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(Pseudopleuronectes americanus) metamorphosis

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Abstract
The aim of this study was to better understand the molecular events governing ontogeny in winter flounder Pseudopleuronectes americanus. The expression of seven genes involved in key metabolic processes during metamorphosis were measured at settlement (S0), at 15 (S15), and 30 (S30) days after settlement and compared to those in pelagic larvae prior to settlement (PL). Two critical stages were identified: 1) larval transit from the pelagic to the benthic habitat (from PL to S0) and 2) metamorphosis maturation, when the larvae stay settled without growth (from S0 to S30). Growth hormone (gh) gene expression significantly increased at S0. At S30, an increase in cytochrome oxidase (cox) gene expression occurred with a second surge of gh gene expression, suggesting that enhanced aerobic capacity was supporting growth before the temperature decrease in the fall. Expression patterns of pyruvate kinase, glucose-6 phosphate dehydrogenase, and bile salt-activated lipase genes indicated that energy synthesis may be mainly supplied through glycolysis in PL, through the pentose–phosphate pathway at settlement, and through lipid metabolism at S30. The expression of the heat-shock protein 70, superoxide dismutase, cox, and peroxiredoxin-6 genes revealed that oxidative stress and the consequent development of antioxidative protection were limited during the PL stage,
reinforced at settlement, and very high at S30, certainly due to the higher growth rate
observed at this period.

Keywords: metamorphosis; growth hormone; antioxidant enzymes; energy metabolism;
winter flounder, *Pseudopleuronectes americanus.*
Introduction

Metamorphosis can be defined in many ways. In the context of the present study, it refers to the “transitions from a larva to a juvenile, including morphological, physiological, and behavioural modifications that proceed while a larva transforms to a juvenile” (Bishop et al. 2006). At least four components of metamorphosis are shared among species: (1) the differentiation of juvenile/adult structures, (2) the degeneration of larval structures, (3) the metamorphic competence, and (4) the change in habitat (Heyland and Moroz 2006). During this period, animals undergo profound physiological and morphological modifications that are controlled by a coordinated change in gene expression (Baolong et al. 2005; Hildahl et al. 2007; Wang et al. 2011). In flatfish, metamorphosis is characterized by a striking anatomical transformation involving a 90° rotation in body position, the development of asymmetrical pigmentation, and the migration of one eye towards the other on the upper side of the fish (Fuiman 1997). This process occurs concomitantly with the transition from the pelagic to the benthic habitat (Fuiman 1997; Gibson 1997; Geffen et al. 2007), bringing modifications in feeding behaviour, type of prey, and digestive physiology (Tanaka et al. 1996; Lagardère et al. 1999; Cañavate et al. 2006).

All these biological and behavioural changes occurring during fish metamorphosis induce a particularly high metabolic demand (Geffen et al. 2007). To meet this demand, fish at early life stages must rapidly develop their metabolic pathways to obtain energy from protein, lipid, and carbohydrate metabolism (Slenzka et al. 1995; Geffen et al. 2007). Several studies reported that the activities of enzymes involved in energy and lipid metabolism may change during metamorphosis depending on fish needs during this
period and on the species considered (Segner and Verreth 1995; Slenzka et al. 1995; Bishop and Torres 1999; Ribeiro et al. 1999; Hoehne-Reitan 2001; Murray et al. 2003).

To meet the high metabolic demand related to the development of all metabolic pathways occurring during metamorphosis, fish must increase exogenous oxygen consumption. This can increase the production of reactive oxygen species (ROS), which are waste products from mitochondrial oxidation and may cause damage to lipids, proteins, and DNA in fish tissues (Fridovich 2004). ROS are continually detoxified and removed from cells by antioxidant enzymes such as peroxiredoxins (Prx), superoxide dismutase (Sod), and cytoprotection enzymes like heat-shock proteins (Hsp). The activity of antioxidant enzymes during fish metamorphosis has been widely studied in several larval fish species, including common dentex, trout, Salmo iridaeus (Aceto et al. 1994), and sprat, Sprattus sprattus (Peters et al. 2001) as well as in flatfish species, such as turbot, Scophthalmus maximus (Peters and Livingstone 1996).

Several authors have studied fish metamorphosis through variations in the activities of enzymes involved in key metabolic pathways, but information regarding the genetic processes underlying these changes is more limited. In a review comparing metamorphosis processes in different animal groups, Heyland and Moroz (2006) showed that, despite significant differences of transcription levels detected by microarray or other molecular methods, some similarities can be observed among taxa. Transcripts related to stress response, immunity, and apoptosis are associated with metamorphosis in all investigated phyla, and regulation signals mediated by hormones and by nitric oxide can act as regulators of metamorphic transitions.
In addition to the traditional morphological, biochemical, physiological, and histological markers, molecular markers could be used to generate useful insight on individual physiological performance during metamorphosis. In this context, the aim of this study was to better understand the molecular events governing ontogeny in flatfishes by measuring the expression of genes involved in key metabolic processes (growth, lipid metabolism, energy metabolism, oxidative stress, and cytoprotection) during flatfish metamorphosis using quantitative PCR (qPCR). By comparing the gene expressions of pelagic larvae with those of settled larvae through the metamorphosis process, we tested the hypothesis that genes coding for hormones or enzymes involved in growth, lipid metabolism, energy metabolism, oxidative stress, and cytoprotection are differentially expressed during the transition from pelagic to benthic life.

The expressions of the gene coding for growth hormone (gh), two genes for antioxidant enzymes (prx6, sod), one for cytoprotection (hsp70), three for enzymes involved in energy metabolism (cytochrome oxidase cox, pyruvate kinase pk, and glucose-6 phosphate dehydrogenase g6pd), and one for an enzyme involved in lipid metabolism (bile salt-activated lipase bal) were measured using qPCR analyses during the first weeks following settlement of a common flatfish species of the Northeast Atlantic coast, the winter flounder *Pseudopleuronectes americanus*.

**Material and methods**

**Biological material**

Winter flounder *Pseudopleuronectes americanus* is a common inshore flatfish that occurs from Labrador (Atlantic Canada, 53° N) to Georgia (southeast United States,
Most studies regarding metamorphosis in this species have investigated size and age at settlement (Chambers and Leggett 1987; Chambers and Leggett 1992; Fraboulet et al. 2009), temperature effects on growth (Chambers and Leggett 1992; Benoît et al. 2000; Fraboulet et al. 2010; 2011), and requirements in nutrient or abiotic parameters for aquaculture production (Ben Khemis et al. 2000; Seychelles et al. 2009; Fraboulet et al. 2011).

**Fish rearing conditions**

All experiments were conducted at the Station aquicole de Pointe-au-Père (ISMER / UQAR; 48° 27' N, 68° 32' W; QC, Canada). Fish manipulations were done according to the Canadian Council of Animal Protection recommendations, and protocols were approved by the University Animal Care Committee.

Egg stripping and fertilization were done according to Ben Khemis et al. (2000). Once hatched (day 0), larvae were transferred into nine 55 L cylindro-conical tanks (density: 250 larvae L\(^{-1}\)) set in a temperature-controlled room (10ºC), and exposed to a 12L:12D photoperiod cycle. Tanks were supplied with flowing filtered ambient sea water except during the feeding period (09:00–17:00), when flow was stopped. A permanent up-welling current was maintained in each tank by the aeration system placed at the bottom of a vertical strainer. From mouth opening at four days post-hatching (dph) until the end of the experiment, larvae were fed rotifers *Brachionus plicatilis* (5 ind. ml\(^{-1}\)) enriched with a mixture of three microalgae that fulfilled the fishes’ energy requirement: *Nannochloropsis oculata*, *Isochrysis galbana*, and *Pavlova lutheri* (see Seychelles et al. 2009 for the enrichment protocol).
When settlement occurred (~ 45 dph), newly settled larvae were collected every three days and transferred into rectangular tanks (35.5 × 65 × 6.5 cm). Each replicate tank contained 300 individuals. Settled larvae were reared according to Fraboulet et al. (2010), using flowing filtered seawater (50 µm, 2 L min⁻¹) and under natural conditions of temperature (10.4 ± 1°C), salinity (28.8 ± 1.3), and photoperiod (artificial light 400lux, 12L:12D). Each day, settled larvae were fed the same diet as during the larval stage (5 rotifers ml⁻¹ at 11:00, 13:00, and 16:00), completed with 10 microdiet meals (Gemma wean; www.skretting.com) in excess every 30 min between 09:00 and 11:00 and between 13:30 and 15:30. Seawater flow was stopped from 09:00–12:00 and 13:00–17:00 to avoid rotifer loss. Water was renewed between 12:00 and 13:00 and overnight. Dead individuals and excess feed were removed every day and tanks were cleaned every two weeks.

**Samplings**

Samplings were done early in the morning before the first meal to allow a 12 h fast prior to sampling. Pelagic larvae (PL) and newly settled larvae (S0) were sampled both on the same day, *i.e.*, at the peak of settlement. Fish were also sampled 15 and 30 days after settlement (S15 and S30). At each sampling period, 10 individuals per tank were collected and anaesthetized (MS 222, 0.05 g L⁻¹) for growth measurements and four subsamples of five or six larvae were fixed in five volumes of RNA*later®* (Applied Biosystems, CA, USA) for 24 h before being frozen at -80°C for further gene expression measurements.
Growth measurements

Total body length, standard length (*i.e.*, notochord length), and maximum body width were measured using a micrometer (± 0.1 mm).

Gene expression measurements

Total RNA was extracted from 30 mg of fish using the RNeasy Plus Mini Kit® (Qiagen, Inc., ON, Canada) according to the manufacturer’s instructions. Total RNA purity and concentration were controlled using the 260/280 nm absorbance ratio measured with a NanoDrop® instrument (NanoDrop ND-1000 spectrophotometer v3.3.0, NanoDrop Technologies, Inc., DE, USA). RNA purity was also assessed by ethidium bromide staining of 28S and 18S ribosomal RNA bands separated by electrophoresis on a 1.2% agarose gel. cDNAs were immediately obtained by reverse transcription (in duplicate) on 1 µg of total RNA from each sample using a Quantitect Reverse Transcription kit® with integrated removal of genomic DNA contamination (Qiagen, Inc., ON, Canada). cDNA concentrations were estimated using a NanoDrop spectrophotometer. Duplicate cDNAs were pooled for each sample and stored at -20°C until analyses. qPCR was performed for each sample on pooled cDNA using the iCycler iQ™ (Bio-Rad Laboratories Inc., ON, Canada).

The mRNA sequences for the *cox* (GenBank accession no. EU752157), *bal* (GenBank accession no. AF512561), *g6pd* (GenBank accession no. AY225097), and *prx6* (GenBank accession no. AY156726) genes were available for *Pseudopleuronectes americanus* in the GenBank® database (Benson et al. 2005), but those for *pk*, *hsp70*, *gh*, *sod*, and *glyceraldehyde phosphate dehydrogenase gapdh* were not. Consequently,
primers were designed from the mRNA sequences of other species to obtain PCR products ranging from 90 to 150 bp. Primers for *hsp70*, *gapdh*, *gh*, and *sod* were designed from sequences available for *Paralichthys olivaceus* (GenBank accession no. AB010871, GenBank accession no. AB029337, GenBank accession no. M23439, and GenBank accession no. EF681883.1, respectively) using Primer Express® software v.3.0 (Applied Biosystems, CA, USA). Primers for *pk* were designed from alignments between mRNA sequences of *Scophthalmus maximus* (GenBank accession no. AF467775) and *Salmo salar* (GenBank accession no. NM_001141703) using Primer 3® software (Rozen and Skaletsky, 2000). The primer sequences used for each gene are summarized in Table 1. For each gene, the amplicon obtained was sequenced to assess the specificity of forward and reverse primers. Sequencing was performed using ligation with the TOPO TA Cloning Kit for Sequencing® (Invitrogen Inc., ON, Canada), and transformation was done using One Shot Chemically Competent *E. coli®* (Invitrogen Inc., ON, Canada). Bacterial cDNA was extracted using the EZNA Plasmid Mini Kit I® (Omega Bio-Tek, GA, USA). Nucleotides were isolated with the Ultra-Step Dye Terminator Removal Kit® (Eazy Nucleic Isolation, EZNA, Omega Bio-Tek, GA, USA) and sequenced in forward and reverse directions using the Big Dye Terminator v3 chemistry® (Applied Biosystems, CA, USA). For each gene, the sequence obtained was compared to the sequence(s) used for the primer design using BLAST® software (Altschul et al. 1990). Sequence lengths and percentages of similarity with the reference sequences are presented in Table 1.

qPCR analyses for each gene were performed in duplicate for each pool of cDNA in a total volume of 15 µl containing 5 µl cDNA (mean initial concentration 20.0 ± 2.4 µg ml⁻¹) diluted by 10⁻², 0.5 µl primers (10 µmol l⁻¹), 1.5 µl of sterile water, and 7.5 µl
2X iQ SYBR Green Supermix® (Bio-Rad laboratories, Inc., ON, Canada). Thermal cycling of real-time PCR consisted of an initial incubation at 95°C for 13.5 min followed by 45 cycles of denaturing at 95°C for 30 s, annealing at 60°C for 1 min, and elongating at 72°C for 30 s. Cycle threshold (CT) values correspond to the number of cycles at which the fluorescence emission monitored in real time exceeded the threshold limit. CT values were automatically calculated on the log curve for each gene. Following PCR amplification, a melting curve was performed for each gene to ensure the accuracy of quantification: 45 cycles for cDNA amplification were followed by one cycle at 95°C for 1 min, one cycle at 55°C for 1 min, and 80 cycles at 55°C for 10 s.

To determine the relative quantity of target gene-specific transcripts present in each subsample, CT were averaged for each duplicate and then for each tank, and relative expression was calculated according to the equation from Livak and Schmittgen (2001):

\[ 2^{-\Delta\Delta^CT} = 2^{-(\Delta CT_e - \Delta CT_c)} \]  

(1)

where \( CT_e = CT_{target\ gene} - CT_{reference\ gene} \) for the sample x and \( CT_c = CT_{target\ gene} - CT_{reference-gene} \) for the calibrator.

In our study, the calibrator was the pelagic larval stage (PL group). \( gapdh \) was used as a reference gene because its expression remained constant between samples and through developmental stages. Standard curves (done in triplicate) were established for each developmental stage by plotting the CT values against the \( \log_{10} \) of five different dilutions (in triplicate) of a pool of representative cDNA sample solutions. The absence of any effect of developmental stage on the reference gene was examined with a test of slope homogeneity \( (F = 0.00; n = 4; p = 0.99 > 0.05) \) followed by an ANCOVA \( (F = 0.11; n = 4; p = 0.95 > 0.05) \) using Statistica® (Statsoft v.6.1, Tulsa, OK, USA).
Statistical analyses

All statistical tests were performed with Statistica®. Normality and homoscedasticity of data were tested using Kolmogorov-Smirnov’s test and Levene’s test, respectively. Gene expression data were transformed using log (x+1) to obtain homoscedasticity. The effects of developmental stage (PL, S0, S15, and S30) on fish growth (total length, standard length, and maximum width) and gene expression were tested using one-way ANOVA. When a significant effect was found, the unequal Tukey test was applied if ANOVA assumptions were met. For prx6 gene expression, homoscedasticity was not met using classic transformation, so Fisher’s LSD was applied on rank-transformed data (Quinn and Keough, 2002). Differences were considered significant at $p < 0.05$.

Results

Standard body length did not increase with later developmental stage, while total length and maximum width were significantly affected ($p < 0.001, F_3 = 12.45$ and $p < 0.001, F_3 = 43.61$, respectively; Fig. 1) and varied similarly. Between PL and S0, body width increased significantly by 1.6 fold, remained unchanged between S0 and S15, and increased again at S30 (Fig. 1A). Total length varied from an average of $6.60 \pm 0.08$ mm in PL, S0, and S15 to $7.48 \pm 0.17$ mm in S30 (Fig. 1B).

The gh gene expression significantly increased with developmental stages (Fig. 2; $p < 0.001, F_3 = 44.61$). It significantly increased by about threefold from PL stage to S0,
and it was 14 times higher than in the PL group 30 days after settlement. There was no
significant difference between S0 and S15.

The \textit{g6pd} gene expression greatly increased from PL to S0, by up to 13 times
(Fig. 3A; \(p < 0.001, F_3 = 70.72\)). It then decreased sevenfold from S0 to S15 and
remained unchanged until S30. The \textit{bal} gene expression was 2.5 times higher at S30 than
in the PL and S0 groups (Fig. 3B; \(p < 0.01, F_3 = 5.13\)). Relative gene expression was
intermediate in the S15 group, indicating that the expression activation had begun at this
stage. Expression of the \textit{pk} gene continuously decreased following settlement to reach the
lowest relative expression level at S30 (Fig. 3C; \(p < 0.05, F_3 = 3.12\)), while \textit{cox} gene
expression was about twofold higher in the S30 group than in the other groups (Fig. 3D;
\(p < 0.01, F_3 = 5.87\)).

The \textit{prx6} gene expression increased at settlement relative to the PL group and
remained relatively unchanged afterward (Fig. 4A; \(p < 0.05, F_3 = 4.15\)). The \textit{sod} gene
expression decreased from settlement to S0 and S15 and then increased to S30 (Fig. 4B; \(p
< 0.01, F_3 = 13.01\)). The \textit{hsp70} gene expression increased at S15 and S30 and was about
sevenfold higher than at settlement and in the PL group (Fig. 4C; \(p < 0.001, F_3 = 29.03\)).

Discussion

The aim of this study was to better understand the molecular events governing
ontogeny in winter flounder \textit{Pseudopleuronectes americanus} by measuring the
expression of seven genes involved in key metabolic processes. The results provided
insight on specific variations of growth, lipid metabolism, energy metabolism, oxidative
stress, and cytoprotection that may occur during the transition from a pelagic to a benthic lifestyle.

Stage development and gh expression

Morphological data clearly indicated different steps in winter flounder growth. A settled larva was significantly wider than a pelagic larva and its length and width did not change until 30 days post settlement, when individual growth in width and length increased again. Based on these observations, we identified two main critical stages related to winter flounder metamorphosis: 1) larval transit from the pelagic to the benthic habitat and 2) the metamorphosis maturation, when the larvae stay settled without growth.

Morphological modifications in length and width during winter flounder metamorphosis are reinforced by gh gene expression data, with gh expression increasing at the same time as the body length and/or width increases. Thus, gh could be a useful indicator of the two main critical stages identified during metamorphosis since it first increased during the settlement phase and then again with an even larger increase at the beginning of the juvenile stage (30 days after settlement). These results are in accordance with the hypothesis of Heyland and Moroz (2006) that specific hormones act as a signal regulating development in larvae and as a regulator of the metamorphic transition. Moreover, the role of thyroid hormones (Infante et al. 2008) and insulin-growth factor-1 (Hildahl et al. 2008) as well as Gh (Hildahl et al. 2008) and Igf-1 receptors (Escobar et al. 2011) in the regulation of metamorphosis has recently been highlighted in different fish species.
Gh is essential for the hepatic production of the insulin-like growth factors (Igf-1 and Igf-2), which mediate the anabolic actions of Gh (for review see Yousefian and Shirzad 2011). We designed primers to study the expression of Igf-1 based on sequences already identified in other fish species. Unfortunately, none allowed the amplification of a homologous sequence.

Changes in metabolic strategy

The pk, cox, g6pd, and bal gene expressions could indicate changes in metabolic strategy during metamorphosis. These changes could be linked with changes in activities related to energy metabolism, in feeding behaviour, and in digestive physiology observed in flatfish during metamorphosis in natural environments (Tanaka et al. 1996; Lagardère et al. 1999; Cañavate et al. 2006). Strong morphological and functional changes in the digestive system have been observed during metamorphosis in Japanese flounder Paralyechtis olivaceus (Tanaka et al. 1996). The authors suggested that these changes were related to a shift in the diet at settlement from zooplanktonic to benthic prey.

From the pelagic larval to the beginning of the juvenile stage (S30), pk gene expression significantly decreased, suggesting that the glycolysis pathway is more a larval pathway than a juvenile one. Pk is an enzyme involved in the last step of glycolysis, allowing the phosphorylation of adenosine di-phosphate (ADP) to adenosine tri-phosphate (ATP). The higher expression of the pk gene in PL than in S30 may indicate a higher anaerobic capacity of larvae compared to juveniles. In pelagic fish, an increase in the anaerobic potential has been associated with the necessity for short-term anaerobically powered swimming bursts during feeding and predator–prey interactions.
that require rapid and efficient production of ATP (Childress and Somero 1990). This
could apply to pelagic marine fish larvae that may be more exposed to predators than
juveniles, which can hide in the bottom substrate. Moreover, the decrease in gene
expression related to the glycolysis pathway in settled juveniles compared to pelagic
larvae combined with the increase in cox gene expression measured at S30 suggest a
higher aerobic capacity in juveniles. This may reflect an increased aerobic metabolic rate
due to tissue reorganization and higher growth rate (gh gene expression, total length, and
maximum width) occurring at the same time. In contrast, Darias et al. (2008) used
microarrays to show an increased expression of genes involved in
neoglucogenesis/glycolysis in larval European seabass, with a significantly higher
anaerobic capacity at the end of the larval stage.

At settlement, g6pd gene expression strongly increased, suggesting a shift from
glycolysis to pentose–phosphate metabolism for this stage. Activation of the pentose–
phosphate pathway would be necessary to supply a large quantity of ribose units for
nucleotide synthesis and thus support the high demand for newly formed RNA and DNA
in fast-growing juveniles. This is in accordance with Munilla-Moran and Stark (1989),
who observed no detectable levels of G6pd enzyme activity in turbot Scophthalmus
maximus larvae, thereby concluding that the pentose–phosphate pathway is inoperative
during early life in turbot. Segner and Verreth (1995) also reported very low levels of
G6pd enzyme activity in early life stages of the pelagic catfish Clarias gariepinus that
increased through development.

At the juvenile stage (from S30), when growth resumes, bal gene expression
dramatically increased, suggesting that winter flounder once again changed its strategy to
rely mainly on lipid metabolism to support its growth and development. Using semi-quantitative PCR, Murray et al. (2003) reported an increase in bal gene expression from the larval to juvenile stage in winter flounder. They showed that this enzyme may use different lipid substrates, including triacylglycerols. While analyzing the fate of lipid classes from metamorphosis to 45 days post-settlement (45 dps) by thin-layer chromatography, Fraboulet et al. (2010) showed that even though they represented only a small fraction of the total lipids, triacylglycerols dropped by 79% during the growth period occurring from settlement to 45 dps. These observations confirmed that lipid metabolism becomes the main pathway to support juvenile development, and changes in gene expression that will lead to lipase production take place two weeks after settlement. Since the Bal enzyme is mostly produced by the pancreas in winter flounder (Murray et al. 2003), the increase in bal gene expression observed in the present study at S30 could indicate that the pancreas continues to develop during the juvenile settlement stage (from S30). Moreover, this increase could not be related to the food spectrum and feeding protocol, as suggested in a previous study (Borlongan 1990), because settled larvae were fed the same diet (with the same lipid composition) using the same feeding protocol throughout the experiment. This increase in bal gene expression at S30 suggests that fish development not only corresponds to the very beginning of metamorphosis in winter flounder, but also that it continues until around a month after settlement.

Antioxidant enzymes

The present study reveals the expression of genes coding for antioxidant enzymes (prx6 and sod) and cytoprotection (hsp70) from pelagic larval to benthic juvenile stages
in *P. americanus*. The results obtained demonstrate that all antioxidant genes tested showed a maximal relative expression at S30, suggesting an increased response to stress and antioxidant protection at the juvenile stage. Accordingly, in the sturgeon *Acipenser naccarii*, an increased activity of antioxidant enzymes (Sod, catalase, glutathione peroxidase, and glutathione reductase) was observed during the juvenile stage (Díaz et al. 2010). In winter flounder, the expression of these genes evolved in different ways during metamorphosis.

The sod gene expression suggested that this enzyme played a major role during the pelagic larval stage, settlement, and the beginning of the juvenile stage. Previous studies reporting Sod enzyme activity in larval fish showed that the enzymatic activity varies according to the species considered: a decrease in Sod activity throughout larval development was demonstrated in turbot (Peters and Livingstone 1996) and in common dentex (Mourente et al. 1999) while an increase was demonstrated in larval trout *Salmo iridaeus* (Aceto et al. 1994). Kalaimani et al. (2007) did not report any difference in Sod activity throughout larval development in the Asian seabass *Lates calcarifer*.

Except for sod, the expression of the two other genes coding for antioxidant enzymes or cytoprotection were lowest during pelagic larval stage. This is in accordance with the use of glycolysis metabolism during this period. Glycolysis is an anaerobic process, and as a consequence it limits oxidative stress (Wu and Wei 2011). The use of this type of metabolism could be an adaptation by fish to limit their oxidative stress during the critical larval period.

At settlement, detoxification seems to be enhanced by Prx6. The Prx6 enzyme plays a protective antioxidant role in cells, reducing and detoxifying hydrogen peroxide,
peroxinitrite, and a wide range of organic hydroperoxides (Wood et al. 2003). The
different gene expression patterns observed for sod and prx6 can be explained by the fact
that the two enzymes use different substrates: Sod catalyzes the dismutation of
superoxide in oxygen and hydrogen peroxide that must be further detoxified by Prx6.
Thus, the large amounts of hydrogen peroxide produced at the PL stage by Sod will be
further detoxified by Prx6 starting at settlement. Prxs are the most recently discovered
group of antioxidant enzymes, and while they have been cloned and characterized in
several fish species (channel catfish Ictalurus punctatus, Yeh and Klesius 2007; Gilthead
seabream, Pérez-Sánchez et al. 2011) including flatfishes (winter flounder, Chapman et
al. 2004; turbot, Zheng et al. 2010), their expression pattern and function are largely
unknown. Our results suggest that this enzyme plays a role as the settlement stage begins.
Furthermore, the higher prx6 gene expression at settlement could indicate that cellular
maintenance under normal physiological conditions in blood, heart, muscle, and kidney is
completely functional from settlement in winter flounder. Indeed, while prx6 was
detected in most organs of the turbot (Zheng et al. 2010, using quantitative PCR) and of
the catfish (Yeh and Klesius 2007, using semi-quantitative PCR), its highest expression
levels were detected in these organs and the lowest in spleen. The detoxification of
hydrogen peroxide at settlement by Prx6 could be reinforced by the G6pd activity since
prx6 and g6pd both showed an increase in gene expression at settlement. G6pd activity
would provide a reductive potential in the form of nicotinamide adenine dinucleotide
phosphate-oxidase (NADPH), which is able to detoxify hydrogen peroxide (Pandolfi et
al. 1995). These results indicate that, in addition to high energy synthesis, the shift from
glycolysis to the pentose–phosphate pathway at settlement provides an additional antioxidant protection during settlement.

Starting from S15, the marked increase in hsp70 gene expression suggested high environmental stress due to marked metabolic and cellular changes caused by ontogenetic metamorphosis processes occurring during settlement in winter flounder (Tanaka et al. 1996; Gibson 1997). The development of cytoprotection and possible oxidative defence through hsp70 toward the end of fish metamorphosis has also been reported by Deane and Woo (2003) in silver sea bream Sparus sarba. These authors found unaltered hsp70 transcript levels from 1–14 days post hatching that further progressively increased until settlement. However, data are not available regarding the hsp70 gene expression after the settlement process.

The different expression patterns measured in our study for sod, hsp70, and prx6, mostly observed at settlement or 15 days later (hsp70, sod), could partly be linked with changes in feeding behaviour and digestive physiology observed during this period in natural environments (Tanaka et al. 1996; Lagardère et al. 1999; Cañavate et al. 2006).

Previous studies demonstrated that dietary components can affect levels of antioxidant enzymes in the larval and adult life stages of organisms (Peters and Livingstone 1996).

This hypothesis would be supported by the changes in energy metabolism that we observed throughout the fish development in this study.

To conclude, our results revealed several important points. (1) Two critical stages could be identified during winter flounder metamorphosis: a) the transition from the pelagic to the benthic habitat and b) the metamorphosis maturation, when the larvae stay
settled without growth. They allow a more precise identification of the start of the
juvenile stage as the period denoted by the resumption of growth and the increased
aerobic capacity that occur approximately 30 days after settlement under the temperature
and photoperiod conditions used in the present experiment. (2) \( gh \) gene expression could
be a useful indicator of these main phases of development because its first expression
increase corresponds to the settlement phase while the second and more intense increase
corresponds to the beginning of the juvenile stage. (3) The results suggest that pelagic
larvae have a higher anaerobic capacity, while juveniles have a higher aerobic capacity
associated with a significant increase in growth rate. (4) The results suggest different
sources of energy synthesis for the different developmental stages: a) through glycolysis
in PL, b) through the pentose–phosphate pathway in settled larvae, and c) through lipid
metabolism in juveniles. (5) During the pelagic larval stage, oxidative stress and the
consequent antioxidative protection may be limited by glycolysis, while an increase in
antioxidative protection reinforced by the shift to the pentose–phosphate pathway seemed
to occur at settlement. Gene expression related to antioxidative protection was very high
in juveniles, certainly due to the higher growth rate observed at this period. (6) The
results suggest that cellular maintenance in organs such as blood, heart, muscle, and
kidney is fully functional from settlement, whereas the pancreas may continue to develop
until 30 days after settlement. The whole set of results obtained provides useful
preliminary indicators for the metamorphosis progress in this species and in larval fishes
through the understanding of molecular events governing ontogeny. However, because
post-transcriptional regulations may occur, it would be interesting to complete these
results with enzymatic measurements. Moreover, a future study should also include the
evaluation of gene expression for a greater number of genes in the different pathways of interest. Finally, other novel factors, including both known and unknown genes and pathways, could be identified by new transcriptomic tools such as the use of RNAseq.

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Figure 2

Fold change in g/h gene expression with respect to pelagic larval group.

Developmental stage:
- PL
- S0
- S15
- S30

Legend:
- a
- b
- c