Interdisciplinary Approaches in Fish Skeletal Biology

Mosaics of the Roman villa of Milreu (Estoi) located 7 km from Faro/Ossonoba in the Roman Lusitania, south of Portugal

First Meeting
27-29 April 2009
Tavira, Portugal

Centre of Marine Sciences

Fish Skeletal Biology

Welcome from the organisers

An increasing number of biological disciplines focus on fish skeletal biology, including paleontology, morphology, developmental biology, systematic, genomics, proteomics, regenerative medicine, and aquaculture. The first International Workshop on "Interdisciplinary Approaches in Fish Skeletal Biology" aims at joining experts from these, often separated, research communities and promote exchange in ideas, concepts and methodological approaches applied to fish skeletal research. This should contribute to the advancement of research in all sub-disciplines and implement the basis for new interdisciplinary collaborations in this field. We heartily welcome all participants and we thank everybody for contributing to make this workshop a reality.

We hope that you will all have an exciting, fruitful and joyful meeting.

M. Leonor Cancela and **P. Eckhard Witten**

Scientific committee

Ann Huysseune
Brian K. Hall
Santosh P. Lall
Jean-Yves Sire
Tom Hansen
António Jacinto
Harald Rosenthal

Local committee

Ricardo B. Leite
Paulo J. Gavaia
Anabela Bensimon-Brito
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Skeletal development and performance of zebrafish *Danio rerio* (Hamilton, 1822) larvae and juvenile fish fed with different diets

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The appearance of skeletal deformities is a major problem associated to intensive, large-scale culture of fish species, affecting growth, development and ultimately, survival of produced fish. Although the mechanisms working behind it are still unclear, it is known that environmental, genetic and nutritional factors play fundamental roles. Given its importance as a vertebrate model to study skeleton-related diseases, the selection of an appropriate dietary protocol is a critical step in order to diminish the nutritional effect on bone and cartilage development. To better understand the effect of diet on skeletal development and deformities, as well as on larvae and juvenile global performance, we have tested four distinct dietary regimes, composed of rotifers *Brachionus* sp., *Artemia nauplii* and dry, formulated food. Mortality and growth were registered for each treatment in order to characterize the general success of each diet. At different life stages, we analyzed the skeleton and characterized the deformities after whole-mount staining with the histological markers alizarin red and alcian blue, which allowed detailed detection of both calcium deposition and cartilaginous structures. The relative expression levels of the skeletal molecular markers matrix Gla protein (*mgp*) and osteocalcin (*oc*) of whole specimens were evaluated by quantitative real-time PCR. The results obtained suggest that diet has a significant effect on skeletal development of zebrafish larvae and juveniles, showing that *Artemia nauplii* and a daily mixture of live and inert food seem to be the most appropriate diets for this species' performance and skeletal development. This work also reinforces the importance of diet optimization to achieve zebrafish optimal development.

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Effect of warfarin in zebrafish (*Danio rerio*) bone formation during caudal fin regeneration

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In the last decade, fish has emerged as an important organism for studies on skeletal development in vertebrates, and evidence has been accumulated showing that zebrafish is a suitable system to perform phenotype-based drug screens. The ability to regenerate epidermal injuries is a general feature of most organisms yet only a few can fully regenerate severed appendages comprising several different tissues. Zebrafish is one of the most used models for regeneration studies, creating a powerful tool to study *de novo* bone formation without affecting vital development processes. Warfarin (sodium warfarin or 4-hydroxycoumarin compound) is a known anticoagulant whose mode of action is based on the interference of carboxylation of γ-carboxyglutamate (Gla) proteins including blood coagulation factors and bone related proteins. When warfarin disrupts normal posttranslational modification of osteocalcin (Oc) and matrix Gla protein (Mgp) it consequently affects bone formation and mineralization at various levels. It has been described to (i) induce excessive mineralization in growth cartilage and arteries but also (ii) decrease osteoblast and osteoid surface, (iii) increase osteoclast surface, (iv) decrease rate of bone formation and (v) increase rate of bone resorption. To determine whether zebrafish *oc* and *mgp* genes are active during regeneration of caudal fin, we performed whole mount *in situ* hybridization experiments on regenerating adult fins at different time points of the regenerating process, and showed a disruption of normal expression patterns for both genes at 24h post amputation. The analysis of control and Warfarin exposed fish stained for mineralized (Alizarin red) fins revealed a delay in bone regeneration as well as the appearance of malformations. By contributing to clarify warfarin effects during bone development in fish, this work should provide further insight into Gla proteins function, known to be crucial for skeletal development.

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Skeletal evaluation and technical improvements to decrease the incidence of skeletal deformities in *Solea senegalensis*, Kaup

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The Senegalese sole (*Solea senegalensis*) is characteristic from Southern Europe and Mediterranean. It has recently been adapted for aquaculture production since it is well accepted by consumers and reaches high commercial values. After the description of the ontogenic events of skeletogenesis, an evaluation on the incidence of malformations was conducted, revealing high levels of skeletal deformities reaching up to 80% incidence. This can represent a constraint for further improving performances in this species. A comparison of rearing methodologies revealed a relatively high incidence of skeletal deformities in early life stages captured in nature (20%) and in individuals raised in mesocosms (20-50%), which indicates that deformities have other causes than just the ones induced in captivity. Several trials have been performed in order to characterise the effects of different rearing methods, feeding strategies and regimes, on the skeletal quality of *S. senegalensis* fingerlings. No significant differences were found in overall rates of deformities when lipidic enrichment content was modulated, showing that the levels of DHA although affecting growth, do not appear to negatively influence the development of skeletal malformations in *S. senegalensis*. The modulation of feeding frequency after weaning also adds no effects on the incidence of skeletal deformities. The modulation of the vitaminic content in larval diets showed marked increase in deformities in vitamin A supplementations, with higher deformity incidences and higher number of affected areas per fish. However, recent experiments indicate that the modulation of phylloquinone levels during growth as a positive effect on skeletogenesis. Although relatively high numbers of deformities were observed in different conditions, the external anatomy is normally not severely affected, meaning that the aquaculture success of this species might not be impaired by skeletal deformities. Still, advances in nutritional and zootechnical conditions should be made to improve the overall skeletal quality of *Solea senegalensis*.

49 Warfarin effects in the skeletal development of zebrafish (*Danio rerio*)

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The vitamin K-dependent (VKD) gamma-glutamyl carboxylase promotes the conversion of glutamic acid residues (Glu) to gamma-carbolxyglutamic acid (Gla) in a post translational modification that is essential for the functionality of VKD proteins. This process affects homeostasis, apoptosis, signal transduction, growth control, arterial calcification and bone development. Among this broad protein family, matrix Gla protein (Mgp) and osteocalcin (Oc) or bone Gla protein are known for their ability to bind calcium which is essential for their biological activity, influencing the normal skeletal development. Warfarin (Wa) is a known anticoagulant, that inhibits the action of vitamin K gamma-carboxylation (VKGC), disrupting the postranslational modification and correct function of oc and mgp. In this work, a series of zebrafish larval developmental stages were used for experimental treatments, in order to better understand the effects of warfarin and consequent inhibition of Oc and Mgp function in early skeletal development events. The analysis of control and Wa-exposed fish stained for mineralized (Alizarin red) and cartilaginous (Alcian blue) structures revealed a delay in calcification in Wa-larvae and a higher incidence of malformations. In addition, oc and mgp relative gene expression levels were upregulated as a response to warfarin treatment, after 9 days post fertilization for oc and for the entire period for mgp, together with a higher protein accumulation in skeletal tissues. This work allowed a better understanding of the role played by Oc and Mgp in zebrafish skeletal development, by observing its inhibition in Wa-exposed larval stages, emphasizing that drug testing is a very useful approach in order to determine functional inhibition of specific proteins.

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Mgp expression and accumulation in heart and kidney of turbot (*Scophthalmus maximus*)

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Matrix γ-carboxyglutamic acid (Gla) protein (Mgp) is a vitamin K-dependent protein normally found associated with the organic matrix of cartilage and bone *in vivo*. After the discovery of Mgp in various soft tissues, this protein was proposed to act as a local inhibitor of mineralization although its molecular mechanisms of action remain incompletely understood. Using the turbot (*Scophthalmus maximus*) as teleost fish model, we proposed to analyse gene expression and protein accumulation of Mgp in soft tissues, since available studies have not shown accumulation in these organs under non-pathological condition. In heart, in addition to the high levels of mgp expression detected by quantitative real-time PCR, we also observed, surprisingly, a strong accumulation of the protein throughout the entire cardiac muscle, outside and inside cardiac cells. Furthermore, we also observed mgp RNA expressed in the kidney, specifically in cells from glomeruli and urinary tubules, as well as protein located within the same sites. In all cases, protein was observed inside and outside the cells, reflecting its secretion and accumulation into the extracellular matrix. No RNA or protein signals were detected in liver tissues. This is the first report where intracellular accumulation of Mgp is clearly observed in a non-pathological condition and these findings could be related to Mgp protective role against ectopic calcification of those soft tissues, where high levels of calcium are present, thus leading to an increased risk of calcification.

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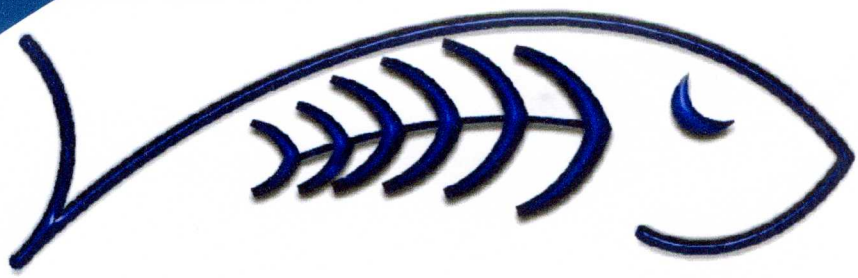

Certificate of Attendance

This is to certify that

Paulo GAVAIA

attended the IAFSB meeting
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The organizers

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