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PB.1

Comparative Gene Expression During Stress in Co-Existing, Ecologically Similar Algal Species

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Cosmopolitan intertidal brown seaweeds in the genus *Fucus* occur on temperate shores throughout the western and eastern North Atlantic. Upper vertical limits in the intertidal zone, as well as the geographical ranges of individual species, may be partially set by tolerance to emersion stresses. Where several species coexist with more or less distinct vertical distributions on rocky shores, it has long been known that these correlate with physiological tolerance to desiccation. We are interested in the molecular basis of desiccation tolerance and in 1) The role of local adaptation in stress tolerance-variation at the population level, and 2) species-specific differences in gene expression that are correlated with their vertical position on the shore. To begin to address these questions, we constructed subtractive cDNA libraries for divergent *Fucus vesiculosus* (L.) populations undergoing desiccation. These libraries were differentially screened to isolate clones representing genes up-regulated during the stress. After sequencing, clones representing partial cDNAs were selected to construct macroarrays, and these were used to investigate stress-responsive gene expression in several populations of *F. vesiculosus*. We also present results from Northern and/or RT-PCR analyses of *F. spiralis* (high-shore, stress-tolerant), *F. vesiculosus* (mid-high shore, stress tolerant), and *F. serratus* (low-shore, stress-susceptible) undergoing desiccation.

PB.2

Expression of the oligopeptide transporter PepT1 (Solute carrier family 15, member 1) in fed and starved larvae of Zebra fish (*Danio rerio*).

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Fish embryos develop while utilizing yolk nutrients supplied by the mother. There normally exists a "first feeding window" between when the larvae is able to ingest exogenous feed, and the "point of no return" where it has exhausted its yolk reserves and also irreversibly degraded critical tissues for energetic purposes if feed is not ingested. In fact, gut tissues have been suggested to be very sensitive to starvation in larval fish. The oligopeptide transporter-1 (PepT1) is responsible for the uptake of small peptides in the gut lumen and has been implicated to serve an important role in the absorption of dietary proteins. The transporter has recently been cloned and functionally characterized in Zebra fish (Verri et al. 2003. FEBS letters 549, 115-122). The aim of this work was to clarify how PepT1 expression levels are influenced by delayed onset of feeding. Total RNA was prepared from two groups (fed and starved) of zebra fish from onset of exogenous feeding. The temporal expression of PEPT1 in the larvae was determined using quantitative real time PCR. The results will be discussed in relation to nutritional requirement and the point of no return in fish.

PB.3

Cloning and ontogenetic expression of the oligopeptide transporter PepT1 (Solute carrier family 15, member 1), in fed and starved larvae of Atlantic cod, *Gadus morhua* L.

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Functional characteristics of marine fish larvae digestive system at the onset of exogenous feeding have long been a subject for discussion. Absorption of digested proteins in vertebrates has been shown to include transporter systems for single amino acids and small peptides. However, molecular and functional descriptions of these carrier systems in fish larvae remain largely unknown (Verri et al. 2003. FEBS letters 549, 115-122). The aim of this work was to clone and study the ontogeny of the peptide transport system present in the digestive system (PepT1) and determine if its levels of expression are influenced by delayed onset of feeding in Atlantic cod larvae. There is normally a "first feeding window" between the moment when larvae is able to start hunting and catching prey, and the "point of no return" where it has exhausted its yolk reserves and also irreversibly degraded critical tissues for energetic purposes. We have cloned by RT-PCR the oligopeptide transporter PepT1 from larva of Atlantic cod, *Gadus morhua* L. following data base mining and sequence comparison. Identification was confirmed by DNA sequence analysis. Total RNA was prepared from fed and starved cultured cod and the temporal expression of PepT1 in the larvae was determined using quantitative real time PCR. Results suggest that PepT1 gene was expressed at onset of first feeding in Atlantic cod but gene expression levels were rapidly reduced under starved conditions.

PB.4

Identification, cloning and regulation of a bifunctional enzyme from the *de novo*" pathway of purine synthesis in *Perkinsus atlanticus*: Functional implications

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Perkinsus (P.) atlanticus is a parasite from the Portuguese clam *Ruditapes decussatus* known by its association with high mortalities in clam beds from Southern Portugal. Despite its relevance for economical reasons, there is a huge lack of information concerning its biology and some stages of *Perkinsus* life cycle are still unknown. Furthermore, the relationship host-parasite is poorly understood. In this work we have identified, for the first time, the presence of enzymes from a "de novo" pathway for purine synthesis in alveolata (which include apicomplexa and dinoflagellates). A cDNA belonging to a bi-functional enzyme was cloned and identified as the transcript of a GARS-AIRS gene, encoding the 2nd and 5th enzymes of this pathway. In an effort to identify drugs which may target this pathway, five different drugs were used to inhibit the purine synthesis. Total RNA was extracted from *Perkinsus* cells in culture and converted into cDNA by reverse transcription. The quantification of the gene GARS-AIRS, and of the control gene L38, was then carried out by Real Time PCR. Since two main domains are present, the analysis was performed using two different probes targeting either the GARS or the AIRS domain. Preliminary results indicate different expression for each domain suggesting the existence of complex regulatory mechanisms within this gene. Results will be presented and discussed. This work was partially supported by a MARE project AMBIPERK

PB.5

Constitutive expression of bone Gla protein in two fish bone-derived cell lines: identification of gene expression variation between chondrocytic-like (VSa13) and bone-like (VSa16) cell lineages

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Two cell lines, VSa13 and VSa16, derived from vertebra of the gilthead sea bream, *Sparus aurata*, and capable of mineralizing their extracellular matrix were recently developed in our laboratory (Pombinho *et al.* 2004. Cell Tissue Res 315:393). VSa cells were found to express matrix Gla protein (MGP) or osteocalcina (Bone Gla protein or BGP), two vitamin K dependent proteins involved in tissue mineralization, in a mutually exclusive manner and according to the status of mineralization of each cell type. In this work, we investigated the effects of BGP forced or over-expression on (i) cell growth rate, (ii) culture mineralization, and (iii) expression of bone- and cartilage-specific genes. In order to produce different clones which constitutively express BGP, each cell line was stably transfected with two different constructs: empty vector alone or an expression vector containing BGP ORF. Expression levels of the transgene were confirmed by Northern analysis in both cell lines whereas the presence of BGP protein in the cell media of VSa16 was confirmed by western blot. Analysis of the BGP-expressing clones provided clear evidence for a different cell-type specific response to the presence of the BGP transgene. This data indicate that the two cell lines differ in their gene expression in response to mineralization and levels of BGP expression. Ongoing experiments aim at understanding the regulatory pathways involved in BGP-induced variation in gene expression in VSa13 and VSa16 cell lines.

PB.6

Effect of Citral on zebrafish skeletal morphology and vascular system integrity during the first stages of development

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Citral (3,7-dimethyl-2,6-octadienal), the aldehyde of geraniol, is a vitamin A antagonist that inhibits the oxidation of retinol to retinoic acid (RA), an important signalling molecule that performs numerous physiological roles during embryonic vertebrate development.. In this study, we used zebrafish larvae has a model system to observe the effect of citral in the development of skeletal structures and maintenance of vascular system integrity. Differences in skeletal structures and vascular system were analysed by histological techniques and complemented with studies of gene expression and protein accumulation by quantitative Real Time PCR, *in situ* hybridization and immunolocalization. Matrix Gla Protein (MGP) and osteocalcin (Bone Gla Protein or BGP) are vitamin K dependent proteins involved in the physiological regulation of tissue mineralization and were used as molecular markers to accesses to effects of Citral in zebrafish development. After the 3 weeks of Citral treatment, a decrease in the expression of both BGP and MGP was observed through real time PCR. In *situ* hybridization and immunolocalization revealed no obvious differences in sites of gene expression or protein accumulation between control and treated animals. The effect on skeletal structures and vascular system is currently being evaluated. PG supported in part by a doctoral fellowship (PRAXIS/BD/19665/1999) and POCTI/CVT/42098/2001

PB.7

Effect of 11-keto-testosterone on the testicular expression of seabream (*Sparus auruta*, Teleostei, Perciformes) estrogen and androgen receptors

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The seabream is a protandic hermaphrodite maturating as a male in the first year of age and undergoing a sex reversal to female around the second and third years. In teleosts fishes the function of androgens promoting masculinization of the gonads and stimulation of spermatogenesis it is well recognized. Furthermore, was verified that the levels of 11-ketotestosterone (11-KT), a potent androgen in male teleost fishes, decrease during sex reversal in the protandrous hermaphrodite Lates calcarifer, seeming to occur a shift in gonadal steroidogenesis from 11-oxygenated androgens to estrogens1. However, the studies envisaging the role of androgens on seabream reproduction are very scarce or inexistent. In order to clarify the molecular mechanisms underlying seabream reproduction we studied the effect of 11-KT, on the expression of androgen receptor (AR) and estrogen receptors, (ERalfa, ERbeta1 and ERbeta2). To achieve this goal, 16 one year of age animals were stimulated with 11KT (10 mg/kg) and the expression of AR and ERs was determined by a semi-quantitative RT-PCR. The 11KT treatment increases the proportion of testis in the gonads. The expression of ERbeta1 and ERbeta2 it is not affected by treatment while the expression of ERalfa and AR is much more variable. However, a shift on the expression of AR and ERalfa seems to occur. 1. Guigen, Y. et al (1995). General and Comparative Endocrinology 100: 106-118

PB.8

Gla proteins as markers for studies of skeletal development and malformations in new aquaculture fish species (*Pagrus auriga*, *Diplodus sargo* and *Scophtalmus maximus*)

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Evaluation of the onset and development of skeletal structures during larval ontogeny of commercially important species in southern Iberian Peninsula and Mediterranean area (*Diplodus sargo*, *Pagrus auriga* and *Scophtalmus maximus*) is essential for the successful production of high quality fish. With that purpose, we have chosen two calcium-binding proteins that can be used as markers for cartilage matrix (MGP, Matrix Gla Protein) and bone/dentin (BGP, Bone Gla Protein or osteocalcin). In order to obtain the necessary sequences for the generation of probes to follow the developmental appearance and expression patterns of the studied proteins, we have amplified by RT-PCR and cloned the 3' ends of BGPs and MGPs cDNAs from those species. Validation of their use as molecular markers of cartilage and bone was performed by *in situ* hybridization. These results will allow us to analyze changes in gene expression as a result of different experimental culture conditions and relate them to alterations in skeletogenesis. These alterations will be detected by histological methods allowing the detection of bone and cartilage in whole specimens. PG and JD supported by a doctoral and postdoctoral fellowship (PRAXIS/BD/19665/1999 SFRH/BPD/7151/2001). This project was founded by the project MCYT/AGL/2003-03558 (SPARUGENES), from the Spanish Ministry for Science and Technology.

PB.9

Development of fluorescent markers for early skeletal development of zebrafish embryo and larvae through in vivo expression of fusion GFP-Gla proteins

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Matrix Gla Protein (MGP) and osteocalcin (Bone Gla Protein, BGP) are two calcium binding, vitamin K–dependent, proteins of the extracellular matrix previously shown to be involved in the regulation of bone (BGP) and cartilage (MGP) mineralization as well as chondrocyte differentiation and maturation (MGP). During development, MGP and osteocalcin preferentially accumulate in mineralized cartilage and bone. Two DNA constructs were produced in which the ORFs for BGP and MGP were introduced into an expression plasmid downstream from the green fluorescent protein (GFP) ORF and under the control of the cytomegalovirus (CMV) promoter. The resulting fusion protein contains GFP at the N-terminus moiety. The DNA constructs where delivered by microinjection into 1-4 cell stage embryos of the zebrafish *Danio rerio*. The embryos were allowed to develop normally and developmental appearance of GFP-MGP and GFP-BGP was followed by fluorescence microscopy. MGP-GFP and BGP-GFP were found to be associated to mineralized structures from the time of formation of these structures. Mineralized skeletal structures formed up to 10 days after fertilization (DAF) remained fluorescent up to 30 DAF while no detectable skeletal alterations where observed in transgenic larvae. This data represents a first approach towards the development of a transgenic line of zebra fish expressing GFP-Gla proteins as markers for skeletal structures and emphasizes the usefulness of this model system to further analyze in vivo skeletal malformations. PG and SM supported by a doctoral and postdoctoral fellowship (PRAXIS/BD/19665/1999 SFRH/BPD/9403/2002)

PB.10

***MUC2* mucin gene regulation in gastric cells**

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Normal gastric mucosa shows little or no expression of the intestinal mucin MUC2. In intestinal metaplasia, a preneoplastic lesion of the stomach characterized by the transdifferentiation of the gastric mucosa to an intestinal phenotype, there are alterations in the mucin expression pattern including *de novo* expression of MUC2, mostly in goblet cells. Thirty percent of gastric carcinomas, including all carcinomas of the mucinous type, also aberrantly express MUC2 intestinal mucin.

- In order to understand the mechanisms underlying the absence of MUC2 expression in normal stomach and its *de novo* expression in intestinal metaplasia and gastric carcinoma we analyzed the methylation levels of nine CpG sites of *MUC2* promoter in mucinous gastric carcinomas and in normal gastric mucosa. Our results suggested that *MUC2* expression in gastric cells is regulated by promoter methylation and further indicated that two specific CpG sites could play a particularly important regulatory role.

- Recently we have also demonstrated direct involvement of the Cdx-2 homeodomain protein in the transcriptional regulation of the *MUC2* gene in gastric carcinoma cells. We observed a higher transactivation effect of Cdx-2 on the *MUC2* promoter construct comprising nucleotides -947 to +27, which was shown to be essential for transcriptional regulation of *MUC2* in gastric carcinoma cell lines.

PB.11

Cloning, characterization and tissue distribution of androgen receptor in seabream, *Sparus aurata* (Teleostei, Perciformes)

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Androgens play important roles in the male reproductive system, particularly in sexual differentiation, maturation and spermatogenesis. Androgens effects are mediated by an androgen receptor (AR), which is expressed in target tissues, acting as a transcription factor regulating the expression of target genes. Although androgens function as a masculization agent in several fish species, its function in seabream reproduction, namely sex reversal, has not been studied. As a step to understand the molecular basis of these processes, we have cloned a seabream AR (sbAR) cDNA. The isolated clone encompassing most of the A/B domain, domains C to E and the entire C-terminal untranslated region, showed highest homologies to other fish ARs. The highest mRNA expression was observed in head kidney and testis. This pattern of tissue distribution is in agreement with the known androgen functions in teleosts. However moderate expression was also detected in the liver. Considering that a role for androgens on vitellogenesis has been suggested1, we have analysed the expression of sbAR, together with estrogen receptors and vitellogenin in response to the fish androgen 11-ketotestosterone. The results indicate that androgen treatment modulates expression of seabream AR and ERα but seems to have no effect on the expression of ERβ. 1) Kim, BH. et al. 2003. Gen. Comp. Endocrinol. 132: 248-255

PB.12

Cloning of matrix Gla protein in a marine cartilaginous fish, *Prionace glauca* : identification of sites of mRNA and protein accumulation at single cell resolution.

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Matrix Gla Protein (MGP) belongs to the family of vitamin K dependent Gla containing protein and, in mammals, birds and Xenopus, its mRNA has been previously detected in bone, cartilage and soft tissue extracts, while the accumulation of the protein was found mainly in bone and calcified tissues. More recently MGP expression was also studied in marine teleosts were it was found associated with chondrocytes and vascular smooth muscle and endothelial cells. MGP as been shown to be a an inhibitor of arterial wall and cartilage calcification. To date no information is available on sites of MGP expression or accumulation in cartilaginous fishes, that diverged from mammals over 400 million years ago. In this work we study the sites of gene expression and protein accumulation by means of in situ hybridization and immunohistochemistry, respectively. MGP gene expression and protein accumulation were localized in cartilage from branchial arches and vertebra. Moreover, MGP gene expression and protein accumulation were detected in the endothelia of vascular system as well as in the tubular renal endothelium. Finally our results corroborate the hypothesis that in *P. glauca*, as previously shown in mammals, MGP protein probably also acts as a calcification inhibitor, protecting soft tissues from abnormal and ectopic calcification. This supported by a postdoctoral fellowship (SFRH/BPD/7151/2001)

PB.13

Differential expression and contrasting roles of two oxalate decarboxylases during spore development in *Bacillus subtilis*.

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In Bacillus subtilis, the spore coat is formed by the ordered synthesis and assembly of several polypeptides whose synthesis is orchestrated by a cascade of mother cell-specific regulators. OxdD, for instance, is an oxalate decarboxylase produced under the control of SigK, and is recruited to the inner layers of the spore coat. B. subtilis produces a second oxalate decarboxylase, OxdC, during growth under acid conditions, but not during sporulation. We show that oxdC is co-transcribed with a downstream gene, yvrL, for a membrane protein of 12 kDa. The separation of oxdC from yvrL or the inactivation of yvrL, results in constitutive expression of oxdC. In contrast, the ectopic expression of an yvrL allele from an inducible promoter represses transcription of an oxdC-lacZ fusion. We conclude that yvrL is a repressor of oxdC. In an yvrL mutant, an OxdC-GFP fusion accumulates during sporulation, but does not localize around the developing spore. Expression of oxdC from the sporulation-specific oxdD promoter also does not result in the assembly of OxdC. Irrespective of its association with the spore, OxdC has a role in spore coat assembly, as an oxdC mutant forms spores with an aberrant coat. Surprinsingly, deletion of oxdD, which per se does not affects the coat composition, restores normal coat assembly to an oxdC mutant. The two proteins are highly similar, but have strikingly different surface potentials. We speculate that this feature contributes to the different roles of OxdC and OxdD in spore morphogenesis.

PB.14

Hormonal regulation of Matrix Gla Protein expression in a *Xenopus laevis* bone-derived cell culture

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Matrix Gla protein (MGP) is a small protein implicated in the regulation of extracellular matrix calcification in higher vertebrates. In mammals expression of the MGP gene is regulated by steroid hormones such as retinoic acid and 1,25-dihydroxyvitamin D3, as well as by growth factors. We have obtained bone derived cell line from *Xenopus laevis* (X1 cells), capable of expressing basal levels of MGP at least 50 fold higher than the established *Xenopus* A6 cell line, as seen by transcription assays with the intact MGP promoter linked to the luciferase reporter gene. We characterized X1 cells through determination of number of chromosomes, *in vitro* mineralization, pattern of secreted proteins and presence of the receptoes for 1,25-dihydroxyvitamin D3 and retinoic acid. We found that MGP mRNA levels are increased by approximately 4-fold after 72 hours of treatment with 1μM retinoic acid and that this effect is probably mediated through the retinoic acid receptor beta isoform (RAR-β) nuclear factor, since it was the only retinoic acid receptor detected by RT-PCR on X1 cells. This result is similar to those obtained for human bone derived cells, suggesting a common pattern of retinoic acid modulation of MGP gene expression for both species.
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PB.15

Excess light stress triggers an antioxidative response upon transfer of in vitro grapevine to ex vitro.

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Environmental stresses, such as light in excess, can exert at least part of their effect through oxidative damage. To understand how the antioxidant defence system is able to overcome photooxidative stress is of considerable interest when working with in vitro propagated plants. In fact, after transfer to ex vitro, these plants are exposed to irradiances several fold higher than in vitro, leading to photooxidative stress. In this work we focus on the response of the ascorbate-glutathione cycle in grapevine plantlets during the first seven days after transfer to ex vitro. For that purpose we have quantified the levels of involved metabolites (hydrogen peroxide; reduced/oxidized ascorbate; reduced/oxidized glutathione) and the total activities of antioxidative enzymes (catalase, superoxide dismutase, ascorbate peroxidase, monodehydroascorbate reductase, dehydroascorbate reductase and glutathione reductase). We have also evaluated the activity of some of those enzymes by activity staining after native PAGE. We present evidence that light in excess triggers an antioxidative response, showing a peak 2 days after transfer to ex vitro and a second one at 5 or 6 days. After each peak, enzyme activities return to lower, steady values. From the results obtained with the present plant model system we will discuss its suitability for dissecting the molecular effect of photooxidative stress as well as its antioxidant efficiency.

PB.16

Regulation of matrix Gla protein gene expression in a fish bone-derived cell line

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Matrix Gla protein (MGP) is an extracellular matrix protein secreted by chondrocytes (bone and cartilage) and vascular smooth muscle cells, and is considered to be a physiological inhibitor of tissue mineralization. In vitro studies have demonstrated that MGP gene expression is highly regulated in mammals, but no data is available for other vertebrates. In the present work, we have studied regulation of *Sparus aurata* MGP gene expression by vitamins (C, K or D), growth factors (FGF, TGF, EGF or VEGF), extracellular calcium (Caext), inorganic phosphate (Pi), retinoic acid (RA), insulin (Ins), b-estradiol (E2) and dexamethasone (Dex) using a bone-derived cell line, VSa13, recently developed in our laboratory (Pombinho et al. 2004. Cell Tissue Res 315:393). MGP gene expression was (1) strongly down-regulated in cells treated with 1 mM of RA or 100 nM of Dex, (2) was slightly up-regulated in cells treated with 2 nM of Ins or 10 nM of E2, (3) was more strongly up-regulated in cells treated with 50 mg/ml of vit. C, 10 nM of vit. D, 50 mg/ml of vit. K or 50 ng/ml of FGF, and (4) unchanged in cells treated with TGF, EGF, VEGF, Caext or Pi. The stimulating effect of vit. C on MGP gene expression was slightly increased in presence of Caext or Pi and strongly increased when both Caext and Pi were present. Some of these results are in contradiction with those obtained in higher vertebrates but could be explained either by a different cell type or degree of cell differentiation, or by different regulatory mechanisms in lower vertebrates. Constructions containing MGP promoter fragments upstream of Luciferase reporter gene are currently being used to validate these results and identify regulatory regions.
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