met (final dilution 1:500) and secondary antibody antirabbit (1:10000) were used. A densitometry analysis is shown above. Results are expressed as arbitrary units of densitometry and mean ± SE. Significant differences (p<0,05) are represented by an asterisc in the figure. In rainbow trout, the profile observed for c-Met coincides with that observed for CD34, a molecule that labels those cells that have not yet been determined to myoblasts (TAMAKI et al., 2002). Similarly, in gilthead sea bream, PCNA analyses, that label the proliferative nuclei on cultured cells, showed the highest levels in the myocyte stage, decreasing progressively from that moment. Little information is available on the presence of these molecules in fish (BRODEUR et al., 2003; JOHNSTON, 2006), but these preliminary results suggest a function similar to that observed in mammals. Finally, studies on MyoD2 in gilthead sea bream myocytes showed the highest

expression during the first stages of development in satellite cells and early myocytes, but this expression decreased with the progress of the culture. Such a pattern of MyoD2 agrees with that observed in gilthead sea bream during myogenesis (TAN and DU, 2002), which would indicate that this myogenic factor plays a primary role in lineage determination.

In vivo studies

Trout fed with an experimental diet with a high level of plant protein showed less growth than in the control fish fed with a diet without substitutes. The control group presented a significantly higher c-Met level in white muscle, which might suggest that best growth is accompanied by a large number of myogenic precursors and higher proliferation. The same tendency was observed in gilthead sea bream.

Juveniles of rainbow trout subjected to forced swimming presented higher values of total AKT compared to control fish, but MAPK did not show variation between groups (Figure 3). This response would agree with the higher level of physical activity in the fish, which would then need a greater supply of metabolites.

Figure 3.

AKT EXPRESSION IN RAINBOW TROUT SUBJECTED TO FORCED SWIMMING. 30µg of protein extract from white muscle were loaded in each lane and subjected to 12% SDS-PAGE under reducing conditions. Primary antibody against Akt (final dilution 1:2000) and secondary antibody anti-rabbit (1:10000) were used. A densitometry analysis of three independent experiments is shown above. Results are expressed as percentage of Akt over control levels and mean \pm SE (n=3 experiments). Asterisc represent significant differences between experimental groups (p<0,05).



Myocyte culture provides a useful tool with which to identify the molecules that may indicate the stage of the muscle development and presence of satellite cells, in an attempt to determine those conditions that result in better muscle growth and flesh quality. Thus the combination of certain molecules (MAPK, c-Met, MyoD...) can suggest a muscle with a strong potency for proliferation and growth. Further studies will be necessary to verify these hypotheses.

Acknowledgements

This work has been supported by AGL2004-06319-C02/ACU from Ministerio de Educación y Ciencia to I.N., AGL2006-03923/ACU to J.G., and Xarxa de Referència en Aqüicultura (XRAq)-2006.

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