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ANIMAL SCIENCE **DOCTORAL PROGRAMME** - II WORKSHOP

18th SEPTEMBER 2015

Salão Nobre, ICBAS-UP

Rua de Jorge Viterbo Ferreira n.º 228

Porto,

PORTUGAL

ANIMAL SCIENCE DOCTORAL PROGRAMME

- II WORKSHOP

18th September 2015

Organizers

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Luísa Maria Pinheiro Valente, CIIMAR & ICBAS
Ana Rita Cabrita, REQUIMTE & ICBAS

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Paulo Vaz Pires, CIMAR & ICBAS-UP
Elisabete Matos, SORGAL

ÍNDICE

PROGRAM

14:00 – Registration

14:15 – Opening Session and presentation of SANFEED Programme Topics 2015/2016:

Scientific Committee of the ANIMAL SCIENCE DOCTORAL PROGRAMME

António Mira da Fonseca, REQUIMTE & ICBAS-UP

Luísa Valente, CIIMAR & ICBAS-UP

Júlio Carvalheira, CIBIO & ICBAS-UP

Ana Rita Cabrita, REQUIMTE & ICBAS-UP

Paulo Vaz Pires, CIIMAR & ICBAS-UP

Jorge Dias, SPAROS

Session I: Chairperson Júlio Carvalheira, CIBIO & ICBAS-UP & ***Marcela Segundo,*** REQUIMTE & FF-UP

14:30 – Plenary session: Markers and proxies for feed efficiency and methane emissions from cattle
Richard Dewhurst, Scotland's Rural College

15:00 – Alkaloids profile of European lupin seeds (Lupinus spp.) used in food and feedstuffs
Sara C. Q. Magalhães, REQUIMTE & ICBAS-UP PhD student

15:15 – Energy-protein interrelations in cattle and sheep: what the intragastric infusion technique tells us
Denis Meehan, REQUIMTE & ICBAS-UP PhD student

15:30 – Metabolic effects of dietary seaweed supplementation in marine fish
Maria João Peixoto, CIIMAR & ICBAS-UP PhD student

15:45 – Feathermeal hydrolysate as a fishmeal substitute in diets for European seabass: effects on growth performance and nutrient utilisation
Inês Campos, CIIMAR & ICBAS-UP PhD student

16:00 – Coffee Break & Posters session

Session II: Chairperson Paulo Vaz Pires, *CIIMAR & ICBAS-UP &* ***Elisabete Matos,*** *Sorgal*

16:30 – Plenary session: Advances in the AA nutrition of aquatic species: research at the interface of industry
Cláudia Silva, Evonik Nutrition & Care GmbH

17:00 – Cell-mediated immune responses of senegalese sole (Solea senegalensis) against Tenacibaculum maritimum
Mahmoud Mabrok, CIIMAR & ICBAS-UP PhD student

17:15 – A cytosolic carbonic anhydrase molecular switch occurs in the gills of sea lamprey, during metamorphosis
Diogo Martins, CIIMAR & ICBAS-UP PhD student

18:00 – Round Table discussion: "Animal Science Doctoral Programme: Future Research and Development Priorities"
Jorge Dias, SPAROS, Cláudia Silva, Evonik Nutrition & Care GmbH, Emídio Gomes, CCDR-N & ICBAS-UP, Richard Dewhurst, Scotland's Rural College, Sara Magalhães & Sónia Batista, PhD students & Members of the Monitoring Committee

ORAL COMMUNICATIONS

Cláudia Figueiredo

Cláudia Figueiredo Silva, PhD degree in Aquatic Sciences, ICBAS-UP, Portugal. Currently working as Senior Manager Technical Support Aquaculture– Evonik Nutrition & Care GmbH, Germany. Professional background in the fields of nutrition, metabolism and physiology of amino acids and fatty acids in fish and shrimp. Responsible for technical and scientific matters, trial employment, literature studies and other studies with existing and development of new feed additives in the field of aquaculture.

Richard Dewhurst

Richard Dewhurst is Head of the Beef & Sheep Research Centre at SRUC, with responsibility for research on greenhouse gases and feed efficiency at the award-winning GreenCow facility. He has wide leadership and management experience – across three countries, Universities and Institutes, laboratories, animal houses and farms and a wide range of disciplines (including molecular and computational biology, genetics, nutrition, fertility, health and welfare). His personal research has focused on ruminant nutrition and production systems with significant contributions at the interfaces between nutrition, product composition and rumen function - notably modelling of forage composition, dry cow feeding strategies, forages and fatty acids, fatty acids and fertility, and rumen diagnostics. Some work has been used directly by the animal feed industry – notably feed evaluation systems, feed values and feeding strategies. Much more work has been used by farmers and advisers – notably to develop advice and models to predict and increase forage intakes, improve the utilisation of feed protein, improve milk composition, and facilitate the use of forage legumes in conventional and organic farming. Current research is developing markers for feed conversion efficiency and greenhouse gas emissions in ruminants to support application of breeding/genomics and development of precision management tools. There are over 3000 cites to his publications (H-index of 30).

ADVANCES IN THE AMINO ACID NUTRITION OF AQUATIC SPECIES: RESEARCH AT THE INTERFACE OF INDUSTRY

Cláudia Figueiredo-Silva*

Evonik Nutrition & Care GmbH, Germany
Email: claudia.silva@evonik.com

Turning successful research in the field of animal nutrition into business success requires a sound knowledge of the global marketplace and a R&D working closely aligned to the needs of customers, including those of aquaculture industry. Evonik is a company continuously working on these strengths by establishing collaborative research partnerships with both academia and feed industry all over the world. This is recognized as a key R&D strategy allowing effective developing, testing and promoting of nutrition concepts and feeding recommendations, which back up the existing and novel products in the field of amino acids and other nutritive additives for aquatic species.

The growth in fish and shrimp production has resulted in an increasing demand for shrimp feed and the ingredients used to make the feeds. This same trend has occurred across aquaculture sectors which currently rely on fish meal as one of the primary protein sources. Fish meal has often been used as a major protein source in aquaculture feed because of its excellent sources of nutrients, e.g., balanced amino acid profiles, essential fatty acids, and mineral content. However, in order to reduce feed cost, finding alternative protein sources to replace costly proteins such as fishmeal is one of the major challenges of nutritionists. Considerable research has demonstrated that substitution of fishmeal with alternative protein sources, like soybean meal and in combination with other ingredients, in fish as well as in shrimp feeds results in similar growth, survival, and feed conversion ratios, as long as nutrient composition, including amino acid profile, are balanced to cover animal requirements. Another major challenge that nutritionists, in particular shrimp nutritionists are faced with is to find effective strategies to minimize leaching losses. The slow feeding behaviour of crustaceans may result in a long residence time of the feed under water and thus of nutrients being dissolved out or leached. Nutritionists, feed manufacturers and farmers have long recognized this issue and the need to improve feed stability in water so that wasting of nutrients due to physical deterioration is minimal. Besides the obvious economic losses, leaching of nutrients and in particular of amino acids and other nitrogen-compounds is long recognized to lead to eutrophication of the water and thus impacting negatively the environment. It is in this context that Evonik Industries started in 2008 a R&D project with the objective of developing a second generation methionine source for shrimp, prawn and other crustaceans. Such a product would remain sufficiently stable in water reducing leaching, and would be digested as slowly as protein-bound amino acids, improving protein synthesis. From several different molecules developed, the dipeptide DL-methionyl-DL-Methionine (methionylmethionine or Met-Met for short) was selected for its exceptional physical and chemical characteristics. The mixture of four different methionine stereoisomers (DL-Met-Met, LD-Met-Met, DD-Met-Met and LL-Met-Met) confers unique characteristics to this product due to its extremely low water solubility when compared to other methionine sources available in the market. Most importantly all four different stereoisomers were shown to be effectively cleaved by fish and crustacean digestive enzymes to free D- and L-methionine, in several in vitro digestion experiments. Several trials have been conducted so far and provide evidence that Met-Met is highly effective in covering methionine requirements of shrimp and might constitute a cost-effective strategy in replacing fish meal and/or reducing dietary protein, without detrimental effects on shrimp growth.

MARKERS AND PROXIES FOR FEED EFFICIENCY AND METHANE EMISSIONS FROM CATTLE

Richard J Dewhurst*

Scotland's Rural College, Roslin Institute Building Easter Bush, Midlothian EH25 9RG, United Kingdom. richard.dewhurst@sruc.ac.uk

Ruminant livestock production is an extremely important part of UK agricultural output, with 9.8 million cattle (including calves) and 32.9 million sheep (including lambs) at the June 2013 census (DEFRA and Devolved Administrations, 2014). However, there is ever-increasing financial pressure due to volatile feed prices, consumer requirements for cheaper produce and competitive beef imports. The UK cattle finishing and cattle genetics industries are facing a "do or die" scenario with an urgent need for a step-change in efficiency, quality and profitability. At the same time, UK ruminant livestock industries are also facing large challenges in relation to greenhouse gas (GHG) emissions – with beef and sheep production being most vulnerable owing to low feed conversion efficiency (FCE). The UK's Climate Change Act (2008) established a legally binding requirement to reduce UK GHG emissions by at least 80% (from 1990 levels) by 2050.

One of the major challenges for GHG research is the observation that many of the successful approaches to reduce methane emissions involve impairing rumen function. For example, feeding high levels of starchy concentrates can be effective in reducing methane – but only at the point where rumen pH falls to a level where fibrolytic bacteria are impaired. Similarly, recent Australian observations about the rumen characteristics of low methane emitting sheep (smaller rumens with higher rumen passage rates; Goopy *et al*., 2014) also point to a reduction in their effectiveness as ruminants. Thus, there is considerable interest to include FCE in cattle breeding programmes in several countries (e.g. Herd *et al*., 2004), both because of potential to reduce feed costs (perhaps by over €100 per animal finished), as well as for the indirect effects on GHG emissions per kg of meat produced (Basarab *et al*., 2013)

The need for proxies

The 'gold standard' methods to estimate FCE or methane emissions from cattle are respiration chambers and long duration growth trials respectively. These are costly and laborious, particularly unsuited to use in livestock breeding programmes. Consequently, my recent research has focussed on development of marker or proxy approaches to predict FCE or methane emissions based on rapid, low-cost approaches that could be applied to large numbers of animals on commercial farms.

Development of proxies for FCE

A number of studies with beef cattle (e.g. Wheadon *et al*., 2014), dairy cattle (e.g. Cabrita *et al*., 2014) and sheep (e.g. Cheng *et al*., 2013) have demonstrated potential to use the phenomenon of N isotopic fractionation as a proxy for FCE. Protein becomes enriched with ^{15}N at each trophic level within a food chain because of isotopic fractionation in pathways such as transamination and deamination associated with N partitioning. There is a corresponding depletion of ^{15}N in urine. There is less isotopic fractionation when animals are more efficient at converting feed protein into milk or meat protein. We have shown the basis for a simple testing regime for FCE based on analysis of samples of blood, hair/wool or muscle that are easily obtained from commercial animals.

Development of proxies for GHG emissions

We have investigated alternative markers for methanogens and methanogenesis on the basis of the distinctive ether lipids of archaeal membranes. Compounds such as archaeol (diether) and caldarchaeol (tetraether; McCartney *et al*., 2013a) have been measured in rumen fluid (McCartney *et al*., 2013b) or faeces (McCartney *et al*., 2014a,b) in studies with beef and dairy cattle. There was a significant relationship between faecal archaeol concentration and methane production across a range of diets when comparing treatment means. However, the relationship appears subject to considerable between-animal variation and the approach is unlikely to be useful to assess methane production from individual animals. This variation was also evident in the weak relationship between archaeol concentrations in rumen digesta and faeces (McCartney *et al*., 2014b). This work has also highlighted the need to describe methanogen abundance in all rumen fractions and the limitations of working with the rumen liquid phase alone. A further source of variation in relationships relates to changes in the composition of archaeal membranes, for example changes in the ratio archaeol:caldarchaeol in response to rumen pH (McCartney *et al*., 2014a).

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ALKALOIDS PROFILE OF EUROPEAN LUPIN SEEDS (*LUPINUS* SPP.) USED IN FOOD AND FEEDSTUFFS

Sara C. Q. Magalhães[1]*, Fátima Fernandes[2], Ana R. J. Cabrita[1], António J. M. Fonseca[1], Patrícia Valentão[2], Paula B. Andrade[2]

[1] REQUIMTE/LAQV, ICBAS, Instituto de Ciências Biomédicas de Abel Salazar, Universidade do Porto, Rua Jorge Viterbo Ferreira, 4050-313 Porto, Portugal
[2] REQUIMTE/LAQV, Laboratório de Farmacognosia, Departamento de Química, Faculdade de Farmácia, Universidade do Porto, R. Jorge Viterbo Ferreira, nº 228, 4050-313 Porto, Portugal.
E-mail: pandrade@ff.up.pt

Introduction

Lupin seeds (*Lupinus* spp.) are low price and non-genetic modified ingredients that constitute good sources of protein (ca. 40%), fiber (ca. 28%), healthy fatty acids, vitamins, minerals and other metabolites with recognized antioxidant properties (e.g., polyphenols). However, they contain alkaloids as main antinutritional factors, which may cause several types of disorders on humans and animals. Considering the recent efforts towards increasing the local production of protein-rich crops, with emphasis on lupins, in the European countries for food and feed purposes, the present work aimed at determining the alkaloids profile of some lupins grown in Mediterranean countries and in Poland. The potential of the studied lupin seeds to be included in food and feed is here briefly discussed, based on our results on seeds alkaloids composition and on relevant information available in the literature.

Material and methods

Eleven varieties (included in the European Plant Variety Database) and one Portuguese ecotype of lupins, corresponding to mature raw seeds of *L. albus* (white lupin, WL; n=5), *L. angustifolius* (narrow-leafed lupin, NLL; n=2) and *L. luteus* (yellow lupin, NLL; n=2), were analyzed. Seeds were dried (65 ºC, 24 h), ground (1 mm) and dry matter (DM) content determined after drying the powdered samples at 103 ºC overnight. Alkaloids were extracted as previously described by Muzquiz *et al.* (1994) and Gresta *et al.* (2010), with slight modifications. Alkaloids identification and quantification in the rich-alkaloid extracts was performed by GC-MS and GC-FID, respectively. Chromatographic conditions were as described by Gresta *et al.* (2010). Using SPSS, mean values were compared by one-way ANOVA and principal component analysis (PCA) was applied for reducing the number of variables to a smaller number of the new derived variables (principal components, PCs) that adequately summarize the original information, i.e., the alkaloids composition of the studied lupin samples.

Major results and discussion

Nine compounds were identified comprising quinolizidine (lupinine, sparteine, angustifoline, α-isolupanine, lupanine, 11,12,-dehydrolupanine and 13α-hydroxylupanine), piperidine (smipine) and indole (gramine) alkaloids. Lupanine was the major alkaloid in samples of WL and NLL whereas sparteine was the most abundant compound in most of YL samples. These two tetracyclic quinolizidine alkaloids are ubiquitous in lupin species. Two PCs explained 73.23% of total data variability (Fig. 1). PC1 represented 47.44% of the variation and was associated with total alkaloids content, and with the compounds angustifoline, lupanine and 13α-hydroxylupanine, whereas PC2, responsible for 25.79% of the variation, was mainly represented by lupinine and sparteine. According to that, three groups of lupin samples could be clearly distinguished (Fig. 1).

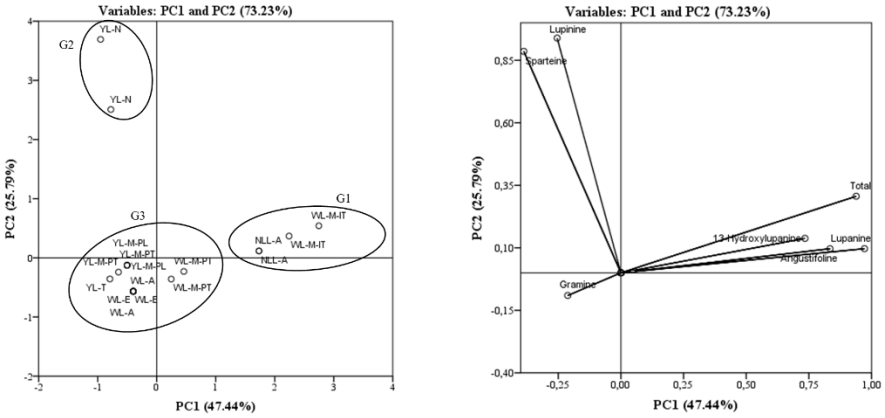

Fig. 1. Projection of lupin samples (variables: WL var. Estoril (WL-E); WL var. Amiga (WL-A); WL var. Multitalia-IT (WL-M-IT); WL var. Multitalia-PT (WL-M-PT); WL var. Lumen; YL ecotype Nacional (YL-N); YL var. Mister-PT (YL-M-PT); YL var. Mister-PL (YL-M-PL); YL var. Dukat (YL-D); YL var. Taper (YL-T); NLL var. Azuro (NLL-A); NLL var. Sonet (NLL-S)) and loadings by alkaloids and total alkaloids content into the plane composed by the principal components PC1 and PC2 containing 73.23% of the total variance.

Table 1 summarizes the total alkaloids content of each lupin sample, indicates which of them are suitable for human consumption and also reports their maximum level of inclusion in feedstuffs based on the maximum recommended concentration of individual alkaloids reported in the literature.

Table 1. Studied lupin samples: total alkaloids content and suitability as food and feed.

Lupin samples	Total alkaloids mg/100 g DM	Human consumption? if < 20 mg/100 g DM	Trout % of inclusion	Pigs
WL-E	19.8 (S)	Yes	-	38-56
WL-A	0.0 (S)	Yes	-	100
WL-M-IT	5169.1 (B)	No	-	< 1
WL-M-PT	1219.2 (B)	No	-	< 1
WL-L	31.5 (S)	No	-	27-40
YL-N	1030.7 (B)	No	~ 1	~ 1
YL-M-PT	26.7 (S)	No	38	19-41
YL-M-PL	70.6 (B)	No	14	7-16
YL-T	77.5 (B)	No	18	24-51
YL-D	12.4 (S)	Yes (yet, not from an edible lupin species)	81	41-89
NLL-A	2440.2 (B)	No	-	< 1
NLL-S	63.9 (B)	No	-	14-20

S, sweet (< 50 mg/100 g DM); B, bitter (> 50 mg/100 g DM)

Besides monogastrics, also ruminants (sheep, cattle) are large consumers of lupin seeds as protein sources. Their biggest advantage regarding dietary alkaloids is that, apparently, prolonged exposure of alkaloids to rumen microorganisms increase their tolerance to such metabolites and may suppress alkaloids deleterious effects (Aguiar and Wink, 2005). Nonetheless, under penalty of affecting feed intake, bitter varieties found in the present work, and especially those containing very high levels of alkaloids (> 1000 mg/100 g DM) should be debittered to ensure a safer consumption and to allow increasing lupins dietary levels in monogastrics and ruminants.

Conclusions

Sweet WL and YL varieties appear as good options to include in food and/or feedstuffs as high intakes or inclusion levels appear to be possible. For the other lupin seeds, a debittering process is recommended before consumption.

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ENERGY-PROTEIN INTERRELATIONS IN CATTLE AND SHEEP: WHAT THE INTRAGASTRIC INFUSION TECHNIQUE TELLS US

Denis J. Meehan*[1], Orskov, E.R.[2], Ana R. J. Cabrita[1], António J. M. Fonseca[1]

[1]REQUIMTE/LAQV/ICBAS, Instituto de Ciências Biomédicas de Abel Salazar, Universidade do Porto, Rua Jorge Viterbo Ferreira, 4050-313 Porto, Portugal.
[2]The James Hutton Institute, Craigiebuckler, Aberdeen AB15 8QH, Scotland UK
E-mail: ajfonseca@icbas.up.pt

Abstract

Volatile fatty acids (VFAs) which are the end-products of microbial fermentation in the rumen represent the main source of energy to ruminants (Orskov and Ryle, 1990) and microbial protein produced from this fermentation accounts for approximately 70% of the amino acids absorbed at the level of the small intestine (Pathak A.K., 2008). Controversy has reigned for years as to the exact reasons behind the lower observed efficiency of utilisation of forage vs. concentrate diets and in particular whether or not this could be attributed to differential efficiencies in utilisation of these VFAs (Acetic acid, Butyric acid and Propionic acid). High forage diets give rise to high molar proportions of acetic acid in the rumen (Orskov and Ryle, 1990) and Armstrong and Blaxter (1957) were the first to observe a poor efficiency of utilisation of acetic acid for fattening (Kf) based on a high heat increment (HI) being observed when acetic acid was infused singly into the rumen.

Different schools of thought exist as to the possible origins of this high HI (thereby lower energetic efficiency) on high forage diets. Some authors related it to thermodynamic losses associated with intermediary metabolism, namely a shortage of Tricarboxylic acid cycle intermediates (Marston, 1948) while others suggested the additional energy costs associated with the mechanical / physical work of digestion (Kellner, 1926).

The development of the Intragastric Infusion technique (Orskov et al., 1979) meant that by by-passing rumen fermentation a wide range of VFA mixtures could be infused at any given level of energy intake with indirect respiration calorimetry being used to simultaneously measure heat production (through O2 consumption) and thereby efficiency of utilisation of these VFA compositions (see Figure 1). In addition, by infusing protein with these VFAs, the energy:protein ratio of nutrients absorbed by the animal (unlike normally fed ruminants) could be varied at will to permit studies on (among other aspects) the extent to which protein is oxidized by the ruminant on low dietary energy levels.

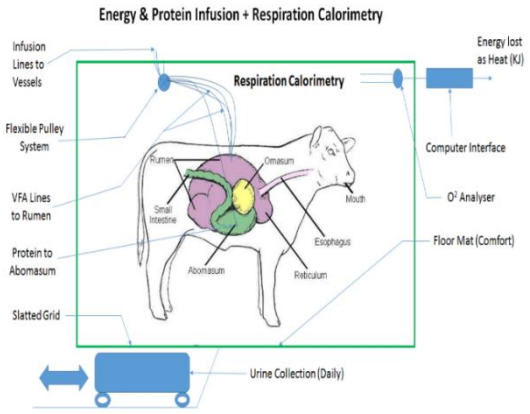

Figure 1: Schematic representation of the Intragastric Infusion Technique.

When lambs were infused with VFA (above maintenance requirement) of varying composition ranging in molar proportions from a high acetic acid / low propionic acid (85:5:10) to low acetic acid / high propionic acid (45:45:10) mixture with butyric acid remaining constant, all VFA mixtures were utilised with a broadly similar efficiency ranging from 0.57 to 0.64 (Orskov et al., 1979). This suggested that differences in utilisation between forage and concentrate diets could not be attributed to the composition of the VFAs absorbed and metabolised by the animal. Further studies revealed that the additional energy costs incurred by the animal in the process of eating (i.e. prehension and mastication of feed and rumination) lie behind the lower efficiency of forage diets (Orskov and MacLeod, 1990).

Chowdhury et al. (1997) infused sub-maintenance glucogenic VFA to sheep (Acetic acid: Propionic acid: Butyric acid of 16:79:5 molar proportions) in conjunction with successive increments of casein-N (from 0 to 3000 mg N/kgW.75) and observed that the animals attained a positive N balance while simultaneously in substantial negative energy balance of 210 KJ / KgW.75. This contradicted conventional thinking which assumed until recently that undernourished animals oxidise amino acids as an energy source when in negative energy deficit. Instead, the undernourished animal has the innate ability to prioritise these important and scarce nutrients (i.e. essential and non-essential amino acids) to maintain or even gain tissue protein.

The implications of these findings are of interest. Firstly, the efficiency of utilisation of forage diets can be improved by chopping or grinding but such treatments are unlikely to prove economically viable at farm level. Secondly, the composition of live weight gain in steers and lambs can be manipulated by supplementing low energy basal diets (i.e. straw / hay) with by-pass proteins (e.g. soya bean) provided the animal has the genetic capacity to deposit lean tissue and adequate body fat reserves (condition score 3 to 4) with which to "fuel" this process.

No Intragastric Infusion studies (involving rumen cannulation plus abomasal catheters) have been conducted in recent years in the UK due to a lack of funding.

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METABOLIC EFFECTS OF DIETARY SEAWEED SUPPLEMENTATION IN MARINE FISH

Maria João Peixoto[*1,2], José Fernando Gonçalves[1,2], Rui Pereira[3], Rodrigo Ozório[1,2].

1 - CIIMAR – Centro Interdisciplinar de Investigação Marinha e Ambiental, Universidade do Porto, Rua dos Bragas 289, 4050-123 Porto, Portugal.
2 - ICBAS – Instituto de Ciências Biomédicas de Abel Salazar, Universidade do Porto, Rua de Jorge Viterbo Ferreira 228, 4050-313 Porto, Portugal.
3 - ALGAPLUS, Lda - Travessa Alexandre da Conceição S/N., 3830-196 Ílhavo, Portugal.
Email: mjoaodiaspeixoto@gmail.com

Seaweeds are considered a potential source for biologically active substances, as well as essential nutrients for human and animal nutrition, but remain an underexploited natural resource. Seaweeds are a source of phytochemicals, advantageous for the control of several diseases, including hyperlipidemia, thrombosis, tumor and obesity. The application of marine bioactives in fish diets may modulate their performance, since life-traits can be modulated by nutrition.

Oxygen consumption rates can be used as a powerful indicator of the modulatory effects of dietary treatments on aerobic scope. Two physiological parameters often used to evaluate the aerobic energy metabolism are standard metabolic rate (SMR) and maximum metabolic rate (MMR), the lower and upper boundaries for oxygen uptake rates, respectively. SMR corresponds to the cost of living, or the minimal maintenance requirement of energy turnover for a post-prandial, unstressed, resting fish at basal physiological function. MMR corresponds to the highest metabolic activity level. MMR is usually expressed for a short period by a physically challenged animal. Deducing SMR from MMR provides the aerobic metabolic scope (AMS), a measure for the total aerobic expanse of simultaneous metabolically demanding processes that fish can withstand. Many fish species show bouts of activity, typically fueled by aerobic energy with metabolism fluctuating around the average level due to endogenous or exogenous factors, the so call routine metabolic rate (RMR). RMR can provide insight to the level of energy that fish spend during daily activities. The objective of this study was to examine the metabolic effects of dietary seaweed supplementation in seabass (*Dicentrarchus labrax*) and meagre (*Argyrosomus regius*), using respirometry technique.

In the first experiment using seabass, a control diet was compared with a Rhodophyta supplemented diet and a mix diet composed of rhodophyta, phaeophyta and chlorophyta genus representatives. A total of 180 fish were reared at 20 ºC and fed until apparent satiation during 49 days. Thereafter, 27 fish were selected for the respirometry trial. No differences were found between treatments for MMR and SMR and AMS (Table 1). In contrast, fish fed diet supplemented with rhodophyta exhibited elevated RMR (Figure 1), providing evidence that rhodophyta supplementation may influence energy metabolism.

In the second experiment, 180 meagre were reared at 21 ºC and fed until apparent satiation during 100 days. Thereafter, 27 fish were selected for the respirometry trial. Fish were fed three diets: a phaeophyta supplemented diet, a rhodophyta supplemented diet, and a non-supplemented (control) diet. No differences were found between treatments for SMR, RMR and AMS (Table 2). MMR (Figure 2) showed differences between fish fed phaeophyta supplemented diet and the control diet ($p = 0.02$).

Table 1 Standard metabolic rate (SMR), maximum metabolic rate (MMR) and aerobic metabolic scope (AMS) (mg O_2 kg^{-1} h^{-1}) in seabass at 20 °C fed three different diets.

Diet	SMR[1]	SMR[2]	MMR	AMS
Control	117.61 ± 4.88	122.62 ± 15.41	420.01 ± 19.29	302.40 ± 0.53
R	124.98 ± 6.00	134.28 ± 17.56	455.06 ± 21.71	330.08 ± 14.98
Mix	112.76 ± 5.43	120.37 ± 14.01	432.41 ± 22.04	319.65 ± 16.36

No statistical differences between diets ($P > 0.05$). Values presented as mean ± S.E.
[1] Determined using the 10 lowest MO_2 values.
[2] Determined using the 10 % lowest MO_2 values.

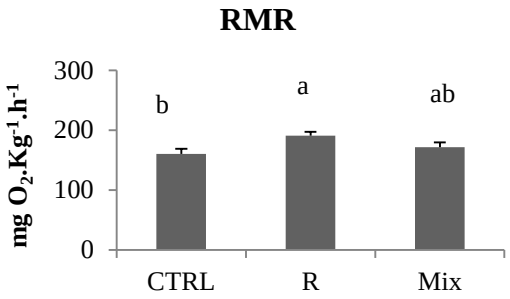

Figure 1 Routine metabolic rate (RMR; mg O2 kg^{-1} h^{-1}) in seabass at 20 °C fed three different diets. Different letters indicate statistical differences ($P < 0.05$). Values presented as mean ± S.E.

Table 2 Respirometry results with standard metabolic rate, routine metabolic rate and aerobic metabolic scope (mg O_2 kg^{-1} h^{-1}) showing no statistical differences between treatments.

Diets	SMR	RMR	AMS
Control	488.68 ± 149.20	779.34 ± 367.72	732.03 ± 196.44
P	613.86 ± 137.27	641.54 ± 97.5	971.60 ± 159.51
R	760.89 ± 149.93	717.63 ± 239.59	803.68 ± 312.73

Values presented as mean ± S.D.

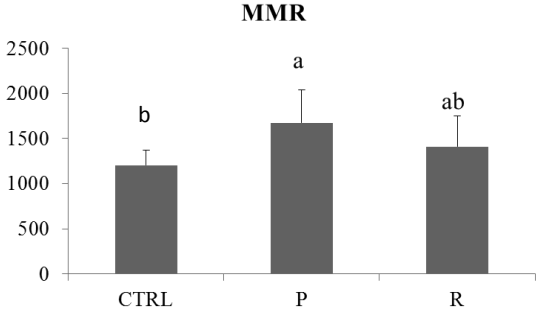

Figure 2 Maximum metabolic rate (MMR; mg O2 kg^{-1} h^{-1}) in meagre. Different letters indicate statistical differences ($P < 0.05$) between diets. Values presented as mean ± S.E.

The applicability of seaweeds to enhance energy metabolism and performance in fish is not entirely ascertained. Many reports demonstrated that dietary seaweed supplementation may improve fish performances. Nevertheless, other studies did not observed such effects. To our best knowledge, the current study is the first reporting the use of respirometry techniques to evaluate the effects of seaweed supplementation on the oxygen consumption in seabass and meagre. Since fish are subjected to temporal shifts in energy demand throughout their life-cycle, the beneficial effects of seaweed supplementation may depend on environmental and physiological effects. In conclusion, seaweed supplementation may alter RMR and MMR in seabass and meagre, respectively, indicating an improvement on the allocation of energy for daily routine (seabass) and for sudden change in energy demand (meagre), as often happens during stress conditions.

FEATHERMEAL HYDROLYSATE AS A FISHMEAL SUBSTITUTE IN DIETS FOR EUROPEAN SEABASS: EFFECTS ON GROWTH PERFORMANCE AND NUTRIENT UTILISATION

I. Campos* [a], E. Matos [b], A. Marques [a], Luisa M.P. Valente [a]

[a] CIIMAR-CIMAR L.A., Centro Interdisciplinar de Investigação Marinha e Ambiental and ICBAS, Instituto de Ciências Biomédicas de Abel Salazar, Universidade do Porto, Rua dos Bragas, 289, 4050-123 Porto, Portugal
[b] SORGAL, Sociedade de Óleos e Rações, Estrada 109 Lugar da Pardala, 3880-728 S. João OVR, Portugal
Email: lvalente@icbas.up.pt

Introduction

The poultry production industries produce large amounts of by-products responsible for environmental pollution if not properly discarded. However, the rendering of these by-products generate useful biological resources (National Research Council, 2011) suitable to be used as new protein sources in animal feed. Due to recent changes in regulation that now allows animal by-products to be used in feeds for monogastric animals (European Commission, 2013), there is an urge for investigating locally produced industrial by-products able to substitute fishmeal. This could help the aqua feed industry to lower the fishmeal imports, and would also provide the industrial poultry facilities with a cost-effective alternative to their wastes.

Some studies have already been made on the replacement of fishmeal by poultry by-products, including poultry by-product meal and feathermeal hydrolysate in several farmed fish species (namely *Sparus aurata*, *Oncorhynchus mykiss*, *Paralichthys olivaceus*, and *Oreochromis niloticus*). However, there is still little information on the use of such by-products in practical diet formulations for the European seabass (*Dicentrarchus labrax*), which is an important saltwater farmed species in the Mediterranean.

In the present study, increasing levels of feathermeal hydrolysate were used to replace fishmeal in diets for European seabass juveniles. The effects of such diets on fish growth performance, nutrient utilization, body composition and flesh quality were evaluated.

Materials and Methods

Based on the known nutrient requirements of the European seabass, four extruded isoproteic (50 %DM), isolipidic (19 %DM) and isoenergetic (21 kj.gDM^{-1}) diets were formulated: a reference commercial based diet (FM) and three experimental diets with increasing levels of feather meal hydrolysate (5 % HP5, 7.5 % HP7.5 and 12.5 % HP12.5) to replace fishmeal. The feathermeal hydrolysate was obtained by steam hydrolysis of feathermeal, a poultry slaughter by-product (Sorgal).

Homogenous groups of 25 fish (initial individual weight 16.7 ± 2.7 g) were kept in twelve 55 L fiber glass tanks inserted in a recirculating saltwater system (salinity 35 ‰, 21 ± 1 ºC) and submitted to a 12-hour light/12-hour dark photoperiod regime. The experimental diets were randomly assigned to the tanks, in triplicate, and fed to apparent satiety three times a day by automatic feeders for 18 weeks. Fish were bulk weighed twice throughout the experiment to monitor weight gain and feed consumption. At the end of the growth trial, fish were individually weighed and measured. Five fish per tank were sampled for whole body composition. Muscle samples were also collected from 6 fish per tank for fatty acid determination.

At the end of the growth trial, the apparent digestibility coefficients of the test diets were evaluated, after including 1 % chromic oxide as an inert marker, according to Cho & Slinger (1979).

Results

The growth performance of fish can be observed in Fig. 1. No significant differences in body weight could be registered among the dietary treatments over time (one way ANOVA, $p < 0.05$). Growth performance, feed intake, feed conversion ratio, protein efficiency ratio and daily growth index were similar for all diets. Furthermore, final whole-body composition was similar among dietary treatments: 35-36 % dry matter, 17 % protein, 14-15 % fat and 9 $kJ.g^{-1}$ gross energy. Protein and energy apparent digestibility coefficients were significantly lower in fish fed HP12.5 diet than for those fed the reference diet (FM).

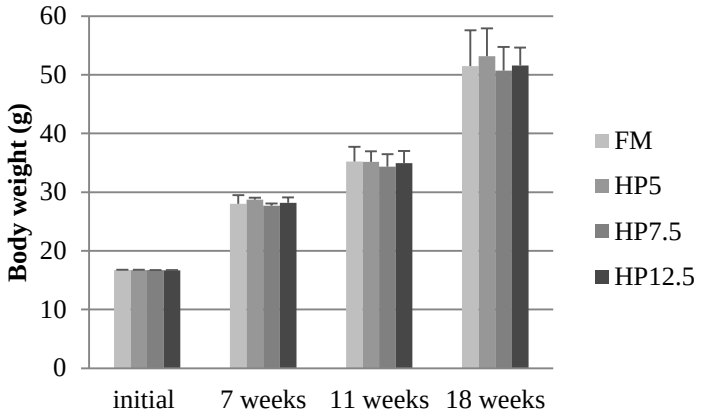

Fig. 1 Body weight of fish fed the experimental diets.
The absence of superscript letters at each sampling point indicates no significant differences between treatments (ANOVA, $p < 0.05$).

Discussion and Conclusion

The present results showed no significant differences in growth performance or nutrient utilization among dietary treatments. This outcome suggests it is possible to incorporate feathermeal hydrolysate up to 12.5 % in European seabass diets without impairing growth and without affecting whole-body composition. However, since the diet with 12.5 % feathermeal hydrolysate inclusion reduced protein and energy digestibility, a longer trial should be conducted in order to confirm these results.

Acknowledgements

This work was subsidized by Project VALORINTEGRADOR, funded by Quadro de Referência Estratégico Nacional (QREN), financed by the European Regional Development Fund (FEDER) through the Operational Competitiveness Programme (COMPETE) - reference number 38861.

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CELL-MEDIATED IMMUNE RESPONSES OF SENEGALESE SOLE (SOLEA SENEGALENSIS) AGAINST TENACIBACULUM MARITIMUM

Mahmoud Mabrok1, 2,*, António Afonso1, 2, Luísa MP Valente1, 2, Benjamín Costas1

1Centro Interdisciplinar de Investigacão Marinha e Ambiental (CIIMAR-CIMAR), Universidade do Porto, Porto, Portugal.
2Instituto de Ciências Biomédicas Abel Salazar (ICBAS–UP), Universidade do Porto, Porto, Portugal.
*Centro Interdisciplinar de Investigacão Marinha e Ambiental, Rua dos Bragas 289, 4050-123 Porto, Portugal. E-mail: dr.mahmoudmabrok@yahoo.com

Introduction

Senegalese sole (*Solea senegaensis*), like other marine organisms, is often exposed to opportunistic pathogens, threatening its production. *Tenacibaculum maritimum* (formerly *Cytophaga marina* and *Flexibacter marinus* or *maritimus*), is a Gram-negative bacterium and the aetiological agent of marine tenacibaculosis or flexibacteriosis, currently considered one of the most threaten bacterial infections limiting the culture of many fish species of commercial value in distinct geographical areas of the world. Obstacles in preparation of homogenised bacterial suspension due to its swarming activity, together with the lack of reliable infection models particularly in Senegalese sole, hindered the progress of research regarding the host-pathogen interaction. Therefore, the present study was undertaken to study the interaction between *T.maritimum* and Senegalese sole phagocytes. Primary head-kidney leucocytes coupled with an improved preparation of homogenised bacterial suspension will indeed be an invaluable tool for investigating the mechanisms of immunity and pathogenesis of *T. maritimum*–host interactions.

Material and methods

Three *T. maritimum* strains (ACC20.1; ACC13.1; ACC6.1) isolated from Senegalese sole in a local fish farm (Póvoa de Varzim, Portugal) were used during experimental assays. These isolates belong to the serotype O3 described for *T. maritimum* (1) and were kindly provided by Professor Alicia E. Toranzo (Departamento de Microbiología y Parasitología, Facultad de Biología, University of Santiago de Compostela, Spain). They are kept frozen at – 70 °C until being used. Bacteria in the late logarithmic phase were adjusted to 1×10^8 bacteria ml^{-1} according to (2). When required, bacteria were killed by ultraviolet (UV) exposure for 4 h. Loss of bacterial viability following UV treatment was confirmed by plating on marine agar plates and MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; Sigma) reduction assay. Head-kidney leucocytes (HKL) were isolated according to (3). Afterwards, cell-mediated immune responses including ROS (Reactive oxygen species**)** and NO (nitric oxide**)** were measured according to [3, 4] respectively following stimulation with different bacterial stimuli. Furthermore, the killing assay was measured by a colorimetric assay based on the reduction of MTT. Briefly, the HKL adherent cell layer was washed twice and 100 µl of different *T. maritimum* strains adjusted at (10^8 CFU ml^{-1}) were added. The plates were then centrifuged at 150 × *g* for 5 min at 18 °C. Afterwards, the plates were incubated at 18 °C for 0 (T_0), 3 (T_3) and 5 (T_5) h. At the end of each incubation period, the supernatant was removed, and the bactericidal activity was stopped by the lysis of leukocytes with 50 µl of cold sterile distilled water. Then, 100 µl of marine broth was added to promote the growth of surviving bacteria. After 48 h at 18 °C, 10 µl of MTT (5 mg ml^{-1}) were added to each well and the optical density (OD) was read after 15 min at 600 nm. The difference between OD readings for each concentration between time T0, T3 and T5 represents the degree of bactericidal activity.

Results and discussion

Senegalese sole HKL increased ROS and NO production following exposure to live and UV killed strains in a different manner (Fig.1. A and B). Exposure to UV radiation could physically alter bacterial cell wall composition as previously mentioned by Lye et al. (4) and therefore, modulate cell innate immune responses. NO data suggest that bacteria viability is not essential for pathogen recognition and also reflected the importance of balanced pH during NO measurement, as prolonged incubation of cells with such bacterial strain resulted in sudden drop in pH up to 6 (data not shown). The MTT assay (data not presented) showed significant differences among strains whereas no differences regarding incubation time were recorded. A potent bactericidal activity was observed against the strain ACC13.1 after 3 and 5 h incubation period, while strains ACC6.1 and ACC20.1 revealed higher survival rates with no differences between them. Overall, results from this study suggest the interactions between the evaluated bacterial strains and sole HKL are diverse, probably related to different degrees of microorganism evading strategies against host cell-mediated immunity.

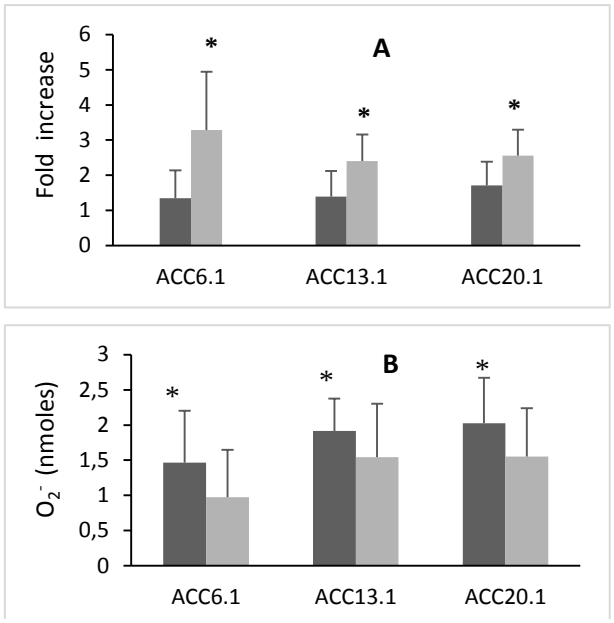

Fig.1. A) Nitric oxide production represented as fold increase. B) Reactive oxygen species production of Senegalese sole, *Solea senegalensis*, head-kidney leucocytes following exposure to UV killed ( ) and live ( ) *T.maritimum* strains (ACC6.1, ACC13.1 and ACC20.1) at concentration 10^8 CFU ml^{-1}. Data are expressed as means ± SD (n = 8). Single asterisk means significant differences between UV killed and live bacterial strains for the same concentration (two-way ANOVA; $P < 0.05$).

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A CYTOSOLIC CARBONIC ANHYDRASE MOLECULAR SWITCH OCCURS IN THE GILLS OF SEA LAMPREY, DURING METAMORPHOSIS.

D. Ferreira-Martins*[1,2], S.D. McCormick[3], A. Campos[1], H. Osório[4], J. Coimbra[1,2], L.F C. Castro[1,5], J. M. Wilson[1,6]
*Dr. Diogo Ferreira Martins, Interdisciplinary Centre of Marine and Environmental Research (CIIMAR/CIMAR), Rua dos Bragas 289, 4050-123 Porto, Portugal. tel +351 22 340 1809 fax +351 22 339 0608; Email:diogofmartins14@gmail.com

[1]Interdisciplinary Centre of Marine and Environmental Research (CIIMAR/CIMAR), University of Porto, Portugal
[2]Instituto de Ciências Biomédicas de Abel Salazar, ICBAS, University of Porto, Portugal
[3]Conte Anadromous Fish Research Center, USGS, Turner Falls MA USA.
[4]Institute of Molecular Pathology and Immunology of the University of Porto (IPATIMUP), Portugal
[5]Department of Biology, Faculty of Sciences, University of Porto, Porto, Portugal
[6]Department of Biology, Wilfrid Laurier University, Canada

Abstract

The sea lamprey *Petromyzon marinus* is an anadromous species with a complex life cycle in which ammocoetes (larvae) are benthic filter feeders and undergo a dramatic metamorphosis into parasitic feeding juveniles. To date only a single carbonic anhydrase (CA) isoform, an enzyme that plays a key role in CO_2 transport and acid-base regulation in vertebrates, has been found in lampreys. In this study we have identified a novel CA sequence in the ammocoetes and early stages of metamorphosis of the sea lamprey. To this, a RT-PCR approach was taken using degenerate and consensus sequence primers with samples from adult, ammocoete and post-metamorphic transformer stages. A novel partial sequence was identified in ammocoete gills and the full length sequence was obtained by RACE PCR. The transcript codes for a 257 amino acid (aa) protein that has 67.5% aa identity with the published carbonic anhydrase isoform from lamprey and 55.9% to 57.4% and 51.3% to 57.8% with rainbow trout and human Cac (Ca2-like) and CA2 isoforms, respectively. The mRNA expression of both isoforms in different life stages was also assessed using real time RT-PCR. In addition, we perform proteomic analysis to characterize this novel sequence and compare with the previously known CA sequence. For this, red blood cells (RBCs) of ammocoetes and post-metamorphic (parasitic) juveniles were used. Immunoblots of lamprey RBC homogenates with a heterologous mammalian CA2 antibody revealed a pair of bands (27 and 29 kDa) with different expression patterns during different life stages. Expression of a 27 kDa immunoreactive band was highest in early stages and decreased significantly during metamorphosis becoming undetectable in post-metamorphic juveniles. In contrast the 29 kDa band expression was significantly lower during the early stages, increasing significantly during latter stages. The identity of the two bands was confirmed with proteomics approach using 2-dimesional gel electrophoresis (2-DE), and spots were examined by MS/MS. High protein identification scores revealed the presence of one spot as the previously described CA in RBCs of adult lamprey (Genbank DQ157849) and six additional spots as the novel isoform expressed in the RBCs of ammocoetes. These findings were confirmed by 2-DE western blotting probed using the heterologous cytosolic CA antibody. Our work suggests that a unique molecular switch occurs during lamprey metamorphosis that results in functionally distinct gill CA activity that is adaptive for altered life mode and habitat. This work was supported by FCT grant PTDC/MAR/98035/2008 to JMW.

ROUND TABLE

II Animal Science Doctoral Program Workshop

September 18th, 2015

ICBAS - Porto

Round Table on "Animal Science Doctoral Program: Future R&D Priorities"

Start: 17.30

End: 19.00

Members of the Discussion Panel
Prof. Emídio Gomes (President of CCDR Norte & Full Professor at ICBAS- UP)
Dr. Jorge Dias (SPAROS Lda & Director of the SANFEED Advisory Board) – moderator
Dr. Cláudia Silva (Aquaculture Manager at Evonik Nutrition & Care GmbH, Germany)
Prof. Richard Dewhurst (Scotland's Rural College, Scotland)
Sara Magalhães (PhD student/Member of the Doctoral Program Monitoring Committee)
Sónia Batista (PhD student & Member of the Doctoral Program Monitoring Committee)

1. Introductory remarks

- As moderator, Dr. Jorge Dias acknowledged the scientific quality and excellence of the various presentations and posters made by the PhD students of the Animal Science Doctoral Program.

- The SANFEED PhD topics for the 2015 call were presented and potential candidates were encouraged to apply.

- The discussion was structured in two main topics:
    - R&D challenges for the area of Animal Science
    - Matching the expectations of PhDs students and Industrial partners.

2. R&D Challenges in Animal Science

Prof. Richard Dewhurst described some current trends and research priorities in the UK. Focus was given to topics and approaches that allow an integrative view of existing and foreseeable problems. The establishment of Innovation Clusters dedicated to Animal

Sciences, with a strong involvement of industrial partners is underway in the UK. The objective is not only to rationalize resources, but also to integrate multidisciplinary teams (from non-traditional areas of animal science) that could bring new processes/technologies to the field. As an example, it was mentioned the need to go beyond animal performance and establish new models for evaluating the response criteria of the animals.

Prof. Emídio Gomes, with his long professional experience in the field of Animal Science, stressed also the importance of consolidating research efforts as opposed to fragmented research activities. In this context, he mentioned and presented some examples of the successes achieved by the "Cluster do Calçado" in Portugal. There is a clear need to establish priorities and action plans to revitalize the sector of Animal Production in Portugal. He mentioned also that opportunities for funding applied research projects fostering the cooperation between Academia and Industrial players exist, and are a priority in the framework of the Programs Portugal 2020 (IDT Co-Promoção) and Horizon 2020 (SME Instruments and Bio-based Industries). However, such projects require a strong participation of industrial partners and a clear alignment with the strategic regional and National development plans. Special credit lines negotiated with the banks are also available as tool to help innovation related investments.

Dr. Jorge Dias made some comments on the activities of SPAROS, which despite being located in Portugal, has a strong involvement in the development of new products and solutions for a global market. Despite the small dimension of our internal market, this should not discourage our companies and academic researcher form searching solutions for global issues.

3. Matching the expectations of PhDs students and Industrial partners

Dr. Cláudia Silva (a former PhD student at the University of Porto) shared with the audience some aspects that she considered as relevant to her successful entry into one of the largest multinational companies in the area of animal nutrition. Key elements pointed out were: i) the scientific excellence of the research work accomplished during the PhD; ii) the research team in which the work is developed, but also the capacity to establish high-quality collaborations; and iii) the willingness to embrace new professional challenges (sometimes abroad).

Sonia Batista & Sara Magalhães, presently PhD students, gave us a feedback of the Animal Science Doctoral Program. The motivation for entering a PhD program are of course linked to the attractiveness of pursuing a research career, but can also be as blunt as not having any other professional options. Their perception of the Program is very positive, since not only it allowed them to pursue a research carrier that they like, but also it gave them also the opportunity to work in various laboratories in Europe. On a personal and scientific level, both classified their PhD experience as very rewarding. However, now the PhDs are close to completion, the professional perspectives are again blur and a reason of great concern. Both students are not part of the SANFEED Program. But expressed their belief that a Doctoral Program that runs in an industrial setting, in which the thesis are directly associated to a company's interest is highly motivating and has better changes in resulting on jobs opportunities.

Prof. Luisa Valente (Director of the SANFEED Scientific Board) mentioned that the Program has attracted few candidates on its first call. This situation may be due to the short dissemination period for dissemination of the PhD topics in the first call, but potentially also to an apparent low interest of students for topics related to Animal Sciences.

Some participants from the audience (Prof. Maria Antónia Salgado and Prof. Marcela Segundo) corroborated this idea that current students are easily attracted by topics explicitly mentioning "omics" approach, but less appealed by areas that involve zootechnical work.

This comment was duly noted and Dr. Jorge Dias, together with Prof. Luisa Valente and Prof. António Mira da Fonseca (Director of the SANFEED Program) took the responsibility of revising the titles of the PhD topics in future calls to enhance its potential to "attract" students. Additional effort will also be directed towards reinforcing the ample dissemination of the PhD calls.

Afterwards, the discussion was focused on the employability of young PhDs.

Prof. Emídio Gomes presented some statistics on the employment rate of PhDs in the private sector in Portugal, which is extremely low when compared to other European countries. The reasons for this situation are multiple. But in the area of Animal Production in Portugal, it seems clear that the management and operational structure of most industrial players is not adequately prepared to incorporate a PhD collaborator in its ranks.

A striking example of this situation came from the audience. When enquired, Dr. Elisabete Matos mentioned that Soja de Portugal, one of largest private groups operating in the area of animal nutrition and animal production in Portugal, only has one PhD in its entire work force of more than 500 collaborators.

Ingrid Van Dorpe (Premix Lda.) mentioned that there is often a mismatch between the highly scientific and theoretical profile of the young PhDs and the practical needs of the industry. New strategies to overcome this difficulty are needed. The SANFEED program, with a strong involvement of the private companies on the genesis of the research topic and the implementation of the research work is a clear positive step to minimize this gap.

Dr. Jorge Dias, mentioned that this "apparent mismatch" requires a careful evaluation. In the field of Aquaculture Nutrition, we could easily list half a dozen of young Portuguese PhDs that are currently working in several large international companies of the sector. Their functions and responsibilities range from research to marketing and management. If given a chance, our PhDs are often regarded as highly qualified collaborators.

The workshop was ended on this optimistic note, but reinforcing the idea that the main objectives and achievements of the SANFEED Program is to contribute towards generating a professional career for the participating PhDs.

POSTERS

THE ROLE OF THE MAIN CORTICOSTEROID HORMONE (11-DEOXYCORTISOL) ON THE EXPRESSION OF ION TRANSPORT PROTEINS IN THE SEA LAMPREY, *Petromyzon marinus*.

D. Ferreira-Martins*[1,2], D. Drake[3], S.D. McCormick[3,4], J. Coimbra[1,2], J. M. Wilson[1,5]
*Dr. Diogo Ferreira Martins, Interdisciplinary Centre of Marine and Environmental Research (CIIMAR/CIMAR), Rua dos Bragas 289, 4050-123 Porto, Portugal. tel +351 22 340 1809 fax +351 22 339 0608; Email:diogofmartins14@gmail.com

[1]Interdisciplinary Centre of Marine and Environmental Research (CIIMAR/CIMAR), University of Porto, Portugal
[2]Instituto de Ciências Biomédicas de Abel Salazar, ICBAS, University of Porto, Portugal
[3]University of Massachusetts, Amherst, MA, USA.
[4]Conte Anadromous Fish Research Center, USGS, Turner Falls MA USA.
[5]Department of Biology, Wilfrid Laurier University, Canada

Abstract

The sea lamprey *Petromyzon marinus* is an anadromous species characterized by three distinctive stages. During the ammocoete (larval) stage sea lampreys are freshwater benthic stream filter feeders, after which they undergo a dramatic morphological and physiological transformation into parasitic feeding juveniles that migrate to the ocean where they become parasitic feeders. The adults re-enter fresh water migrating upstream until they find a suitable place to terminally spawn. In vertebrates, corticosteroid hormones play an important role in ion homeostasis, growth, metabolism, reproduction, immunity and stress. Corticosteroids bind to cytosolic receptors, which are then transported to the nucleus acting either as positive or negative transcription factors. As a result, the expression or repression of regulatory protein has effects on response to external stressors, thereby maintaining homeostasis. The steroid hormone, cortisol has a well-documented impact on teleost osmorregulation, increasing tolerance to salinity in euryhaline species. In lamprey, 11-deoxycortisol (11-DOC) has been identified as the main corticosteroid, and a highly specific corticosteroid receptor is present in the gill cytosol of sea lamprey. For this study we used ammocoetes and post-metamorphic juveniles to address the hypothesis that 11-DOC has an important role in modulating Na^+/K^+ -ATPase activity and expression using gill organ culture system *(in vitro*) and *in vivo* approaches. This study provides evidence for the role of 11-DOC as an osmoregulatory hormone during seawater acclimation in the sea lamprey in addition to further insight into the function of this ancestral steroid hormone. This work was supported by FCT grant PTDC/MAR/98035/2008 to Jonathan Mark Wilson and a Fulbright Research grant awarded to Diogo Ferreira Martins.

LOCALLY PRODUCED BY-PRODUCTS AS POTENTIAL PROTEIN SOURCES FOR EUROPEAN SEABASS (*DICENTRARCHUS LABRAX*)

I. Campos* [a], E. Matos [b], M. Pintado [c], Luisa M.P. Valente [a]

[a] CIIMAR-CIMAR L.A., Centro Interdisciplinar de Investigação Marinha e Ambiental and ICBAS, Instituto de Ciências Biomédicas de Abel Salazar, Universidade do Porto, Rua dos Bragas, 289, 4050-123 Porto, Portugal
[b] SORGAL, Sociedade de Óleos e Rações, Estrada 109 Lugar da Pardala, 3880-728 S. João OVR, Portugal
[c] CBQF – Centro de Biotecnologia e Química Fina, Laboratório Associado, Escola Superior de Biotecnologia, Universidade Católica Portuguesa/Centro Regional do Porto, Rua Arquitecto Lobão Vidal, Apartado 2511, 4202-401 Porto, Portugal,
Email: lvalente@icbas.up.pt

Introduction

The agro-food industries generate large amounts of by-products which are often discarded, causing not only environmental pollution but also the loss of useful biological resources (National Research Council, 2011). Locally produced by-products could be valuable and sustainable novel sources of protein and lipids for fish feed and even though the use of animal meal had been banned in 2001, after its role in the bovine spongiform encephalopathy (BSE) crisis, it has been allowed in fish feed in Europe since 1 July 2013 (European Commission, 2013). There is a growing interest in safe protein-rich alternatives to fish meal that could reduce the dependency of imported protein sources reducing fishing pressure and the carbon footprint of aqua feeds, since these protein sources are locally available. With this in mind, the present study was conducted to evaluate the apparent digestibility of several locally produced rendered agro-food by-products in European seabass (*Dicentrarchus labrax*).

Materials and Methods

Seven by-products from the Portuguese agro-food industry were selected and processed to maximize their potential as protein sources for aqua feeds: beta-lactoglobulin fraction (79 % protein) obtained after whey protein hydrolysis by *Cynara cardunculus* (BETA); the peptide fraction >3000 Da (86 % protein) obtained from fish processing by-product meal (FISH); the peptide fraction >3000 Da (39 % protein) obtained from spent brewer's yeast (YEAST); whole wheat germ (28 % protein) (GERM); steam hydrolyzed feather meal (85 % protein) (FEATH); hydrolyzed poultry by-product meal (53 % protein) (POULT); and whole okara meal (41 % protein), a by-product from soya milk (OKA). Based on known nutritional requirements of European seabass, a commercial-based basal mixture was formulated and added 1 % chromic oxide as inert digestibility marker. The reference diet contained 100 % of this basal mixture (REF) and the seven test diets had 30 % of each test by-product and 70 % of the basal mixture. All mixtures were then dry pelleted through a 3.2 mm die at 50 ºC (CPM, C-300 model).
Homogeneous groups of 20 European seabass juveniles (mean initial weight 15 g) were distributed by six 55L tanks inserted in a recirculating salt water system (salinity 35 ‰, 20 ± 1 ºC) designed as described by Cho & Slinger (1979) to collect the feces and subjected to a 12-hour light/12-hour dark photoperiod regime. The experimental diets were randomly assigned to the tanks, being the experiment divided into three periods of fifteen days, for replication of results (n=2). Apparent Digestibility Coefficients (ADC) of dry matter (105 ºC for 24 h), protein (N x 6.25, using a Leco nitrogen analyzer, Leco), energy (adiabatic bomb calorimeter, IKA), and lipids (petroleum ether extraction using a Soxtherm Multistat/SX PC, FOSS) were determined in the test diets and test ingredients.

The ADC's of the test ingredients were estimated as proposed by the National Research Council (2011).

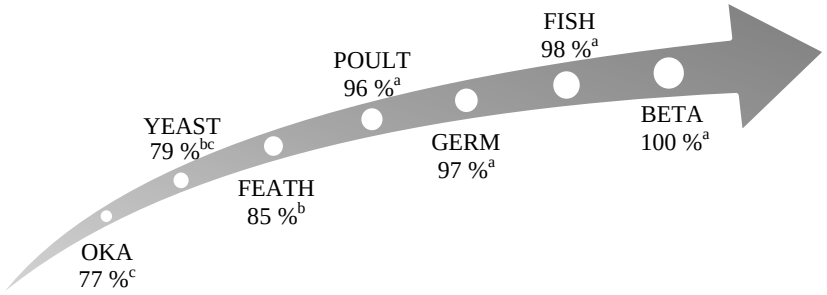

Fig. 2 Protein ADC of test ingredients (OKA – okara meal; YEAST – yeast extract; FEATH – Steam hydrolyzed feather meal; POULT– poultry by-product meal; GERM – wheat germ; FISH – fish processing by-product; BETA – beta-lactoglobulin).
Different superscript letters indicate significant differences ($P < 0.05$).

Results

Dry matter digestibility varied from 42 to 97 % among the various test ingredients, being highest in beta-lactoglobulin and lowest in okara meal. The results of protein ADC are portrayed in Fig. 1. The highest digestibility values (96-100 %) were obtained in beta-lactoglobulin followed by fish processing by-product meal (FISH), wheat germ (GERM) and poultry by-product meal (POULT); the hydrolyzed feathermeal showed intermediate values for protein ADC (85 %); and the lowest protein digestibility was observed in okara meal (77 %) and spent brewer's yeast peptides (79 %). The energy ADC was lowest in spent brewer's yeast peptides and okara meal (61 and 64 % respectively) and highest in POUL and BETA (91 and 95 % respectively).

Discussion and Conclusion

Nutrient digestibility results were high for most test ingredients. Okara meal and the peptide fraction >3000 Da obtained from spent brewer's yeast seem the less interesting protein sources among those evaluated in seabass. Protein ADC of the feathermeal hydrolysate was slightly lower than values reported for fishmeal (91 %) by Lanari & D'Agaro (2005) in the same species, but the remaining test ingredients showed even higher protein digestibility values.
The present results show that feathermeal hydrolysate, poultry by-product meal, wheat germ, fish processing by-product meal and beta-lactoglobulin are promising protein sources for aqua feeds. However, the availability and price of some of these rendered by-products may compromise their further use in feed formulation.

Acknowledgements

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DIETARY PROTEIN FOR SENEGALESE SOLE: STRATEGIES TO IMPROVE LARVAL GROWTH

Paula Canada1,2,3, Sofia Engrola3, Luisa M.P. Valente1,2 and Luís E.C. Conceição4

1 CIIMAR/CIMAR – Centro Interdisciplinar de Investigação Marinha e Ambiental, Rua dos Bragas 289, 4050-123 Porto, Portugal.
2 ICBAS – Instituto de Ciências Biomédicas de Abel Salazar, Universidade do Porto, Rua de Jorge Viterbo Ferreira 228, 4050-313 Porto, Portugal.
3 CCMar/CIMAR - Centro de Ciências do Mar, Universidade do Algarve – Campus de Gambelas, 8005-139 Faro, Portugal.
4 SPAROS Lda., Área Empresarial de Marim, Lote C, 8700-221, Olhão, Algarve, Portugal.

ABSTRACT

When it comes to protein quality, formulating microdiets for larvae becomes quite challenging. Typical protein sources are not easily digestible and do not seem to meet the high and changing amino acids (AA) requirements of altricial larvae that undergo a complex metamorphosis. Different diet formulation strategies are available to improve Senegalese sole larvae capacity to utilize and deposit protein throughout metamorphosis and maximize growth potential: (1) meeting the indispensable amino acids (IAA) requirements, by increasing the content of IAA; (2) meeting the ideal IAA profile by adjusting the dietary AA profile to the larval body AA profile; and (3) decreasing the complexity (molecular weight) of dietary protein, as to make it more digestible. These different formulating strategies were used and had different results on the development of the larvae capacity to utilize protein and direct it for growth.
Three growth trials were run to test different formulating strategies in order to improve Senegalese sole larvae capacity to utilize and deposit protein throughout metamorphosis and maximize growth potential. In all the experiments, Senegalese sole larvae were reared under the same conditions, upon a co-feeding regime from mouth opening, followed by weaning approx. 10 days after the metamorphosis was completed. The larvae capacity to utilize protein was determined using an in vivo method of controlled tube-feeding during relevant stages throughout development. For this purpose, 14C labelled model peptides of different molecular weights (1.0KDa and 7.2KDa), were tested to allow estimate absorption, evacuation, catabolism and retention. Growth was monitored during the whole trial.
Different formulating strategies had different effects on the development of the larvae capacity to utilize and retain protein for growth purposes. We suggest that the formulation of dietary protein in microdiets for Senegalese sole larvae should be changed or adjusted according to the developmental stage.

This work was supported by FEDER thought COMPETE – Programa Operacional Factores de Competitividade (POFC) and by FCT/MCTES (PIDDAC) under the project EPISOLE (PTDC/MAR/110547/2009). P Canada and S. Engrola are supported by grants from FCT (Portugal) SFRH/BD/82149/2011 and SFRH/BPD/49051/2008.

CHANGES IN INTESTINAL MICROBIOTA, IMMUNE- AND STRESS-RELATED TRANSCRIPT LEVELS IN SENEGALESE SOLE (*SOLEA SENEGALENSIS*) FED PLANT INGREDIENTS DIETS INTERCROPPED WITH PROBIOTICS

Batista S[a,b,c], Ozório ROA[a,b], Kollias S[c,d], Dhanasiri AK[c], Lokesh J[c], Kiron V[c], Valente LMP[a,b], Fernandes JMO*[c]

[a] ICBAS – Instituto de Ciências Biomédicas de Abel Salazar, Universidade de Porto, Rua Jorge Viterbo Ferreira, 228, 4050-313 Porto, Portugal
[b] CIMAR/CIIMAR – Centro Interdisciplinar de Investigação Marinha e Ambiental, Rua dos Bragas, 289, 4050-123 Porto, Portugal
[c] FBA – Faculty of Biosciences and Aquaculture, University of Nordland, 8049 Bodø, Norway
[d] Norwegian Sequencing Centre, Centre for Ecological and Evolutionary Synthesis, Department of Biosciences, University of Oslo, PO Box 1066, NO-0316, Oslo, Norway

Keywords: Probiotics, innate immune response, plant ingredient, Senegalese sole, gene expression, microbiota

Abstract

Senegalese sole (*Solea senegalensis*) is a highly valued flatfish that grows well with diets containing plant ingredients but their effects on immune competence are still a matter of debate. The current study aimed to examine changes in innate immune parameters and gut microbiota in Senegalese sole fed with 35% (CTRL) or 72% (PP) of plant ingredients with or without probiotic supplementation. Our data revealed that inclusion of probiotics and plant ingredients in the diet was associated with differences in immune- and stress-related gene expression. Overall, fish fed PP diets showed lower transcript levels of key immune- and stress-related genes in distal intestine, rectum and head-kidney than the CTRL diets. In particular, *hsp90b* mRNA levels in distal intestine were down-regulated by 70% and 60% in PP diet compared to CTRL containing probiotics PRO_1 (*Pediococcus* sp, *Enterococcus* sp, *Bacillus* sp, *Lactobacillus* sp) and PRO_2 (*Saccharomyces cerevisiae*), respectively. In particular, multispecies bacteria supplementation may have activated an antioxidative stress response, as indicated by the up-regulation of *gpx* and *cat* transcript levels in distal intestine, concomitantly with the down-regulation of *ftm* mRNA in rectum.

Denaturing gradient gel electrophoresis showed lower similarity values for distal intestine than rectum. Also fish fed PP diets displayed lower similarity value compared with fish fed CTRL diets, pointing to a difference in the microbial populations between fish fed different plant ingredients content on the diet. Our data revealed that inclusion of plant ingredients was associated with differences in gene expression and a more diverse microbiota profile with no effect on growth performance. Moreover, probiotic supplementation resulted in up-regulation of *hsp90b*, *gpx*, *cat* and *apoa1* transcript levels in distal intestine concomitantly with a growth rate reduction compared to non-supplemented fish.

Acknowledgements

S. M. G. Batista is supported by FCT – SFRH/BD/76668/2011. This work was also supported by PROBIOSOLEA project with the financial support of Quadro de Referência Estratégico Nacional – QREN and Programa Operacional Regional do Norte – ON2 (Ref. no. 13551), supported by the European fund for regional development FEDER. We would like to thank to CIIMAR/ ICBAS (UP) and FBA (University of Nordland) for the use of the facilities and equipment and for technical support.

USING MULTIPLE FLUORESCENT STAINS TO ASSES BONE GROWTH IN FISH LARVAE

Viegas, MN[134]*, Cardeira, J[2], Pinto, W[1], Aguiar, C[3], Salgado, MA[4], Engrola, S[2], Gavaia, P[2], Dias, J[1]

[1] SPAROS Lda, Area Empresarial de Marim, Lote C, 8700-221, Olhão, Portugal.
[2] CCMAR, Universidade do Algarve, Campus Gambelas, 8005-139, Faro, Portugal.
[3] INVIVONSA Portugal S.A., Zona Industrial de Murtede, 3060-372 Cantanhede, Portugal.
[4] CIIMAR, ICBAS, R. Jorge Viterbo Ferreira, 228, 4050-313 Porto, Portugal.
Contact: up201409164@icbas.up.pt

Introduction

Production of high quality and healthy larvae and juvenile fish is one important target for a successful and competitive expansion of the aquaculture industry. Although trace minerals are essential nutrients with a critical role in several physiological and metabolic pathways, knowledge on the qualitative and quantitative requirements of dietary minerals of fish larvae and juveniles is still extremely scarce. Establishing reliable estimations of mineral requirements in fish is difficult since it depends on a correct quantification of the potential contribution of minerals from the water, leaching of minerals from the diet prior to consumption and reliable indicators of mineral bioavailability. Fluorescent compounds that label mineralizing bone have been used in research for many years and by utilizing multiple fluorescent tracers it is possible to measure, at a micron level, mineral apposition and bone formation rates.

Materials & Methods

Senegalese sole larvae were fed from 26 to 90 DAH with diets containing different forms of inorganic phosphorus (sodium phosphate and monoammonium phosphate) at various doses. At 70 DAH larvae were immersed in a calcein (green calcium marker) staining solution and after 20 days the larvae were sacrificed, the vertebral columns removed and stained with alizarin red (red calcium marker). Columns were subsequently observed under a fluorescent microscope and analyzed to evaluate changes in vertebral bone growth. Additionally, whole larvae ash and phosphorus contents were measured.

Results

Calcein (Fig. 1A) and Alizarin Red (Fig. 1B) fluorescence were observed in vertebral bone samples. These images were then combined (Fig. 1C) making it then possible to determine the length of vertebral bone growth (Fig. 1C, white bar).
Fish fed different amounts and forms of phosphorus presented altered vertebral bone growth (Fig. 2).

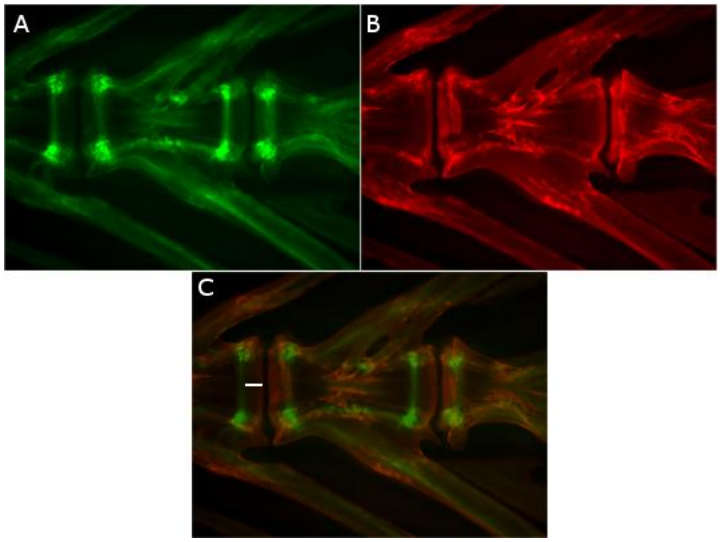

Figure 1- Multiple fluorescence micrographs of stained vertebrae from Senegalese sole larvae. A- Calcein; B- Alizarin Red; C- Combination of A and B. White bar in C represents the length of vertebral bone growth.

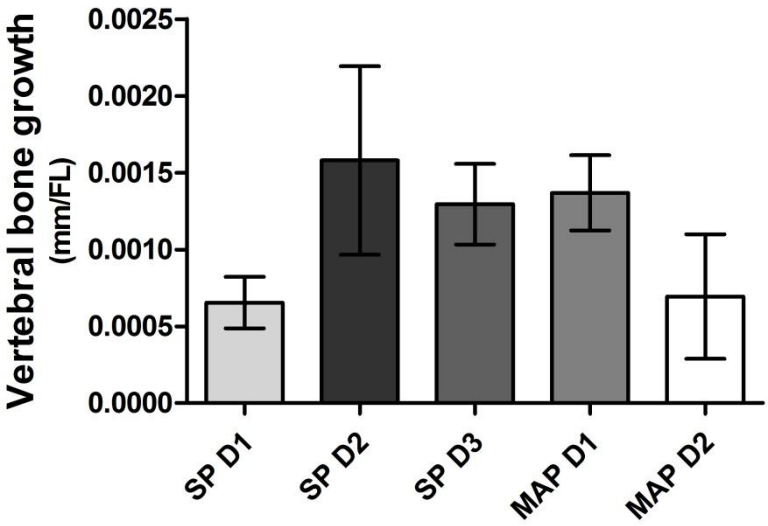

Figure 2- Vertebral bone growth of Senegalese sole. FL = average length of fish per treatment. SP D1, 2 and 3: Fish fed sodium phosphate at dose 1, 2 and 3; MAP D1 and 2: Fish fed monoammonium phosphate at dose 1 and 2.

Conclusion

By employing multiple fluorescent dyes, at sequential timepoints during growth, it was possible to measure changes in the bone formation rates of fish larvae fed with different amounts and forms of phosphorus. This work contributes towards the establishment of reliable analytical methods to assess mineralization patterns in fish larvae, a key element to optimize mineral nutrition.

META-ANALYSIS STUDY OF THE ELECTRICAL STIMULATION EFFECT ON BEEF MEAT TENDERNESS

Xavier, C.*, Gonzales-Barron, U., Muller, A. y Cadavez, V.A.P.

Centro de Investigação de Montanha (CIMO), ESA – Instituto Politécnico de Bragança. Campus de Santa Apolónia, Bragança. Portugal.
E-mail: *cristinaxavier@ipb.pt*

ABSTRACT: The objective of this work was to use meta-analysis to estimate the effect size of the electrical stimulation on beef tenderness through the study of the measurements of shear force. Eight independent studies were used based on comparison of shear force measurements on the *Longissimus dorsi* and *Longissimus lumborum* in cattle carcasses subject to electrical stimulation and unstimulated carcasses. For each study, the mean effect size and standard error was calculated in order to apply a random-effects meta-analysis model. The meta-analysis demonstrated that the electrical stimulation on beef carcasses decreases the values of shear-force of meat by an average of 1.34 kgf. Thus, this study confirmed the positive effect of the electrical stimulation on the beef meat tenderness. However, the effect size displayed high variation among studies which can be attributed to differences in their experimental conditions.

Keywords*:* beef meat, shear force, electrical stimulation, meta-analysis.

INTRODUCTION

The tenderness of beef is the main quality attribute (Hopkins & Fogarty, 1998) and its variation results in impairment to be rejected by consumers. This attribute can be objectively assessed by the shearing force, simulating the action of chewing, and is generally determined by the method of Warner-Bratzler (Huff-Lonergan Lonergan, 2005). Thus, the cutting force required to cut a meat sample is inversely proportional to the tenderness of the meat.

The electrical stimulation (ES) is based on sending an electric current to the channel, which accelerates rigor mortis. Using ES prevents hardening from cold, it induces ATPs using before the onset of *rigor mortis*, accelerate anaerobic glycolysis and increases the pH drop. Thus, the meat subject to ES is more tender as a result of decreased muscle contraction in *rigor mortis*. Several authors (Aalhus *et al.*, 1994; Strydom & Frylinck, 2014; Strydom *et al.*, 2005; Hwang & Thompson, 2001; Kim *et al.*, 2007; Kuttinarayanan & Ramanathan, 2010; White *et al.*, 2006; Hopes-Jones *et al.*, 2010) studied the effect of ES on tenderness of beef; however, no work so far summarized the results obtained with this method. The meta-analysis is a statistical methodology to compare the results obtained by different studies on a given topic (Viechtbauer, 2010), and combines the results of several independent works and recognize patterns in the results of these studies (Gonzales-Barron *et al*., 2012). This study aimed to conduct a meta-analysis on the effect of the ES in the tenderness of the beef.

MATERIAL AND METHODS

This meta-analysis was performed using the package *metafor* (Viechtbauer, 2010) R (R Development Core Team, 2011) software. For this, 8 items with information on the mean and standard error of the cutting force of the meat obtained from unstimulated (Control) and electrically stimulated (Treatment) channels were used. Thus, the study is based on the comparison of the mean difference of *Longissimus dorsi* muscle tenderness and *Longissimus lumborum* of beef subject to ES or not. The overall average effect size was determined using meta-analytic models random effects and the results are visualized through a "forest plot". The existence of heterogeneity among the published studies was

assessed by the I^2 index, defined as the proportion of the total variation that is attributable to the variation between studies (Higgins & Thompson, 2002).

RESULTS AND DISCUSSION

For this meta-analytical study, an effect size as the average difference between the average meat tenderness stimulated channels (Treatment) and those tenderness average unstimulated (Control) was defined. Thus a negative effect size indicates that meat carcasses electrically stimulated presented strength values lower court, while a positive value indicates otherwise. Although the random effects model indicated the presence of heterogeneity (I^2 = 83.4%) among studies, the ES contributes to increase (P <0.001) the tenderness of beef with an estimated effect size of -1.34 kgf. The "forest plot" (Figure 1) shows that the studies had different precision (different confidence interval) and the study of Strydom *et al.* (2005) presented a major contribution to the average size effect, as seen by the larger square. Furthermore, the work of White *et al.* (2006) had the highest effect size (-3.15 kgf), but also exhibited the greatest variation (CI: -7.37 to 1.07).

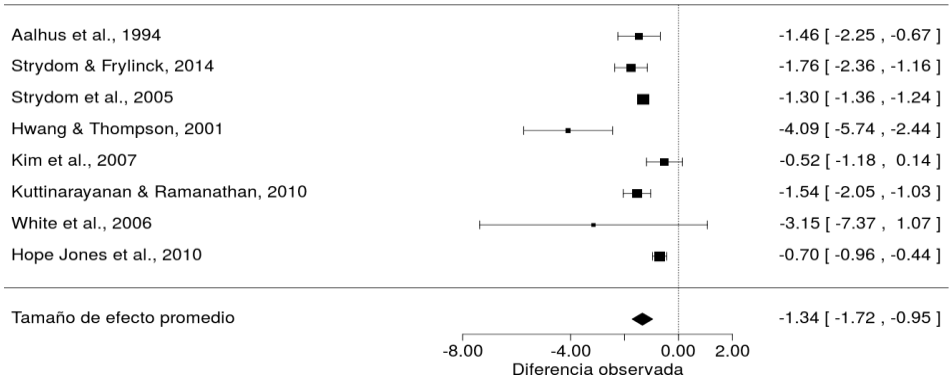

***Figure 1**. Graphic "forest plot" of electrical stimulation effect on the shear force of beef. In parenthesis presents the 95% confidence interval.*

The ES contributes to an overall increase in the tenderness of beef, because it directly stimulates the muscle after the death of the animal, causing the acceleration of rigor mortis, and the immediate drop in pH (Lombard, 2009). The ES contributes to *rigor mortis* occurs at an elevated temperature and avoids the occurrence of cold hardening in the muscle. Likewise, it also accelerates the process of maturation of meat (Simmons *et al.*, 2008). In short, the ES improved quality characteristics tenderness of beef and can be used to reduce variations in the quality attributes of meat resulting from environmental effects such as age, nutrition and animal stress (Lombard, 2009).

CONCLUSION

The effect size displayed great variation between studies; it is expected variation of the different experimental conditions thereof. However, applying a model of random type, this variability between studies was extracted, and the final average effect size confirmed the positive effect of electrical stimulation on tenderness of beef.

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CHARACTERIZATION OF THE SALT SECRETING ORGAN OF THE STRIPED MARINE CATFISH *PLOTOSUS LINEATUS*

Salman Malakpour Kolbadinezhad* (CIIMAR, Porto, Portugal) and Jonathan Mark Wilson (CIIMAR, Porto, Portugal)

salman_malakpoor@yahoo.com

Abstract

The Plotosidae catfishes are one of the few catfish families with marine, brackishwater and freshwater members. They are also unique amongst the teleosts in possessing a specialized salt secreting organ, the dendritic organ (DO). In contrast, salt secretion in all other marine teleosts is typically preformed by gill ionocytes. The evolution of this specialized ionoregulatory organ likely permitted the successful habitation of marine environments by Plotosidae catfishes. This study was directed at the molecular characterization of the DO ion transport mechanisms in *Plotosus lineatus*, the stripped marine catfish, and contrasted the expression pattern of this salt secreting organ with the other main teleost ion transporting organ, the gill. Na^+/K^+-ATPase (NKA) activity was 4.5x higher in DO in contrast to gill which correlated with immunoblot (IB) data for NKA α subunit and immunohistochemical localization of NKA-immunoreactive (NKA-IR) cells (ionocytes). In the gill there were few NKA-IR cells present whereas the parenchymal cells of the DO showed strong expression. The secretory $Na^+:K^+:2Cl^-$ cotransporter (NKCC1) expression in gill was not detectable in contrast to DO which displayed high levels (IB and IHC). Cystic fibrosis transmembrane conductance regulator (CFTR) was detected in DO but not gill by IB, however, it was possible to confirm these results by IHC. Taken together, these results indicate that there is a clear shift of NaCl secretion mechanisms from the gills to the DO of *P. lineatus* firmly establishing this organ as key for hypo-osmoregulation.

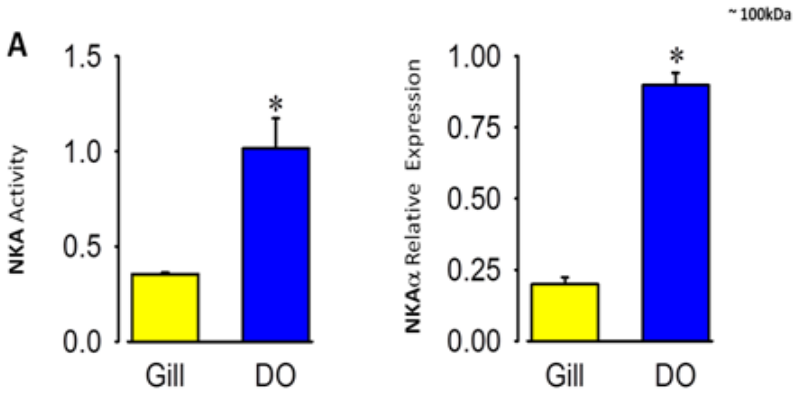


Fig.1. Na^+/K^+-ATPase (**NKA**) activity (**A**) and relative expression of **NKA** α subunit (**B**) protein in the **gill** and **DO** tissues of *P. lineatus*. Representative western blots bands shown (~100 kDa). Means ± SEM (n=6). *Asterisk* indicates a significant difference ($P < 0.05$).

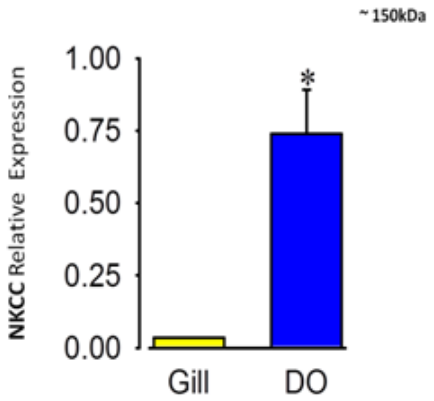


Fig.2. Relative expression of **NKCC** protein in the **gill** and **DO** tissues. Representative western blots images shown (~150 kDa). *Asterisk* indicates a significant difference ($P < 0.05$). Note, the high level of expression in **DO**.

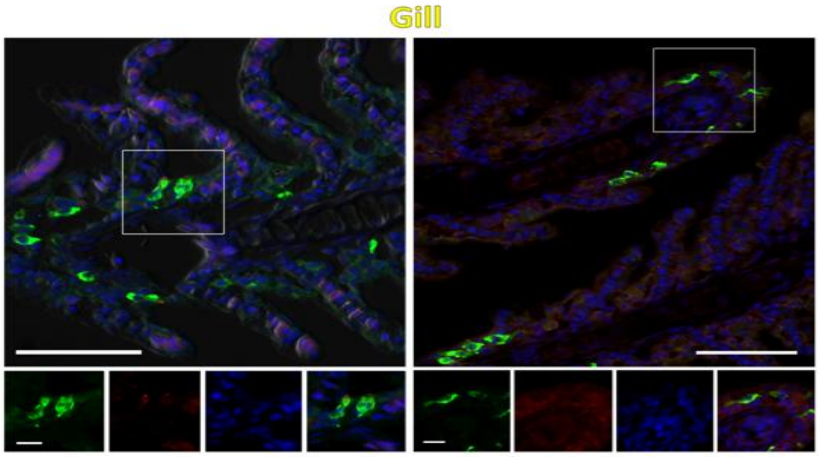


Fig.3. Double immunoflurorescence localization of **NKA** with either (**A**) **CFTR** or (**B**) **NKCC1** in the **gill**. Note, that few NKA-IR cells were present and were heterogeneously distributed within filament epithelium and absent from the lamella. A subpopulation of NKA-IR had apical localization of **CFTR** (arrow). No detectable **NKCC1** expression was found in gill. Sections were counter stained with **DAPI** nuclear staining and overlaid with the contrast image. Scale bar 50 µm in upper panel and 10 µm in inset.

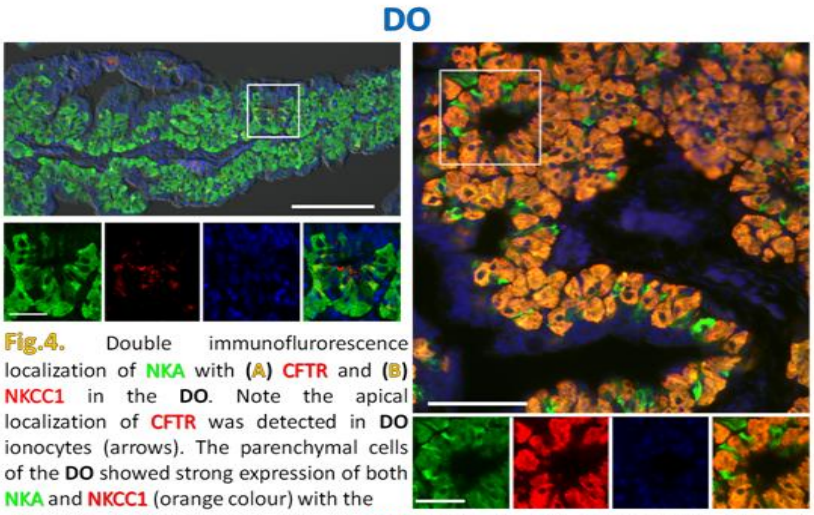
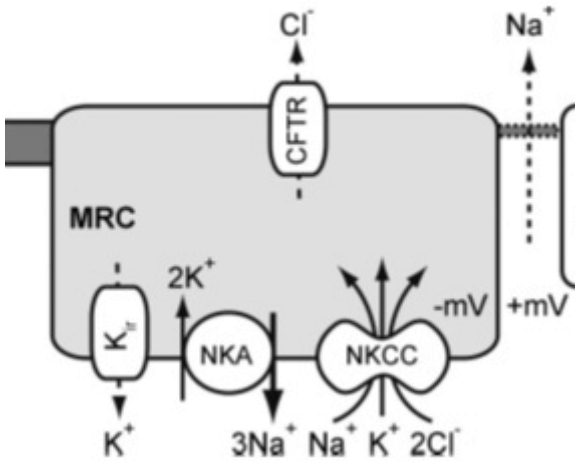


Fig.4. Double immunofluroescence localization of NKA with (A) CFTR and (B) NKCC1 in the **DO**. Note the apical localization of CFTR was detected in **DO** ionocytes (arrows). The parenchymal cells of the **DO** showed strong expression of both NKA and NKCC1 (orange colour) with the exception a few ionocytes with no NKCC1 (arrowheads) revealing two types of ionocytes in DO. Sections were counter stained with DAPI nuclear staining and overlaid with the contrast image. Scale bars 50 µm in upper panel and 10 µm in the insets.

8



LIST OF PARTICIPANTS

Abigail Baptista (FCUP-UP)
Alexandra Marques (CIIMAR)
Ana da Conceição M. Gomes(CAVC)
Ana Raquel Rodrigues (REQUIMTE/ICBAS-UP)
Ana Rita Brás (ICBAS/FCUP-UP)
Ana Rita Cabrita (REQUIMTE/ICBAS-UP)
António M. Fonseca (REQUIMTE/ICBAS-UP)
Ayoub Azizi-Shotorkhoft (Ramin University)
Beatriz (ICBAS-UP)
Benjamin Costas (CIIMAR)
Carla Aguiar (InvivoNsa Portugal)
Cláudia Silva (Evonik Nutrition & Care GmbH)
Cristina Xavier (ICBAS-UP)
Daniela Gomes (ICBAS-UP)
Denis Meehan (REQUIMTE/ICBAS-UP)
Diogo Alexandre Rocha e Silva (FCUP/ICBAS-UP)
Diogo Martins (CIIMAR/ICBAS-UP)
Eliane Pimenta da Silva (ICBAS-UP)
Elisabete Matos (SORGAL)
Emídio Gomes (CCDR-N/ICBAS-UP)
Fernando R. A. Magalhães (CIIMAR/ICBAS-UP)
Filipa Brás da Cruz Faria Curinha (FCUP-UP)
Filipa Fontinha (CIIMAR)
Flávia Teixeira Mendes (FCUP-UP)
Francisca (CIIMAR)
Francisco Guardiola Abellán (CIIMAR)
Gaspar Lobo (ICBAS-UP)
Hugo Oliveira (REQUIMTE/ICBAS-UP)
Inês Campos (CIIMAR/ICBAS-UP)
Inês M. Valente (REQUIMTE)
Ingrid Van Dorpe (Premix Lda)
Isabel Costa Ramos (CAVC)
Isabel Moreira Lopes Santos (CAVC)
Joana Pedro F. C. P. Oliveira (ICBAS-UP)
João Albuquerque
João Ferreira (ICBAS-UP)
João Pacheco (Segalab, S. A.)
Jorge Dias (SPAROS Lda)
José António Rodrigues (FCUP-UP)
José F. M. Gonçalves (ICBAS-UP)
José Pedro Ribeiro (ICBAS-UP)
Júlio Carvalheira (CIBIO/ICBAS-UP)
Lourenço Ramos Pinto (ICBAS-UP)
Luis Ferreira (AGROS)
Luísa M. P. Valente (CIIMAR/ICBAS-UP))
Mahmoud Mabrok (CIIMAR/ICBAS-UP)
Marcela Segundo (REQUIMTE/FF-UP)
Margarida Maia (REQUIMTE/ICBAS-UP)
Maria Antónia Salgado (CIIMAR/ICBAS-UP)
Maria João Dias Peixoto (CIIMAR/ICBAS-UP))
Mariana Monteiro (FCUP/ICBAS-UP)
Marieta Passos (LAVQ/REQUIMTE/FF-UP)
Marina Machado Casimiro (CIIMAR)
Michael Viegas (SPAROS Lda/CIIMAR)
Miguel Silva (ICBAS-UP)
Nertila Malushi (ICBAS-UP)
Patrícia Rodrigues Correia (FCUP/ICBAS-UP)
Paula Canada (CIIMAR/ICBAS-UP)
Paulo Vaz Pires (CIIMAR/ICBAS-UP)
Pedro Eloi Ferraz Carvalho (ICBAS-UP)
Raquel Vizcaino (CIIMAR)
Renato José L. M. Escada (FCUP-UP)
Richard Dewhurst (Scotland's Rural College)
Roberto Branco (Betacapital)
Rui Pereira (ALGAplus Lda)
Salette Reis (REQUIMTE/FFUP)
Salman Malakpour (CIIMAR/ICBAS-UP)
Samuel Herlander (FCUP/ICBAS-UP)
Sandra Isabel Pinto Nunes (FCUP/ICBAS-UP)
Sandra Silva (ICBAS-UP)
Sara Magalhães (REQUIMTE/ICBAS-UP)
Sílvia Azevedo (REQUIMTE/ICBAS-UP)
Sílvia Coelho (ICBAS-UP)
Sónia Batista (CIIMAR/ICBAS-UP)
Teresa Costa (INDUKERN PORTUGAL LDA)

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