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Expression of genes involved in key metabolic processes during winter flounder (*Pseudopleuronectes americanus*) metamorphosis

Marie Vagner, Benjamin de Montgolfier, Jean-Marie Sévigny, Réjean Tremblay, and Céline Audet



Abstract: The aim of this study was to better understand the molecular events governing ontogeny in winter flounder (*Pseudopleuronectes americanus* (Walbaum, 1792)). 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 with 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 (*pk*), glucose-6-phosphate dehydrogenase (*g6pd*), and bile salt activated lipase (*bal*) 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 (*hsp70*), superoxide dismutase (*sod*), *cox*, and peroxiredoxin-6 (*prx6*) 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 owing to the higher growth rate observed at this period.

Key words: metamorphosis, growth hormone, antioxidant enzymes, energy metabolism, winter flounder, *Pseudopleuronectes americanus*.

Résumé : L'étude avait pour but d'établir une meilleure compréhension des événements moléculaires qui régissent l'ontogénèse chez la plie rouge (*Pseudopleuronectes americanus* (Walbaum, 1792)). L'expression de sept gènes intervenant dans des processus métaboliques clés durant la métamorphose a été mesurée au moment de la déposition (S0) et après 15 (S15) et 30 (S30) jours suivant cette dernière, et comparée à celle de larves pélagiques avant la déposition (PL). Deux stades critiques ont été cernés : (1) la transition des larves de l'habitat pélagique à l'habitat benthique (de PL à S0) et (2) la maturation de métamorphose, durant laquelle la larve demeure déposée sans croître (de S0 à S30). L'expression génique de l'hormone de croissance (*gh*) a augmenté de manière significative à S0. À S30, une augmentation de l'expression génique de la cytochrome oxydase (*cox*) a eu lieu, de concert avec une deuxième augmentation de l'expression génique de la *gh*, ce qui laisse croire qu'une capacité aérobie accrue supportait la croissance avant la baisse de température automnale. Les patrons d'expression des gènes de la pyruvate kinase (*pk*), de la glucose-6-phosphate déshydrogénase (*g6pd*) et de la lipase stimulée par les sels biliaires (*bal*) indiquaient que la synthèse énergétique pourrait être principalement assurée par la glycolyse au stade PL, par la voie pentose-phosphate à la déposition et par le métabolisme des lipides à S30. L'expression des gènes de la protéine de choc thermique 70 (*hsp70*), de la superoxyde dismutase (*sod*), de la *cox* et de la peroxyrédoxine-6 (*prx6*) a révélé que le stress oxydatif et le développement d'une protection anti-oxydation en découlant étaient limités durant le stade PL, renforcés à la déposition et très élevés à S30, sans doute en raison du taux de croissance accru observé à cette période. [Traduit par la Rédaction]

Mots-clés : métamorphose, hormone de croissance, enzymes antioxydantes, métabolisme énergétique, plie rouge, *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 modifica-

tions 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).

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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 et al. 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 peroxidases (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 (*Dentex dentex* (L., 1758)) (Mourente et al. 1999), rainbow trout (*Salmo iridaeus* Gibbons, 1855 = *Oncorhynchus mykiss* (Walbaum, 1792)) (Aceto et al. 1994), and European sprat (*Sprattus sprattus* (L., 1758)) (Peters et al. 2001), as well as in flatfish species such as turbot (*Scophthalmus maximus* (L., 1758)) (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 polymerase chain reactions (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* (Walbaum, 1792)).

Materials and methods

Biological material

Winter flounder is a common inshore flatfish that occurs from Labrador (Atlantic Canada, 53°N) to Georgia (southeast United

States, 33°N) (Scott and Scott 1988). Most studies regarding metamorphosis in this species have investigated size and age at settlement (Chambers and Leggett 1987, 1992; Fraboulet et al. 2009), temperature effects on growth (Chambers and Leggett 1992; Benoit 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, Quebec, Canada; 48°27'N, 68°32'W). Fish manipulations were done according to the Canadian Council on Animal Care 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 (10 °C), and exposed to a 12 h light : 12 h dark photoperiod. Tanks were supplied with flowing filtered ambient sea water except during the feeding period (0900–1700), 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 4 days after hatching until the end of the experiment, larvae were fed rotifers *Brachionus plicatilis* (Müller, 1786) (5 individuals·mL⁻¹) enriched with a mixture of three microalgae that fulfilled the fishes' energy requirement: *Nannochloropsis oculata* (Droop) D.J. Hibberd, *Isochrysis galbana* Parke, and *Pavlova lutheri* (Droop) J.C. Green (for the enrichment protocol see Seychelles et al. 2009).

When settlement occurred (~45 days after hatching), newly settled larvae were collected every 3 days and transferred into rectangular tanks (35.5 cm × 65 cm × 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 400 lx, 12 h light : 12 h dark). Each day, settled larvae were fed the same diet as during the larval stage (5 rotifers·mL⁻¹ at 1100, 1300, and 1600), completed with 10 microdiet meals (Gemma wean; available from www.skretting.com) in excess every 30 min between 0900 and 1100 and between 1330 and 1530. Seawater flow was stopped from 0900 to 1200 and from 1300 to 1700 to avoid rotifer loss. Water was renewed between 1200 and 1300 and overnight. Dead individuals and excess feed were removed every day and tanks were cleaned every 2 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 RNAlater® (Applied Biosystems, Foster City, California, 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., Mississauga, Ontario, 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

Table 1. Primers used for winter flounder (*Pseudopleuronectes americanus*) in quantitative polymerase chain reaction (qPCR) analysis.

Gene	Primer set (5' → 3')	Sequence used for primer design	Sequence similarity (%)	PCR amplicon size (bp)
<i>bal</i>	F: GGACAACGCCTACTCCACAT R: GCCTGTGTAGGAACCAAGAA	<i>P. americanus</i> (AF512561)	98	116
<i>cox</i>	F: TCACTGCCGTCCTTCTTCTT R: GTTGGTAGAGGATGGGGTCA	<i>P. americanus</i> (EU752157)	98	129
<i>g6pd</i>	F: CCACCAGATAGAGGGAGAGAAGAA R: TCGGACCACGACCTCCATA	<i>P. americanus</i> (AY225097)	100	64
<i>gapdh</i>	F: CAACGGCGACACTCACTCCTC R: TCGCAGACACGGTTGCTGTAG	<i>Paralichthys olivaceus</i> (AB029337)	85	87
<i>gh</i>	F: CCTGAAGCTGATAGAGGCCAAT R: GGAGCACCGAACTCTCAGAGA	<i>P. olivaceus</i> (M23439)	96	76
<i>hsp70</i>	F: CGAGTATGAGCACCAGCAGAAG R: GGTGCCGCTCCCTGGTA	<i>P. olivaceus</i> (AB010871)	90	86
<i>pk</i>	F: CCATGCTGAGCCATCAAGA R: TTTCTGGTCCTTGGGTCC	Blast between <i>Scophthalmus maximus</i> (AF467775) and <i>Salmo salar</i> (NM_001141703)	81	106
<i>prx6</i>	F: CACCGTTGGGAAGAGAG R: GGATGGATTGCCCTCACC	<i>P. americanus</i> (AY156726)	100	247
<i>sod</i>	F: TGGAGACAACAAACGGG R: CATTGAGGGTGAGCATCTTG	<i>P. olivaceus</i> (EF681883.1)	95	138

Note: The GenBank accession number identifies the sequence of the species used for the primer design. The percentage of similarity obtained between the sequence of the PCR amplicon and that of the GenBank species and the PCR amplicon size (no. of base pairs (bp)) are also provided. Abbreviations: *bal*, bile salt activated lipase; *cox*, cytochrome oxidase; *gapdh*, glyceraldehydes phosphate dehydrogenase; *gh*, growth hormone; *g6pd*, glucose-6-phosphate dehydrogenase; *hsp70*, heat-shock protein 70; *pk*, pyruvate kinase; *prx6*, peroxiredoxin-6; *sod*, superoxide dismutase.

spectrophotometer version 3.3.0; NanoDrop Technologies, Inc., Wilmington, Delaware, 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.). 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., Mississauga, Ontario, 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 Japanese flounder (*Paralichthys olivaceus* (Temminck and Schlegel, 1846)) (GenBank accession Nos. AB010871, AB029337, M23439, and EF681883.1, respectively) using Primer Express® software version 3.0 (Applied Biosystems). Primers for *pk* were designed from alignments between mRNA sequences of *Scophthalmus maximus* (GenBank accession No. AF467775) and Atlantic salmon (*Salmo salar* L., 1758) (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., Burlington, Ontario, Canada) and transformation was done using One Shot Chemically Competent *E. coli*® (Invitrogen Inc.). Bacterial cDNA was extracted using the EZNA Plasmid Mini Kit I® (Omega Bio-Tek, Norcross, Georgia, USA). Nucleotides were isolated with the Ultra-Step Dye Terminator Removal Kit® (Eazy Nucleic Isolation, EZNA; Omega

Bio-Tek) and sequenced in forward and reverse directions using the Big Dye Terminator version 3 chemistry® (Applied Biosystems). For each gene, the sequence obtained was compared with 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 2× iQ SYBR Green Supermix® (Bio-Rad Laboratories, Inc.). 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 (C_T) values correspond to the number of cycles at which the fluorescence emission monitored in real time exceeded the threshold limit. C_T 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 1 cycle at 95 °C for 1 min, 1 cycle at 55 °C for 1 min, and 80 cycles at 55 °C for 10 s.

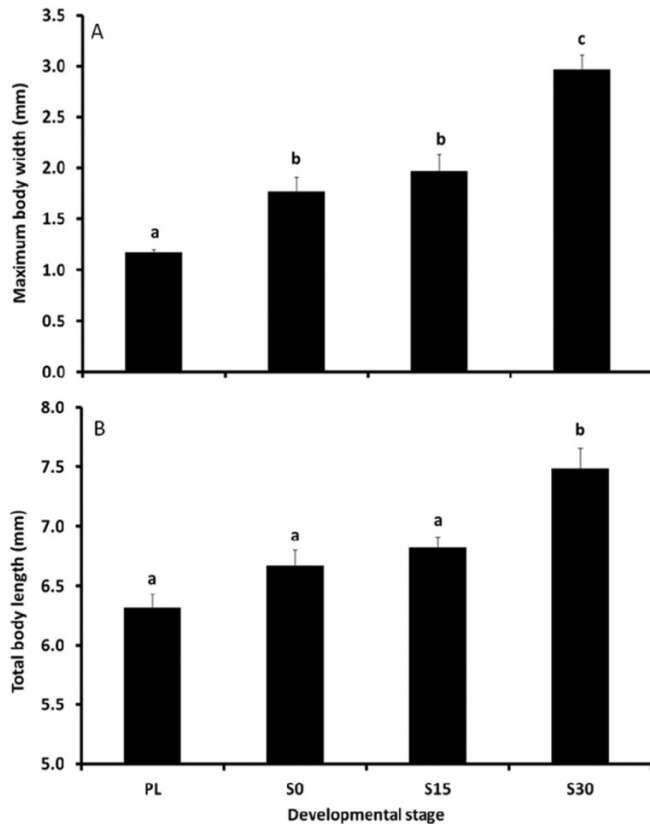
To determine the relative quantity of target gene-specific transcripts present in each subsample, C_T were averaged for each duplicate and then for each tank, and relative expression was calculated according to the equation from Livak and Schmittgen (2001):

$$2^{-\Delta\Delta C_T} = 2^{-(\Delta C_{T_e} - \Delta C_{T_c})}$$

where $C_{T_e} = C_T$ target gene - C_T reference gene for the sample x and $C_{T_c} = C_T$ target gene - C_T 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 C_T values against the log₁₀ of five different dilutions (in triplicate) of a pool of represen-

Fig. 1. (A) Maximum body width and (B) total body length (mean \pm SE) by developmental stage (PL, pelagic larvae; S0, at settlement; S15, 15 days after settlement; S30, 30 days after settlement) of winter flounder (*Pseudopleuronectes americanus*). Developmental groups not sharing a common letter are significantly different (one-way ANOVA; $\alpha = 0.05$).



tative 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® version 6.1 (Statsoft Inc., Tulsa, Oklahoma, USA).

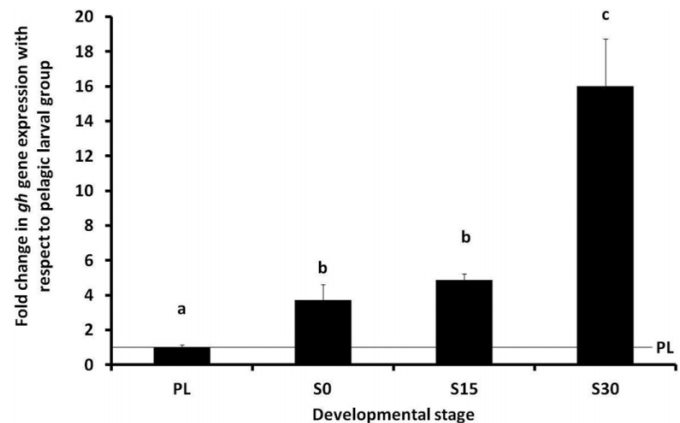
Statistical analyses

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’s 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 ($F_{(3,30)} = 12.45$, $p < 0.001$ and $F_{(3,30)} = 43.61$, $p < 0.001$, 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 6.60 ± 0.08 mm (mean \pm SE) in PL, S0, and S15 to 7.48 ± 0.17 mm in S30 (Fig. 1B).

Fig. 2. Relative change in growth hormone (*gh*) gene expression in winter flounder (*Pseudopleuronectes americanus*) using the pelagic larval group as the reference (mean \pm SE). Developmental groups not sharing a common letter are significantly different; the black horizontal line indicates the level of gene expression in the reference group (PL) from which the gene expressions of other groups were calculated (one-way ANOVA; $\alpha = 0.05$). PL, pelagic larvae; S0, at settlement; S15, 15 days after settlement; S30, 30 days after settlement.



The *gh* gene expression significantly increased with developmental stages (Fig. 2; $F_{(3,30)} = 44.61$, $p < 0.001$). It significantly increased by about 3-fold 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 *g6pd* gene expression greatly increased from PL to S0, by up to 13 times (Fig. 3A; $F_{(3,30)} = 70.72$, $p < 0.001$). It then decreased 7-fold from S0 to S15 and remained unchanged until S30. The *bal* gene expression was 2.5 times higher at S30 than in the PL and S0 groups (Fig. 3B; $F_{(3,30)} = 5.13$, $p < 0.01$). Relative gene expression was intermediate in the S15 group, indicating that the expression activation had begun at this stage. Expression of the *pk* gene continuously decreased following settlement to reach the lowest relative expression level at S30 (Fig. 3C; $F_{(3,30)} = 3.12$, $p < 0.05$), while *cox* gene expression was about 2-fold higher in the S30 group than in the other groups (Fig. 3D; $F_{(3,30)} = 5.87$, $p < 0.01$).

The *prx6* gene expression increased at settlement relative to the PL group and remained relatively unchanged afterward (Fig. 4A; $F_{(3,30)} = 4.15$, $p < 0.05$). The *sod* gene expression decreased from settlement to S0 and S15 and then increased to S30 (Fig. 4B; $F_{(3,30)} = 13.01$, $p < 0.01$). The *hsp70* gene expression increased at S15 and S30 and was about sevenfold higher than at settlement and in the PL group (Fig. 4C; $F_{(3,30)} = 29.03$, $p < 0.001$).

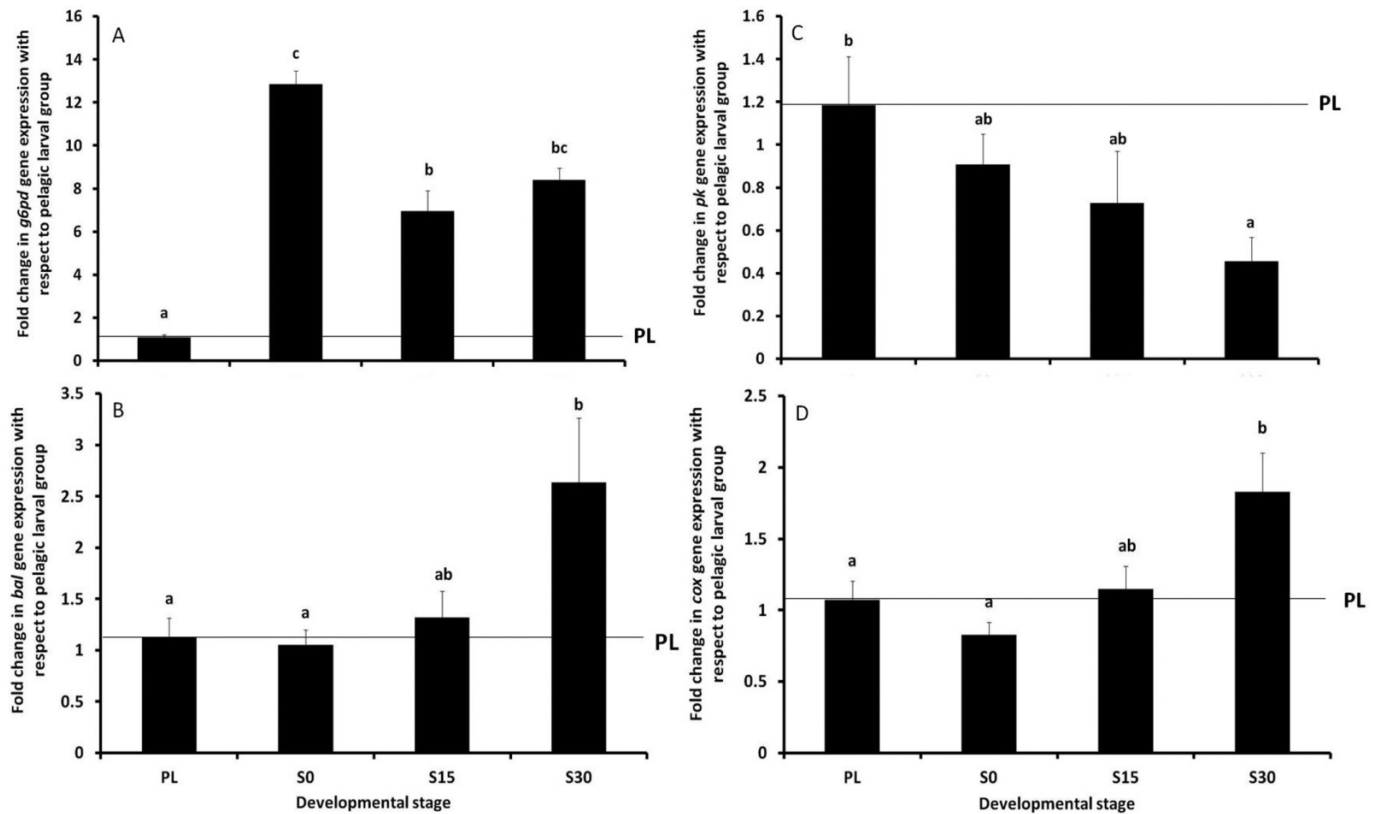
Discussion

The aim of this study was to better understand the molecular events governing ontogeny in winter flounder 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 after 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

Fig. 3. Relative changes in expressions of four genes involved in metabolic strategy in winter flounder (*Pseudopleuronectes americanus*) using the pelagic larval group as the reference (mean \pm SE): (A) glucose-6-phosphate dehydrogenase (*g6pd*), (B) bile salt activated lipase (*bal*), (C) pyruvate kinase (*pk*), and (D) cytochrome oxidase (*cox*) gene expression. Developmental groups not sharing a common letter are significantly different; the black horizontal line indicates the level of gene expression in the reference group (PL) from which the gene expressions of other groups were calculated (one-way ANOVA; $\alpha = 0.05$). PL, pelagic larvae; S0, at settlement; S15, 15 days after settlement; S30, 30 days after settlement.



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 (Igf-1) (Hildahl et al. 2008), growth hormone (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.

Growth hormone is essential for the hepatic production of the insulin-like growth factors (Igf-1 and Igf-2), which mediate the anabolic actions of growth hormone (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.

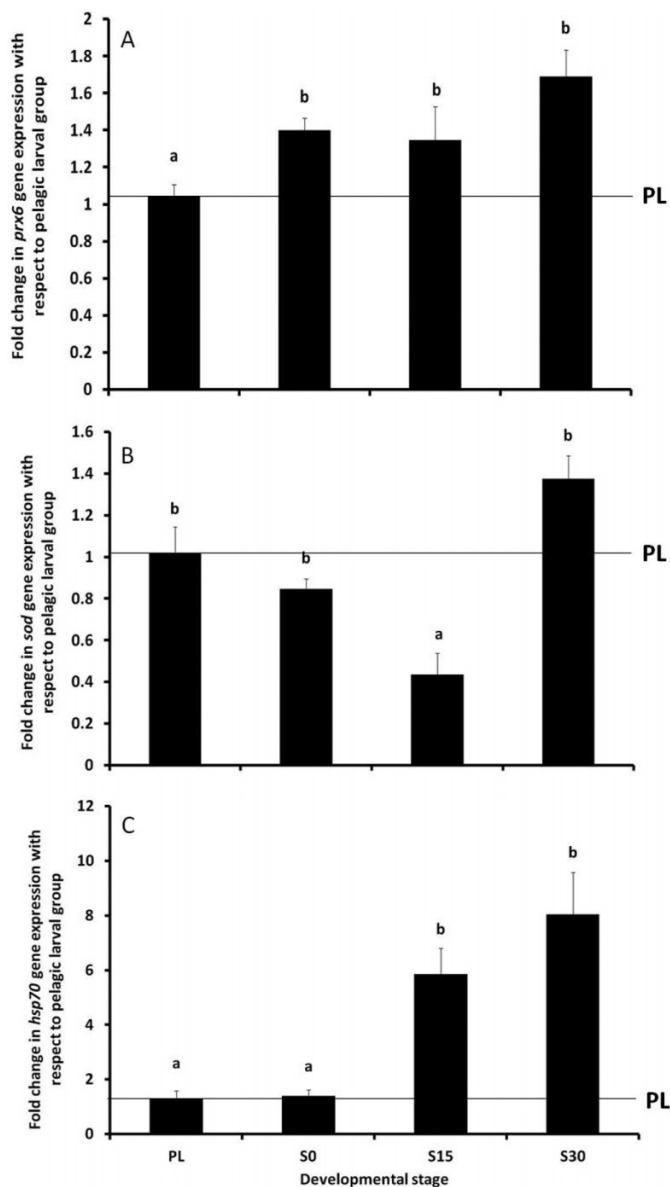
Changes in metabolic strategy

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 physiolo-

gy 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 (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. Pyruvate kinase is an enzyme involved in the last step of glycolysis, allowing the phosphorylation of adenosine diphosphate (ADP) to adenosine triphosphate (ATP). The higher expression of the *pk* gene in PL than in S30 may indicate a higher anaerobic capacity of larvae compared with 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 with 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

Fig. 4. Relative changes in expressions of three genes involved in antioxidant protection in winter flounder (*Pseudopleuronectes americanus*) using the pelagic larval group as the reference (mean \pm SE): (A) peroxiredoxin-6 (*prx6*), (B) superoxide dismutase (*sod*), and (C) heat-shock protein 70 (*hsp70*) gene expressions. Developmental groups not sharing a common letter are significantly different; the black horizontal line indicates the level of gene expression in the reference group (PL) from which the gene expression of other groups were calculated (one-way ANOVA; $\alpha = 0.05$). PL, pelagic larvae; S0, at settlement; S15, 15 days after settlement; and S30, 30 days after settlement.



expression of genes involved in neoglucogenesis–glycolysis in larval European seabass (*Dicentrarchus labrax* (L., 1758)), 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 de-

tectable levels of glucose-6-phosphate dehydrogenase (*G6pd*) activity in turbot 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* activity in early life stages of the pelagic catfish (*Clarias gariepinus* (Burchell, 1822)) 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 after settlement 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 days after settlement. 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 2 weeks after settlement. Since the bile salt activated lipase 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 the winter flounder. 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 Adriatic sturgeon (*Acipenser naccarii* Bonaparte, 1836), 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 at the beginning of the juvenile stage. Previous studies reporting *Sod* 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 dextr (*Mourente et al. 1999*), whereas an increase was demonstrated in larval rainbow trout (Aceto et al. 1994). Kalaimani et al. (2008) did not report any difference in *Sod* activity throughout larval development in the Asian seabass (*Lates calcarifer* (Bloch, 1790)).

Except for *sod*, the expression of the two other genes coding for antioxidant enzymes or cytoprotection was lowest during pelagic larval stage. This is in accordance with the use of glycolysis metabolism during this period. Glycolysis is an anaerobic process, and consequently, 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, peroxynitrite, 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 into 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* (Rafinesque, 1818); Yeh and Klesius 2007; Gilthead seabream, *Sparus aurata* L., 1758; 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, although *prx6* was detected in most organs of the turbot (Zheng et al. 2010, using qPCR) 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 goldlined seabream (*Sparus sarba* (Forsskål, 1775)). These authors found unaltered *hsp70* transcript levels from 1 to 14 days after 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: (i) the transition from the pelagic to the benthic habitat and (ii) 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: (i) through glycolysis in PL, (ii) through the pentose-phosphate pathway in settled larvae, and (iii) 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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