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# Fitness difference between cryptic salinity-related phenotypes of sea bass (*Dicentrarchus labrax*)

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**Summary**: The existence of cryptic salinity-related phenotypes has been hypothesized in the "euryhaline" sea bass (*Dicentrarchus labrax*). How differential osmoregulation costs between freshwater and saltwater environments affect fitness and phenotypic variation is misunderstood in this species. During an experiment lasting around five months, we investigated changes in the whole body mass and in the expression of growth-related genes (insulin-like growth factor 1 [IGF-1]; growth hormone receptor [GHR]) in the intestine and the liver of sea bass thriving in sea water (SSW), successfully acclimated to freshwater (SFW), and unsuccessfully acclimated to freshwater (UFW). Albeit non-significant, a trend toward change in body mass was demonstrated among SSW, UFW and SFW fish, suggesting that SSW fish were a mixture of the other phenotypes. Several mortality peaks were observed during the experiment, with batches of UFW fish showing higher expression in the osmoregulatory intestine due to down-regulation of genes in the liver and significant up-regulation of GHR in the intestine compared with SFW fish. Energy investment toward growth or ion homeostasis hence partly mediates the fitness difference between cryptic SFW and UFW phenotypes. The use of a genetic marker located within the IGF-1 gene showed no genotype-phenotype relationship with levels of gene expression.

**Keywords**: phenotype; gene expression; growth hormone receptor; insulin-like growth factor 1; sea bass.

**Diferencia de eficacia biológica entre fenotipos crípticos relacionados con salinidad en la lubina (*Dicentrarchus labrax*)**

**Resumen**: En la especie "eurihalina" de la lubina (*Dicentrarchus labrax*) se ha planteado la existencia de fenotipos crípticos relacionados con la salinidad. En esta especie los costes diferenciales de osmoregulación a la adaptación en de agua dulce y salada son aún desconocidos. Durante un experimento de aproximadamente 5 meses, se investigó los cambios en la masa corporal y en la expresión de genes relacionados con el crecimiento (factor de crecimiento similar a la insulina 1 [IGF-1]; receptor de la hormona del crecimiento [GHR]) en el intestino y el hígado de lubina en individuos que prosperan en agua de mar (SSW), individuos aclimatados con éxito con el agua dulce (SFW), e individuos no aclimatados al agua dulce (UFW). Aunque no es significativa, se observa una tendencia de cambio en la masa corporal entre individuos SSW, UFW y SFW. Estos resultados sugieren que los individuos SSW son una mezcla de los otros fenotipos. Se observaron varios picos de mortalidad durante el experimento, con lotes de peces UFW que presentan una expresión génica más elevada en el intestino osmoregulador, debido a la regulación a la baja de genes en el hígado y regulación hacia arriba en la GHR del intestino cunado se compara con los peces SFW. Por lo tanto, la inversión de energía hacia el crecimiento o la homeostasis iónica explica en parte la diferencia de adaptación entre los crípticos fenotipos SFW y UFW. El uso de un marcador genético localizado dentro del gen de IGF-1 no demuestra relación genotipo-fenotipo con los niveles de expresión génica.

**Palabras clave**: fenotipo; expression de genes; receptor de la hormona del crecimiento; factor de crecimiento similar a la insulina 1; lubina.

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## INTRODUCTION

Marine species often display phenotypic diversity to acclimate or to adapt to their local environment. Phenotypic variation within species can especially drive patterns in the distribution, abundance, and ecological roles of organisms (Pfennig et al. 2010, Sotka 2012). At the intraspecific level, phenotypic variation as illustrated by the existence of different morphs, developmental pathways, life-history strategies or behaviours is thought to be adaptive and to generally translate into better phenotype-environment matching in response to fluctuating environmental conditions (e.g. Van Valen 1965, Ghalambor et al. 2007, but see Marshall et al. 2010). However, the assessment of phenotypic diversity relies on situations in which different and discrete phenotypes are easily observed or situations in which phenotypes are cryptic. In the latter case, the array of phenotypes is obviously underestimated and can obscure interpretation of performance and fitness of individuals in distinct habitats. When an environmental or stress-induced stimulus is applied, one formerly cryptic phenotype may react to the stimulus and become defined by other attributes, increasing its fitness. This phenotypic switch may occur only after stress has accumulated over individuals until reaching the level inducing the stress response, i.e. the time at which previously cryptic phenotypes are uncovered (Hoffmann and Parsons 1991, Gabriel et al. 2005). Delayed response depends on environmental tolerance, on stress intensity, and for how long cryptic phenotypes are submitted to the stressor environment to unravel performance/fitness differences in each phenotype, rather than simple variation reflecting short-term acclimation response (Palaima 2007).

The distributional range of euryhaline European sea bass (*Dicentrarchus labrax*) extends from Mauritania to Norway and the Mediterranean Sea. It is an economically important fish that naturally inhabits marine, lagoon and estuarine environments (e.g. Dufour et al. 2009, Vasconcelos et al. 2010). Only minor meristic and morphological phenotypic differences necessitating detailed studies to be identified exist in sea bass, and their interaction with fitness is unknown (Barnabé 1973, Corti et al. 1996, Loy et al. 1999, Bahri-Sfar and Ben Hassine 2009, Costa et al. 2010). Concurrently, sea bass has repeatedly demonstrated different capabilities to acclimate freshwater (FW) in both experimental and natural conditions (Chervinski 1974, Dendrinos and Thorpe 1985, Cataudella et al. 1991, Venturini et al. 1992, Allegrucci et al. 1994, Marino et al. 1994, Jensen et al. 1998, Eroldogan and Kumlu 2002, Varsamos et al. 2002, Nebel et al. 2005, Boutet et al. 2007, Giffard-Mena et al. 2008, Dufour et al. 2009). Sea bass could then be a mixture of cryptic phenotypes with distinct environmental tolerance and fitness regarding salinity, rather than a single unconditional, plastic, euryhaline phenotype as traditionally reported in textbooks (Pickett and Pawson 1994, Sánchez Vázquez and Muñoz-Cueto 2014). Despite numerous reports of differential sea bass mortality when facing FW, observations were often a posteriori interpretations of experiments with very diverse objectives, and not studies dedicated to understanding fitness differences among individuals or phenotypes. The dynamics and the root of fitness difference have been very poorly assessed in sea bass, despite recent studies reporting histological observations (Nebel et al. 2005), or variation in patterns of gene expression that differ among juvenile sea bass successfully or unsuccessfully adapted to FW (Boutet et al. 2007, Giffard-Mena et al. 2008).

Indeed, fitness differences may have roots in the differential expression of the genes and variation in patterns of gene expression represents itself an expression of phenotypic variation (Larsen et al. 2011). Fitness differences also emerge from competing demands that forces organisms like fish to adjust their metabolism to environmental conditions without compromising homeostasis and energetic budgets (Guderley and Pörtner 2010). Growth and body mass are major fitness-related traits (Roff 1992). The control of growth involves a multifaceted system of regulation, using cellular controls that are modulated by the various endocrine signals of the growth hormone-insulin-like growth factor 1 (GH–IGF-I) axis (Reinecke 2010, Reindl and Sheridan 2012). Indeed, though they are not the only hormones involved in the control of growth, GH and IGF-1 interact together in a complex manner, likely mediated through binding of GH to the GH receptor (GHR) (Wood et al. 2005). In the liver, this association induces the expression of target genes, including IGF-I, which is responsible for most of the growth effects of GH (Wood et al. 2005, Reinecke 2010). Aside from its role in somatic growth, the GH–IGF-I axis also plays a role in osmoregulation, mediating a wide range of cellular, tissue and physiological adjustments in fish (Duan 1997, Reinecke 2010). As osmoregulation is an energy-demanding process, it naturally competes with growth (Bœuf and Payan 2001), and especially with the roles of GHR and mostly IGF-I in growth (Duan 1997, Moriyama et al. 2000, Calduch-Giner et al. 2003, Côté et al. 2007). How IGF-I simultaneously acts on osmoregulatory potential and growth is poorly understood in fish because studies have primarily concentrated on extrahepatic IGF-I expression in tissues sensitive to nutritional status (muscle; e.g. Montserrat et al. 2007, Fox et al. 2010), rather than concentrating on tissues that may modulate this status (e.g. intestine). It then appears that contrasting gene expression of growth-related genes in an essential relay to growth such as the liver and an osmoregulatory organ such as the intestine could provide a snapshot of energy investment toward growth and ion homeostasis.

The aim of this study was to investigate whether juveniles of the euryhaline European sea bass may be composed of a mixture of cryptic phenotypes exhibiting different physiological and fitness costs during long-term FW stress. Phenotypes were tested by analysing levels of expression for the GHR and IGF-1 genes in the liver and intestine of juvenile individuals in SW and FW conditions over a long-term experiment (~5 months) allowing stress to accumulate in individuals. In parallel, changes in body mass taken as a proxy of fitness were also investigated.

## MATERIALS AND METHODS

### Fish rearing conditions and treatments

In June 2008, young juveniles (n=800; body mass 14.8±2.0 g; age: approx. six months) of *D. labrax* issued from a mass spawning protocol were obtained from the Écloserie Marine de Gravelines (France). Fish were randomly divided in eight groups of size n=100 and reared in eight 0.5-$m^3$ tanks at the Station Méditerranéenne de l'Environnement Littoral in Sète (France; 43°23'33"N, 3°39'51"E). Fish were randomly assigned to two treatments: (i) fish maintained in SW (37‰, filtered seawater from the neighbouring Thau lagoon; four tanks each seeded with n=100 fish); and (ii) fish acclimated to FW (0.5‰; four tanks seeded with n=100 fish). Following Nebel et al. (2005), fish were progressively acclimated to FW during a two-week period with an increment of 2‰ every day. They were reared at prevailing seasonal photoperiod and temperatures (range: 20 to 27.5°C) over the study period (early June to late October 2008), and were fed with commercial pellets according to Varsamos et al. (2006).

### Sampling

Fish that survived in the SW and the FW treatments will be designed as successful seawater (SSW) and successful freshwater (SFW), respectively. At the end of the experiment (October 23, 2008), 20 SSW and 20 SFW were randomly sampled weighed, killed, dissected and organs (liver, and approx. 1.5 cm of intestine after the last pyloric cæcum) were stored at −80°C in 1 ml RNA Later® (Ambion, Austin, TX, USA). Pieces of fin and/or white muscle were collected for each individual and stored in 90% ethanol for DNA analyses of SFW and SSW individuals. As in Nebel et al. (2005), sea bass that demonstrated abnormal behaviour (e.g., swimming out of the shoal, with little or no response to external disturbance) during the freshwater acclimation were categorized as unsuccessfully adapted to freshwater (UFW). They began to appear ~10 days after exposure to full FW (Nebel et al. 2005, Boutet et al. 2007). It has been demonstrated that such individuals generally die within 48 h after such behaviours appear (Nebel et al. 2005). This abnormal behaviour was not recorded for sea bass maintained in SW, and a fourth category of fish could not be investigated. We therefore randomly sub-sampled UFW individuals found dying over the course of the experiment (n=30; with ten fish sampled around July 1, August 27 and October 23, 2008, respectively; see below for justification) for the gene expression study. Due to the low sample size considered in each sampling period, testing for a tank effect is not feasible and results in low statistical power. Individuals found as already dead were never sampled for tissues. UFW fish were immediately dissected. The liver and anterior intestine were extracted and stored at −80°C in 1ml of RNA Later® for further molecular analysis. Pieces of fin were also collected for the 30 UFW individuals and stored in 90% ethanol for DNA analyses. A total of 70 fishes were then included in molecular analyses. Because of the bad conservation of tissues for UFW individuals sampled on ca. July 16, none of the individuals sampled at this date was included in the gene expression analysis.

The variation in body mass of SW, FW and UFW fish was monitored at three times during the course of the experiment (July 1, July 16; August 27; note that SW and FW fish cannot be qualified as SSW of SFW on those dates, as those acronyms are valid only for fish that survived at the end of the experiment), and at the end of the experiment (October 23). Thirty SW and FW fish per group were sampled on the first three dates; then 20 SFW and SFW individuals also used for the gene expression analysis were collected at the end of the experiment to investigate changes in body mass. SW and FW fish sampled on intermediate dates were not sacrificed. The first date (July 1) was retained as it matched the estimated date when the first UFW fish may appear (i.e. the two-week acclimation period followed by ~10 days after exposure to full FW; Nebel et al. 2005). Other dates in July and August corresponded to dates on which higher rates of appearance of UFW fish (i.e. mortality peaks) were recorded during the experiment (not shown). For those dates, we grouped UFW individuals that were found dying three days before or after those dates and performed tests to check for significant body mass differences among UFW, SW, and FW fish during the experiment. The duration of three days was retained as it allowed a sufficient number of UFW individuals in each sample for reliable testing. The last UFW fish labelled as 'October 23' were fish that were collected over the ten days before this date as no mortality peak was observed over this period. Despite its ad hoc status, this sampling was the only way to obtain an estimate of body mass of UFW fish at the end of the experiment. Mortality peaks decreased sea bass density and, accordingly, food input was proportionally decreased in FW tanks to be kept roughly constant over the course of the experiment. Tanks were regularly cleaned for non-ingested food. However, regular control of feed intake was not feasible in this study. UFW fish randomly retained for body mass monitoring were not all included in the study of gene expression.

### Total RNA extraction

Total RNA was extracted from liver and anterior intestine tissues from all samples collected in sea bass using TRIzol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions; the phase preparation step was done twice in the intestine tissue to remove excess lipids. A treatment with DNAseI (Invitrogen) was applied to all RNA samples to prevent genomic DNA (gDNA) contamination. The DNase was further removed by phenol chloroform extraction. The quantity and quality ($A_{260/230}$ and $A_{260/280}$) of total RNA were determined using a Nanodrop spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). Only RNA samples with $A_{260/280}$ ratios above 1.6 and $A_{260/230}$ ratios above 1.8 indicating minimal protein contaminants were used for further analysis.

Table 1. – Specific primers used for real-time PCR analyses (S, sense strand; AS, antisense strand).

| Gene | | Sequence |
|---|---|---|
| IGF-1 | S | 5'-ACCTAAGGTTAGTACCGCAG-3' |
| | A | 5'-CTGATGCACTTCCTTGAAGG-3' |
| GHR | S | 5'-ACAACAGGAAAAGTTGATGG-3' |
| | A | 5'-GTTGTTGTACAGCTCTGGC-3' |
| EF1α | S | 5'-AGGTCAATCTGTGGAGATG-3' |
| | A | 5'-TTCAGGATGATGACCTGGGC-3' |

## Reverse transcription and quantitative real-time polymerase chain reaction (qPCR)

In each tissue, we looked at the expression of GHR and IGF-1 by first generating their cDNAs with the Protoscript® II RT-PCR kit (NE BioLabs® Inc, Ipswish, MA, USA) according to the manufacturer's recommendations. Expression of GH was not investigated as it is only expressed in the pituitary, and variation of GH gene expression was formerly studied in Varsamos et al. (2006). Primers for qPCR (Bustin et al. 2009) are reported in Table 1, including primers for elongation factor-1 (EF1α) retained as reference gene in this study (rationale below). Gene amplifications were carried out with a LightCycler®480 (Roche Applied Science, Mannheim, Germany), using the PCR kit suggested by the manufacturer (LightCycler®480 SYBR Green I Master; Roche Applied Science; Mannheim Germany). In this study, all qPCRs, including reference genes (see below), were performed using three technical triplicates per individual in order to assess the intra-individual variability of gene expression, and then the reproducibility of individual gene expression. EF1α was preliminarily retained as a reference gene as it had been previously used in studies investigating gene expression variation in response to salinity in sea bass (e.g. Varsamos et al. 2006, Boutet et al. 2007, Giffard-Mena et al. 2008). Another reference gene traditionally used in salinity-based studies of gene expression in fish was also screened (β-actin; e.g. Tine et al. 2008). In order to retain a reference gene, we adopted a criterion developed in Avarre et al. (2014). Briefly, we randomly sampled a set of 15 fish (five fish per condition: SSW, SFW and UFW) and measured their cycle of quantification value (Cq, i.e. the cycle at which fluorescence from amplification exceeds the background fluorescence) with the EF1α and β-actin genes. We used the NormFinder software (Andersen et al. 2004) to investigate the repeatability (i.e. stability of gene expression) of their Cq values by measuring the standard deviations of Cq ($SD_{Cq}$) over the 15 individuals (EF1α: $SD_{Cq}$=0.337; β-actin: $SD_{Cq}$=0.646). Avarre et al. (2014) set up a criterion of $SD_{Cq}$<0.5 to qualify genes as 'good' reference genes. We did not use other reference genes in this study as there is no rationale to increase the number reference genes when investigating for gene expression variation at only two target genes. Hence, results are hereby presented for EF1α, which recovered good efficiencies (97.5%) and higher repeatability of Cq values.

Primers used for IGF-1 were designed according to the full genomic sequence of IGF-1 provided by Quéré et al. (2010) (Genbank accession number: GQ924783). Note that IGF-1 primers used in this study were preferred to IGF-1 primers used in former sea bass gene expression studies (Genbank accession: AY800248; e.g. Varsamos et al. 2006, Terova et al. 2007, Mazurais et al. 2008), as they retrieved, e.g., better repeatability of $C_q$ values and efficiencies (details not reported). GHR expression was not previously investigated in sea bass. Two transcripts (Genbank accessions: AF438177 and AY642116) were available in databases as GHR1 and GHR2, respectively. Fukamachi and Meyer (2007) demonstrated that sequences available in databases and reported as GHR (1 or 2) could be somatolactin receptors (SLR). We performed alignments of sequences, and unambiguously linked the sea bass GHR sequence to the transcript with accession AY642116 (GHR2, the alternate transcript being SLR; details not shown). This transcript was then used to design GHR primers (Table 1). Gene amplifications were also carried out with a LightCycler®480. The amplification of each sample was performed in a total final volume of 5 µl (2 µl cDNA diluted to 1/4, 0.25 µl of each primer [10 µM], and 2.5 µl of buffer 2X SYBR Green I Master). The PCR was done in triplicate for each individual with a denaturing cycle of 95°C/10 min, followed by 45 (50 for GHR) cycles of 95°C/10 s, 63°C (58°C for GHR)/15 s (10 s for GHR), 72°C/10 s (8 s for GHR). For each gene, a number of previous trials were carried out in order to determine their Cq value, while remaining within the linear PCR amplification limits. Agarose gel electrophoresis was performed to verify amplicon size and absence of primer-dimers, and to check the absence of cross-amplification of SLR (GHR1) (results not shown). Amplification products were also checked for the shape of their melting curves. In addition, to determine the qPCR efficiencies (E) of each primer pair used, standard curves were generated using five serial dilutions (1/2, 1/4, 1/8, 1/16, 1/32). We also evaluated the intra-assay variability of triplicate qPCRs at different transcript levels (no significant within-individual differences were detected; not shown). Negative controls (i.e. reverse transcriptase and RNA free samples) were also included to assess the reliability of results.

## Statistical analyses of gene expression data

The relative expression of both GHR and IGF-1 normalized to the retained reference gene was assessed following a method presented by Pfaffl (2001). Relative expression (RE) is determined using the equation:

$$RE = [(E_{target})^{\Delta Cqtarget\,(control\text{-}sample)}] / [(E_{ref})^{\Delta Cqref\,(control\text{-}sample)}]$$

where $E_{target}$ is the amplification efficiency of the target (i.e. gene of interest) and $E_{ref}$ is the amplification efficiency of EF1α. The corresponding qPCR efficiency of one cycle in the exponential phase of amplification was calculated according to the equation $E = 10^{[-1/slope]}$ (Pfaffl 2001). The qPCR assays were optimized with linear standard curve with $R^2 \geq 0.98$ (EF1α: $R^2$=0.99; GHR: $R^2$=0.98; IGF-1: $R^2$=0.99). A Kruskal-Wallis test was first used to compare gene expression differences between the three samples of UFW individuals

sampled on July 1, August 27 and October 23 in each organ. Then, parametric analysis of variance was used to compare mean levels of gene expression among fish categories (SSW, SFW, UFW) within each organ. Analyses were performed with the R software (v2.8.1; www.r-project.org). Post-hoc corrections for multiple tests were applied when necessary.

### Genotype-phenotype relationship

Quéré et al. (2010) developed a microsatellite marker labelled as µIGF-1 and located in the IGF-1 gene in sea bass. Polymorphism of all SSW, SFW and UFW individuals was screened at locus µIGF-1 according to PCR protocol and primer reported by these authors. Amplifications were performed on a PTC-200 (MJ Research). Genotyping of individuals was performed by allele sizing on an ABI PRISM® 3130xl Genetic Analyser (Life Technologies, St Aubin, France), using 5'-labelled reverse primers and the GeneScan™ 600 LIZ® Internal Line Standard (Life Technologies) as internal size standard. Allele scoring was performed using the GeneMapper software v.4.0 (Life Technologies). Genetic differentiation at locus µIGF-1 among SSW, SFW and UFW individuals was estimated using $F_{ST}$ (Weir and Cockerham 1984), and significance tested by permutation (1000 replicates) in Genetix v4.05 (kimura.univ-montp2.fr). For each organ, we further investigated the genotype-phenotype relationship of IGF-1 gene expression variation using a nested analysis of variance (ANOVA) with mixed effects in which SSW, SFW and UFW fish were used as the main factor (treatment) and genotypic classes as the nested factor. Individuals were pooled in fixed genotypic classes according to size of their alleles to get sufficient number of individuals in each genotypic class. The procedure to pool genotypes is described in the 'Results' section. It was based on allele size as, for microsatellite and other types of loci linked to functional candidate genes (e.g. IGF-1), gene expression variation was found to correlate with the respective size of the alleles (Gemayel et al. 2010; for fish see, e.g. Streelman and Kocher 2002). Analyses were performed with the R software v2.8.1.

## RESULTS

### Survival and body mass differences

The mean mortality rate of fish that stayed in SW was 22.25% [range: 13-32% over the four SW tanks] while that of fish that were submitted to FW was 53.25% [range: 39-76% over the four FW tanks]. Ranges do not overlap, and differences in mean survival rates between the SW and FW experiments was therefore *ca.* 30%, a gross estimate of UFW fish present in our study. However, variability of survival rates among SW tanks was similar to the difference among treatments, possibly indicating a batch effect due to undetected reasons such as sub-optimal husbandry. After five months of acclimation, the mean (±SD) masses of the two final experimental groups had more than tripled, being 45.15±9.42 g and 53.82±12.60 g in SSW and SFW, respectively (t-test: P=0.41). Hence, no significant cost to FW was demonstrated between groups of surviving sea bass in this study.

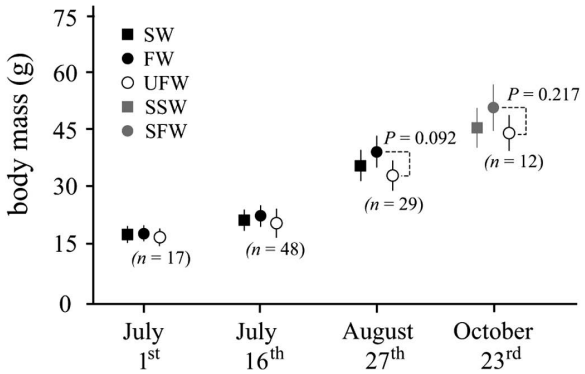


Fig. 1. – Evolution of mean body mass (±SE) of UFW sea bass (*Dicentrarchus labrax*) compared with the body mass of 30 SW and FW fish (July 1, July 16, August 27), or 20 SSW and SFW fish (October 23) (see text for details). Samples of UFW fish represent pools of individuals that demonstrated signs of abnormal behaviour three days before and after those dates, except for the final date October 23, with UFW individuals collected over the 10 days before this date because no mortality peak was observed at that time. Sample sizes of UFW pools are indicated in brackets. No significant differences in body mass were found among groups of fish at each date. Only the lowest observed p-values recorded between UFW fish and fish sampled in FW during the last two surveys are indicated. Note that (S)SW fish always have an intermediate average body mass compared with other fish categories, suggesting that they could be a mixture of cryptic phenotypes.

Similarly, UFW did not demonstrate significantly lower body mass than fish sampled in FW on the four dates used for this study (Fig. 1). While not significant (lowest observed p-value: P=0.092 on August 27), the mean difference in body mass increased during the experiment, indicating a trend toward impaired growth in UFW compared with SFW fish (Fig. 1). Individuals that stayed in seawater showed an intermediate body mass to SFW and UFW on each date (Fig. 1), suggesting they could be a mixture of other phenotypes.

### Gene expression variation

The Kruskal-Wallis test revealed no significant changes for gene expression of GHR and IGF-1 among sub-samples of UFW individuals taken on July 1, August 27 and October 23 (all P's>0.05; 2 df; details not shown). This suggested that identical changes in gene expression occurred on several occasions during development. To increase statistical power and for comparison with gene expression of GHR and IGF-1 in SFW and SSW fish sampled on October 23, individual gene expression data of the three UFW sub-samples (each n=10) were gathered in a single sample (n=30) for each organ. The Kolmogorov-Smirnov (K-S) tests were previously performed to check the normality of gene expression data in UFW, SSW (n=20), and SFW (n=20) for each gene and each organ. K-S tests were not significant except for the expression of GHR in liver of SSW fish (P<0.01). Despite this observation, gene expression variation was then analysed using a parametric set-up.

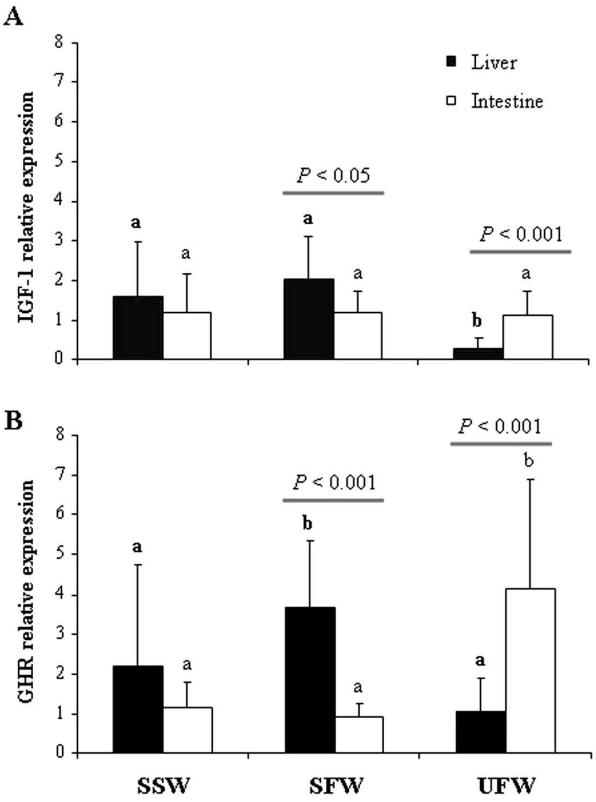


Fig. 2. – Real-time PCR analysis of mRNA expression of (A) IGF-1 and (B) GHR in liver and intestine of European sea bass (*Dicentrarchus labrax*) for SSW (n=20), SFW (n= 0) and UFW (n=30). Each value represents means + SD. Significant differences in gene expression among tissues within each group of fishes are indicated by a horizontal grey line with the significance level of each test. When tests between tissues were not found significant, this line is not reported (SSW). Different letters indicate significant differences in gene expression among the three groups of fishes. For each gene, different boldface characters report significance among SSW, SFW and UFW for liver, whereas standard characters are for the intestine (P<0.001 in each case, except for GHR in liver: P<0.05).

Results pertaining to the gene expression of both IGF-1 and GHR in the two tissues after pooling of UFW sub-samples are reported in Figure 2. Use of triplicates reported no significant intra-individual differences in gene expression for each gene, whatever the category of fish (SSW, SFW and UFW) or organ (details not shown). Measurements of gene expression were therefore reproducible for each individual.

IGF-1 - There was expression of IGF-1 in both the liver and the intestine (Fig. 2). Furthermore, fishes from the FW exposure expressed IGF-1 differently in the liver and in the intestine. On the one hand, SFW had significantly greater expression (mean±SD) in the liver than in the intestine (2.04±1.07 and 1.17±0.55, respectively; P<0.05), whereas the inverse relationship was found in UFW, with lower expression levels in liver than in the anterior intestine (0.29±0.25 and 1.12±0.59, respectively; P<0.001). SSW individuals showed no significant difference in IGF-1 expression between liver and intestine (1.60±1.36 and 1.20±0.95, respectively; not significant [NS]). No significant difference was found in intestinal IGF-1 expression among groups of fish.

For liver, a significantly lower expression of IGF-1 was detected in UFW individuals than in SSW and SFW individuals (P<0.001 in each case).

GHR - No significant difference in GHR gene expression (mean±SD) was recorded between intestine and liver for the SSW individuals (2.18±0.35 and 1.15±1.69, respectively; NS). There was significant over-expression of GHR in the liver of SFW individuals compared with their intestine (3.66±2.56 and 0.92±0.64, respectively; P<0.001). Conversely, the mean relative expression of GHR was significantly lower in the liver than in the intestine of UFW (1.04±0.86 and 4.16±2.72, respectively; P<0.001).

In the liver, there were significant GHR gene expression differences between SFW and UFW individuals (P<0.001) and between SFW and SSW individuals (P<0.05), but not between SSW and UFW individuals. In the intestine, UFW demonstrated higher relative GHR gene expression than the other two groups (P<0.001 in both cases).

## Genotype-phenotype relationship

Six distinct μIGF-1 alleles were found in this study with allele sizes being 232, 240, 246, 248, 254 and 258 base pairs (bp). Allele 240 showed the highest overall allele frequency of the 60 individuals considered in this study (38.25%; details not shown). No significant genetic differentiation was recorded among SSW, SFW, and UFW individuals at locus μIGF-1 (fw=0.007; P=0.617). Individuals were grouped into four distinct genotypic classes with respect to allele size and observed distribution of genotypes: $240/240^{-}$, 240/240, $240/240^{+}$ and $240^{+}/240^{+}$ (i.e. heterozygous individuals with one allele with size <240 bp, homozygous individuals for the 240 bp allele, heterozygous individuals with one allele of size >240 bp, homo- or heterozygous individuals with no 240bp allele, respectively). Taking these genotypic classes into account, no significant genotype-phenotype relationship was found in the nested ANOVA, either in liver (among treatments P=0.022, among genotypic classes P=0.181), or in the intestine (among treatments P=0.612, among genotypic classes P=0.255). As a nested factor, genotypes were not significantly involved in observed gene expression variation in IGF-1. The significant p-value for IGF-1 expression in liver among treatments (SSW, SFW, UFW) refers to the previously mentioned under-expression of IGF-1 in UFW individuals (see above, and Fig. 2).

## DISCUSSION

Hidden phenotypic diversity is undoubtedly present in sea bass and the results challenged the unconditional euryhalinity recognized in this species. Individuals with the SFW phenotype appear to be euryhaline individuals able to withstand large salinity variation, while UFW sea bass can be seen as representing a cryptic phenotype that can be first perceived when the salinity stress is long enough to cause acclimation failure (i.e. ~3 weeks after initiation of the salinity challenge; Nebel

et al. 2005, Giffard-Mena et al. 2008). However, other mortality peaks were observed, and juvenile UFW sea bass were not shown to die during a single mortality episode. These additional mortality peaks were not previously observed in sea bass, because experiments were too short for this observation be reported (~2 months; Nebel et al. 2005, Boutet et al. 2007, Giffard-Mena et al. 2008). Multiple mortality peaks hence question the homogeneity of the UFW phenotype itself. Overall, the mortality rate associated with UFW individuals in this study is ~30%, suggesting that UFW individuals may represent a significant proportion of juvenile sea bass.

How phenotypic variation affect fitness may be studied using the differential expression of the genes (Larsen et al. 2011). It is, however, impossible to establish causal links between fitness and gene regulation with the study of two genes and two organs. Multiple causalities are likely to be at work. In sea bass, Giffard-Mena et al. (2008) demonstrated that gene expression difference of two aquaporin genes (AQP1, AQP3) in gut was probably involved in the death of UFW-like individuals. Boutet et al. (2007) reported a similar observation for relative gene expression of prolactin (PRL) in the gills and to a lesser extent in the intestine of SFW- and UFW-like fish. We hence do not speculate that results reporting variability in gene expression of GHR and IGF-1 summarize the complexity of the regulation of the GH–IGF-1 axis in fish, or other molecular, cellular, histological and physiological mechanisms leading UFW individuals to death. Nevertheless, it is clear that changes in gene expression and gene expression dysfunction may reveal differential selection and fitness differences in fish (Larsen et al. 2011), and that such results have recently been common in sea bass (Nebel et al. 2005, Boutet et al. 2007, Giffard-Mena et al. 2008). As illustrated by Côté et al. (2007) in brook charr (*Salvelinus fontinalis*), changes in the expression of IGF-1 and GHR may provide a snapshot of this complexity and illustrate how, at a molecular level, SFW and UFW fish could manage the essential trade-off between growth and ion homeostasis during osmotic stress as the liver represents an essential relay for growth while the intestine is involved in osmoregulation. This trade-off was not considered in the aforementioned studies on sea bass, which concentrated only on osmoregulatory organs. In this study, SFW individuals preferentially expressed both IGF-1 and GHR in liver, while UFW individuals showed higher expression in the intestine. IGF-1 and GHR expressions are down-regulated in the liver of UFW compared with SFW fish, while GHR is over-expressed in the intestine. The difference in gene expression patterns between SFW and UFW fish may illustrate increased physiological investment toward osmoregulation in UFW compared with SFW. Nevertheless, patterns of GHR expression observed in the study in the gut of SFW and UFW fish are intriguing. Indeed, increase in GHR abundance in osmoregulatory organs was observed when salmonids colonized SW and not the reverse (Sakamoto and Hirano 1991, see also Lerner et al. 2012). The patterns found in UFW (increased GHR expression in FW compared with SW) and SFW (stable GHR expression in FW compared with SW) contradict what is observed in salmonids. This must be investigated further, but, e.g. Bodinier et al. (2009) already reported differences or commonalities in gene expression patterns between sea bass and other euryhaline marine fish species, indicating that gene expression variation might be strongly species-dependent. Studies regarding more genes and organs must be performed to investigate the detailed mechanisms of the physiological imbalance observed in UFW.

It is worth noting that the results were made possible because sea bass were submitted to stress over months. Duration of studies regarding metabolic or gene expression variation in response to an osmoregulatory stress often span from a few days to *ca.* one month (Havird et al. 2013; for specific studies regarding the genes considered in this study see, e.g. Riley et al. 2003, Magdeldin et al. 2007, Link et al. 2010, but see Côté et al. 2007). This observation also holds true for studies searching for a better understanding of the mechanisms promoting euryhalinity (e.g. Scott et al. 2008). Such studies are undoubtedly relevant for deciphering the mechanistic basis of osmoregulation and for studying short-term acclimation performance (Havird et al. 2013). However, these studies poorly illustrate how the long-term fitness of individuals might be affected by a long periods of salinity stress and how gene expression differences translate in adaptive differences. In a rare study monitoring response of individuals to salinity up to five months as performed herein for sea bass, Côté et al. (2007) demonstrated heritable variation of liver gene expression both for IGF-1 and GHR in brook charr. This takes gene expression from an acclimation (short-term) to an adaptive (long-term) context, explaining the success of distinct phenotypes in distinct habitats. Such long-term studies should be promoted.

Concurrently, if differential gene expression was observed between UFW and SFW individuals, no significant osmoregulatory cost between fish was observed in this study, when body mass was used as a proxy. Rubio et al. (2005) and Giffard-Mena et al. (2008) already reported no significant difference in specific growth rate for juvenile sea bass that experienced FW or SW in ~4 and ~1.5 month study, respectively. While non-significant, a trend toward increased growth rate or body mass in freshwater or brackish water similar to the one observed in this study has been demonstrated in several studies in sea bass (e.g. Chervinski 1975, Alliot et al. 1983, Corti et al. 1996, Saillant et al. 2003, Rubio et al. 2005, Giffard-Mena et al. 2008; but see Dendrinos and Thorpe 1985, Conides and Glamuzina 2006). The observation of mortality induced by gene expression dysfunction in the absence of significant body mass response to osmoregulation illustrates the absence of a straightforward correlation between a phenotype monitored at the molecular level (gene expression, which reveals a costly situation and specialist phenotypes regarding response to a salinity challenge), and one monitored at the organismic level (body mass, which instead illustrates a no-cost, generalist phenotype). More generally, assessing the physi-

ological costs of salinity at the organismic level seems difficult in sea bass (Claireaux and Lagardère 1999). It may be suggested that this dichotomy between inferred costs at the molecular and organismic levels is only nascent, and has not yet reached a new physiological equilibrium because of recent divergence between UFW and SFW sea bass phenotypes. This has been suggested for stickleback (*Gasterosteus aculeatus*) colonizing the freshwater and the estuarine environments from the ancestral sea environment (McCairns and Bernatchez 2010).

The existence of intraspecific variation in FW tolerance through two distinct cryptic phenotypes may be linked to alternative life strategies or opportunities to face different environmental challenges in sea bass. The role of variation in osmoregulatory performances in shaping fish distributions is documented at the interspecific level (e.g. Lasserre and Gallis 1975; Plaut 1998; Rigal et al. 2008). Lagoons, estuaries and lower parts of rivers act as nurseries withstanding large salinity variation (Vasconcelos et al. 2010), possibly promoting intraspecific variation in sea bass. Inter-individual variation in habitat use has been shown to favour the emergence of alternative phenotypes in order to increase niche width and/or resilience of populations to environmental impact (Räsänen and Hendry 2008). Purely environmental, epigenetic and genetic mechanisms may be responsible for alternative phenotypes (Gienapp et al. 2008, Angers et al. 2010). In sea bass, several studies have demonstrated a possible genetic basis related to habitat use. Indeed, lagoon individuals were found to be genetically differentiated from individuals inhabiting the buffered open sea environment (Allegrucci et al. 1997, Lemaire et al. 2000). Further studies of other marine, coastal, euryhaline fish species by, e.g., Blel et al. (2010; *Mugil cephalus*), Chaoui et al. (2012; *Sparus aurata*), González-Wangüemert and Pérez-Ruzafa (2012; *Diplodus sargus*) and González-Wangüemert and Vergara-Chen (2014; *Pomatoschistus minutus*) reported similar (but still not fully understood) observations. However, the absence of recognized polymorphic markers in GHR, and the non-significant relationship between gene expression level of IGF-1 and genotypes at the μIGF-1 locus in UFW, SSW and SFW individuals does not allow us to go any further with regard to a possible genotype-phenotype relationship in sea bass. However, targeted genome scans must be performed in sea bass, following, e.g. the study by Shikano et al. (2010) on nine-spined stickleback (*Pungitius pungitius*) inhabiting contrasted freshwater and marine habitats.

The molecular basis of freshwater tolerance/adaptation must be investigated further in sea bass. Indeed, many gene networks may have shifted their expression profiles. The observed changes in IGF-1 and GHR may be part of such failures, downstream effects or parallel effects and not necessarily a direct response to low salinity or the cause of the observed deaths. The genomic resources now available for sea bass (Kuhl et al. 2010; Magnanou et al. 2014) and dedicated molecular techniques such as microarrays (Ferraresso et al. 2010) and RNA sequencing (Wang et al. 2009) may help to achieve this goal at scales far above the levels of expression of a few genes (e.g. Norman et al. 2011, Avarre et al. 2014). This should especially motivate future studies (i) in cultured fish for which selective breeding programmes have been initiated (Chatain and Chavanne 2009) and which may need to control for cryptic phenotypes, and (ii) in wild juvenile sea bass distributed naturally over a large range of salinity conditions, but also trophic or temperature conditions that also greatly vary between habitats, to investigate molecular mechanisms involved in the lagoon/estuarine life compared with the coastal/pelagic life in this species.

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