Expression of genes involved in key metabolic processes during winter flounder (*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*, g*lucose-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 *Dentex dentex* (Mourente 1999), 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.

96 In addition to the traditional morphological, biochemical, physiological, and
97 histological markers, molecular markers could be used to generate useful insight on
98 individual physiological performance during metamorphosis. In this context, the aim of
99 this study was to better understand the molecular events governing ontogeny in flatfishes
100 by measuring the expression of genes involved in key metabolic processes (growth, lipid
101 metabolism, energy metabolism, oxidative stress, and cytoprotection) during flatfish
102 metamorphosis using quantitative PCR (qPCR). By comparing the gene expressions of
103 pelagic larvae with those of settled larvae through the metamorphosis process, we tested
104 the hypothesis that genes coding for hormones or enzymes involved in growth, lipid
105 metabolism, energy metabolism, oxidative stress, and cytoprotection are differentially
106 expressed during the transition from pelagic to benthic life.
107 The expressions of the gene coding for growth hormone (*gh*), two genes for
108 antioxidant enzymes (*prx6*, *sod),* one for cytoprotection *(hsp70)*, three for enzymes
109 involved in energy metabolism (*cytochrome oxidase cox*, *pyruvate kinase pk,* and
110 g*lucose-6 phosphate dehydrogenase g6pd*), and one for an enzyme involved in lipid
111 metabolism (*bile salt-activated lipase bal*) were measured using qPCR analyses during
112 the first weeks following settlement of a common flatfish species of the Northeast
113 Atlantic coast, the winter flounder *Pseudopleuronectes americanus*.
114
115 **Material and methods**
116 **Biological material**
117 Winter flounder *Pseudopleuronectes americanus* is a common inshore flatfish that
118 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; 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 400 lux, 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, 0.05g 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.

165 **Growth measurements**

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

168

169 **Gene expression measurements**

170 Total RNA was extracted from 30 mg of fish using the RNeasy Plus Mini Kit®
171 (Qiagen, Inc., ON, Canada) according to the manufacturer's instructions. Total RNA
172 purity and concentration were controlled using the 260/280 nm absorbance ratio
173 measured with a NanoDrop® instrument (NanoDrop ND-1000 spectrophotometer v3.3.0,
174 NanoDrop Technologies, Inc., DE, USA). RNA purity was also assessed by ethidium
175 bromide staining of 28S and 18S ribosomal RNA bands separated by electrophoresis on a
176 1.2% agarose gel. cDNAs were immediately obtained by reverse transcription (in
177 duplicate) on 1 µg of total RNA from each sample using a Quantitect Reverse
178 Transcription kit® with integrated removal of genomic DNA contamination (Qiagen, Inc.,
179 ON, Canada). cDNA concentrations were estimated using a NanoDrop
180 spectrophotometer. Duplicate cDNAs were pooled for each sample and stored at -20°C
181 until analyses. qPCR was performed for each sample on pooled cDNA using the iCycler
182 iQ™ (Bio-Rad Laboratories Inc., ON, Canada).

183 The mRNA sequences for the *cox* (GenBank accession no. EU752157), *bal*
184 (GenBank accession no. AF512561), *g6pd* (GenBank accession no. AY225097), and
185 *prx6* (GenBank accession no. AY156726) genes were available for *Pseudopleuronectes*
186 *americanus* in the GenBank® database (Benson et al. 2005), but those for *pk*, *hsp70*, *gh*,
187 *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 *Paralichtys 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

211 2X iQ SYBR Green Supermix® (Bio-Rad laboratories, Inc., ON, Canada). Thermal
212 cycling of real-time PCR consisted of an initial incubation at 95°C for 13.5 min followed
213 by 45 cycles of denaturing at 95°C for 30 s, annealing at 60°C for 1 min, and elongating
214 at 72°C for 30 s. Cycle threshold (CT) values correspond to the number of cycles at
215 which the fluorescence emission monitored in real time exceeded the threshold limit. CT
216 values were automatically calculated on the log curve for each gene. Following PCR
217 amplification, a melting curve was performed for each gene to ensure the accuracy of
218 quantification: 45 cycles for cDNA amplification were followed by one cycle at 95°C for
219 1 min, one cycle at 55°C for 1 min, and 80 cycles at 55°C for 10 s.
220 To determine the relative quantity of target gene-specific transcripts present in
221 each subsample, CT were averaged for each duplicate and then for each tank, and relative
222 expression was calculated according to the equation from Livak and Schmittgen (2001):
223 $$2^{-\Delta\Delta^{\wedge}CT} = 2^{\wedge-(\Delta CTe - \Delta CTc)} \quad (1)$$
224 where CTe = $CT_{target\ gene} - CT_{reference\ gene}$ for the sample x and
225 CTc = $CT_{target\ gene} - CT_{reference\text{-}gene}$ for the calibrator.
226 In our study, the calibrator was the pelagic larval stage (PL group). *gapdh* was
227 used as a reference gene because its expression remained constant between samples and
228 through developmental stages. Standard curves (done in triplicate) were established for
229 each developmental stage by plotting the CT values against the $\log_{10}$ of five different
230 dilutions (in triplicate) of a pool of representative cDNA sample solutions. The absence
231 of any effect of developmental stage on the reference gene was examined with a test of
232 slope homogeneity ($F = 0.00$; $n = 4$; $p = 0.99 > 0.05$) followed by an ANCOVA ($F =$
233 0.11; $n = 4$; $p = 0.95 > 0.05$) using Statistica® (Statsoft v.6.1, Tulsa, OK, USA).

234

235 **Statistical analyses**

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

246

247 **Results**

248

249 Standard body length did not increase with later developmental stage, while total
250 length and maximum width were significantly affected ($p < 0.001$, $F_3 = 12.45$ and $p <$
251 0.001, $F_3 = 43.61$, respectively; Fig. 1) and varied similarly. Between PL and S0, body
252 width increased significantly by 1.6 fold, remained unchanged between S0 and S15, and
253 increased again at S30 (Fig. 1A). Total length varied from an average of 6.60 ± 0.08 mm
254 in PL, S0, and S15 to 7.48 ± 0.17 mm in S30 (Fig. 1B).

255 The *gh* gene expression significantly increased with developmental stages (Fig. 2;
256 $p < 0.001$, $F_3 = 44.61$). It significantly increased by about threefold from PL stage to S0,

257 and it was 14 times higher than in the PL group 30 days after settlement. There was no
258 significant difference between S0 and S15.
259 The *g6pd* gene expression greatly increased from PL to S0, by up to 13 times
260 (Fig. 3A; $p < 0.001$, $F_3 = 70.72$). It then decreased sevenfold from S0 to S15 and
261 remained unchanged until S30. The *bal* gene expression was 2.5 times higher at S30 than
262 in the PL and S0 groups (Fig. 3B; $p < 0.01$, $F_3 = 5.13$). Relative gene expression was
263 intermediate in the S15 group, indicating that the expression activation had begun at this
264 stage. Expression of the *pk* gene continuously decreased following settlement to reach the
265 lowest relative expression level at S30 (Fig. 3C; $p < 0.05$, $F_3 = 3.12$), while *cox* gene
266 expression was about twofold higher in the S30 group than in the other groups (Fig. 3D;
267 $p < 0.01$, $F_3 = 5.87$).
268 The *prx6* gene expression increased at settlement relative to the PL group and
269 remained relatively unchanged afterward (Fig. 4A; $p < 0.05$, $F_3 = 4.15$). The *sod* gene
270 expression decreased from settlement to S0 and S15 and then increased to S30 (Fig. 4B; p
271 < 0.01, $F_3 = 13.01$). The *hsp70* gene expression increased at S15 and S30 and was about
272 sevenfold higher than at settlement and in the PL group (Fig. 4C; $p < 0.001$, $F_3 = 29.03$).
273
274 **Discussion**
275
276 The aim of this study was to better understand the molecular events governing
277 ontogeny in winter flounder *Pseudopleuronectes americanus* by measuring the
278 expression of seven genes involved in key metabolic processes. The results provided
279 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.

303 Gh is essential for the hepatic production of the insulin-like growth factors (Igf-1
304 and Igf-2), which mediate the anabolic actions of Gh (for review see Yousefian and
305 Shirzad 2011). We designed primers to study the expression of Igf-1 based on sequences
306 already identified in other fish species. Unfortunately, none allowed the amplification of
307 a homologous sequence.
308
309 **Changes in metabolic strategy**
310 The *pk*, *cox, g6pd,* and *bal* gene expressions could indicate changes in metabolic
311 strategy during metamorphosis. These changes could be linked with changes in activities
312 related to energy metabolism, in feeding behaviour, and in digestive physiology observed
313 in flatfish during metamorphosis in natural environments (Tanaka et al. 1996; Lagardère
314 et al. 1999; Cañavate et al. 2006). Strong morphological and functional changes in the
315 digestive system have been observed during metamorphosis in Japanese flounder
316 *Paralychtis olivaceus* (Tanaka et al. 1996). The authors suggested that these changes
317 were related to a shift in the diet at settlement from zooplanktonic to benthic prey.
318 From the pelagic larval to the beginning of the juvenile stage (S30), *pk* gene
319 expression significantly decreased, suggesting that the glycolysis pathway is more a
320 larval pathway than a juvenile one. Pk is an enzyme involved in the last step of
321 glycolysis, allowing the phosphorylation of adenosine di-phosphate (ADP) to adenosine
322 tri-phosphate (ATP). The higher expression of the *pk* gene in PL than in S30 may indicate
323 a higher anaerobic capacity of larvae compared to juveniles. In pelagic fish, an increase
324 in the anaerobic potential has been associated with the necessity for short-term
325 anaerobically powered swimming bursts during feeding and predator–prey interactions

326 that require rapid and efficient production of ATP (Childress and Somero 1990). This
327 could apply to pelagic marine fish larvae that may be more exposed to predators than
328 juveniles, which can hide in the bottom substrate. Moreover, the decrease in gene
329 expression related to the glycolysis pathway in settled juveniles compared to pelagic
330 larvae combined with the increase in *cox* gene expression measured at S30 suggest a
331 higher aerobic capacity in juveniles. This may reflect an increased aerobic metabolic rate
332 due to tissue reorganization and higher growth rate (*gh* gene expression, total length, and
333 maximum width) occurring at the same time. In contrast, Darias et al. (2008) used
334 microarrays to show an increased expression of genes involved in
335 neoglucogenesis/glycolysis in larval European seabass, with a significantly higher
336 anaerobic capacity at the end of the larval stage.
337 At settlement, *g6pd* gene expression strongly increased, suggesting a shift from
338 glycolysis to pentose–phosphate metabolism for this stage. Activation of the pentose–
339 phosphate pathway would be necessary to supply a large quantity of ribose units for
340 nucleotide synthesis and thus support the high demand for newly formed RNA and DNA
341 in fast-growing juveniles. This is in accordance with Munilla-Moran and Stark (1989),
342 who observed no detectable levels of G6pd enzyme activity in turbot *Scophthalmus*
343 *maximus* larvae, thereby concluding that the pentose–phosphate pathway is inoperative
344 during early life in turbot. Segner and Verreth (1995) also reported very low levels of
345 G6pd enzyme activity in early life stages of the pelagic catfish *Clarias gariepinus* that
346 increased through development.
347 At the juvenile stage (from S30), when growth resumes, *bal* gene expression
348 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 triacylgycerols. 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

372 in *P. americanus*. The results obtained demonstrate that all antioxidant genes tested
373 showed a maximal relative expression at S30, suggesting an increased response to stress
374 and antioxidant protection at the juvenile stage. Accordingly, in the sturgeon *Acipenser*
375 *naccarii,* an increased activity of antioxidant enzymes (Sod, catalase, glutathione
376 peroxidase, and glutathione reductase) was observed during the juvenile stage (Díaz et al.
377 2010). In winter flounder, the expression of these genes evolved in different ways during
378 metamorphosis.

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

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

393 At settlement, detoxification seems to be enhanced by Prx6. The Prx6 enzyme
394 plays a protective antioxidant role in cells, reducing and detoxifying hydrogen peroxide,

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

417 glycolysis to the pentose–phosphate pathway at settlement provides an additional
418 antioxidant protection during settlement.

419 Starting from S15, the marked increase in *hsp70* gene expression suggested high
420 environmental stress due to marked metabolic and cellular changes caused by ontogenetic
421 metamorphosis processes occurring during settlement in winter flounder (Tanaka et al.
422 1996; Gibson 1997). The development of cytoprotection and possible oxidative defence
423 through *hsp70* toward the end of fish metamorphosis has also been reported by Deane
424 and Woo (2003) in silver sea bream *Sparus sarba*. These authors found unaltered *hsp70*
425 transcript levels from 1–14 days post hatching that further progressively increased until
426 settlment. However, data are not available regarding the *hsp70* gene expression after the
427 settlement process.

428 The different expression patterns measured in our study for *sod*, *hsp70*, and *prx6*,
429 mostly observed at settlement or 15 days later (*hsp70*, *sod*), could partly be linked with
430 changes in feeding behaviour and digestive physiology observed during this period in
431 natural environments (Tanaka et al. 1996; Lagardère et al. 1999; Cañavate et al. 2006).
432 Previous studies demonstrated that dietary components can affect levels of antioxidant
433 enzymes in the larval and adult life stages of organisms (Peters and Livingstone 1996).
434 This hypothesis would be supported by the changes in energy metabolism that we
435 observed throughout the fish development in this study.
436

437 To conclude, our results revealed several important points. (1) Two critical stages
438 could be identified during winter flounder metamorphosis: a) the transition from the
439 pelagic to the benthic habitat and b) the metamorphosis maturation, when the larvae stay

440 settled without growth. They allow a more precise identification of the start of the
441 juvenile stage as the period denoted by the resumption of growth and the increased
442 aerobic capacity that occur approximately 30 days after settlement under the temperature
443 and photoperiod conditions used in the present experiment. (2) *gh* gene expression could
444 be a useful indicator of these main phases of development because its first expression
445 increase corresponds to the settlement phase while the second and more intense increase
446 corresponds to the beginning of the juvenile stage. (3) The results suggest that pelagic
447 larvae have a higher anaerobic capacity, while juveniles have a higher aerobic capacity
448 associated with a significant increase in growth rate. (4) The results suggest different
449 sources of energy synthesis for the different developmental stages: a) through glycolysis
450 in PL, b) through the pentose–phosphate pathway in settled larvae, and c) through lipid
451 metabolism in juveniles. (5) During the pelagic larval stage, oxidative stress and the
452 consequent antioxidative protection may be limited by glycolysis, while an increase in
453 antioxidative protection reinforced by the shift to the pentose–phosphate pathway seemed
454 to occur at settlement. Gene expression related to antioxidative protection was very high
455 in juveniles, certainly due to the higher growth rate observed at this period. (6) The
456 results suggest that cellular maintenance in organs such as blood, heart, muscle, and
457 kidney is fully functional from settlement, whereas the pancreas may continue to develop
458 until 30 days after settlement. The whole set of results obtained provides useful
459 preliminary indicators for the metamorphosis progress in this species and in larval fishes
460 through the understanding of molecular events governing ontogeny. However, because
461 post-transcriptional regulations may occur, it would be interesting to complete these
462 results with enzymatic measurements. Moreover, a future study should also include the

463 evaluation of gene expression for a greater number of genes in the different pathways of
464 interest. Finally, other novel factors, including both known and unknown genes and
465 pathways, could be identified by new transcriptomic tools such as the use of RNAseq.
466
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468
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475
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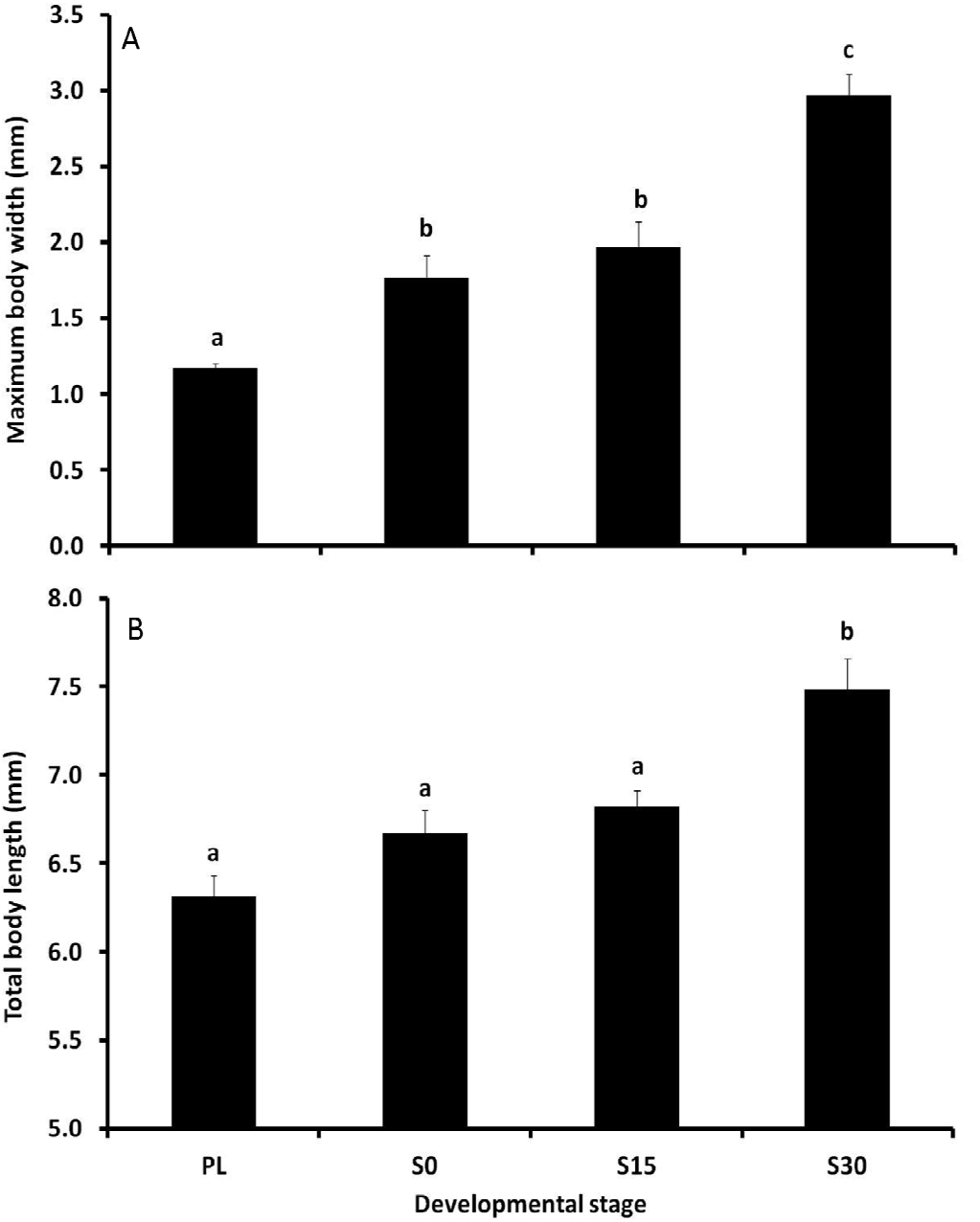
1 Figure 1



2

3

4

Figure 2

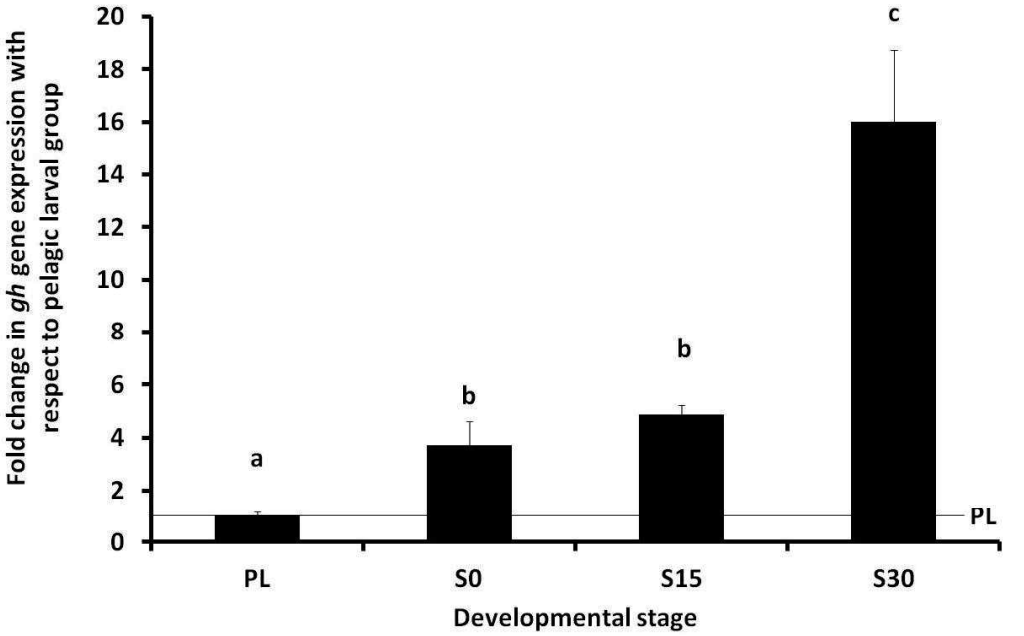
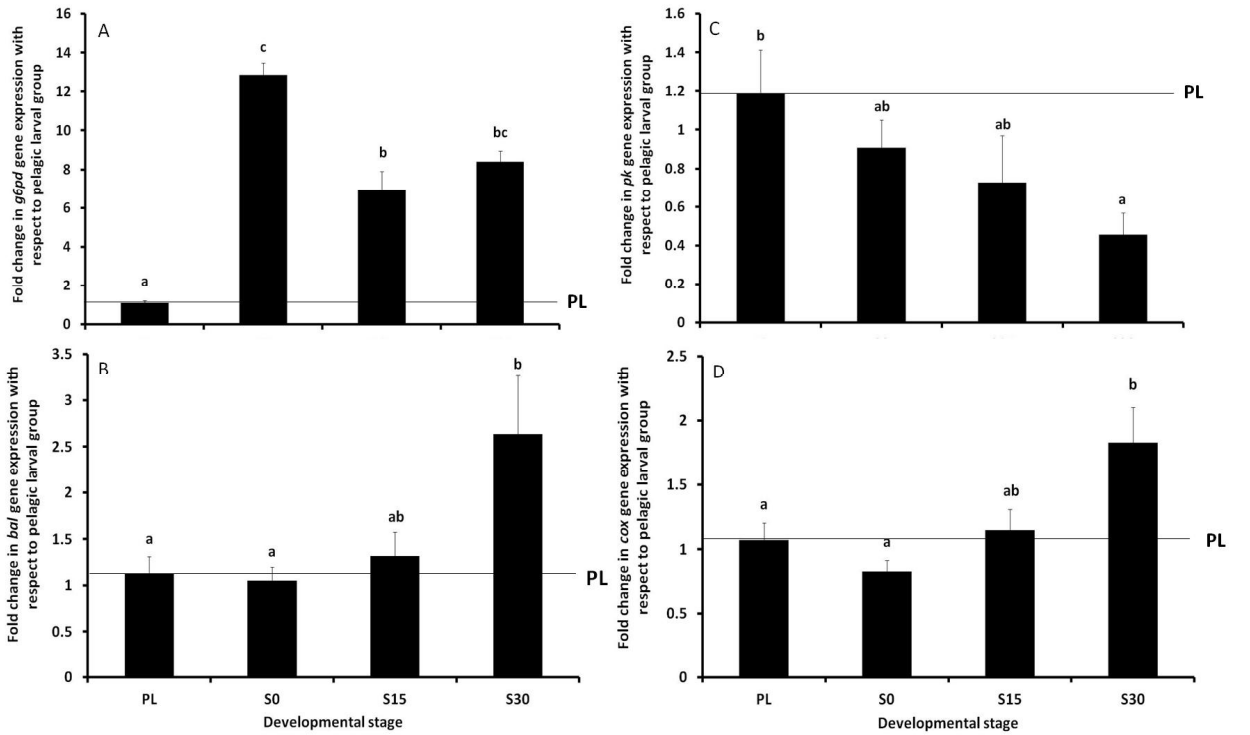
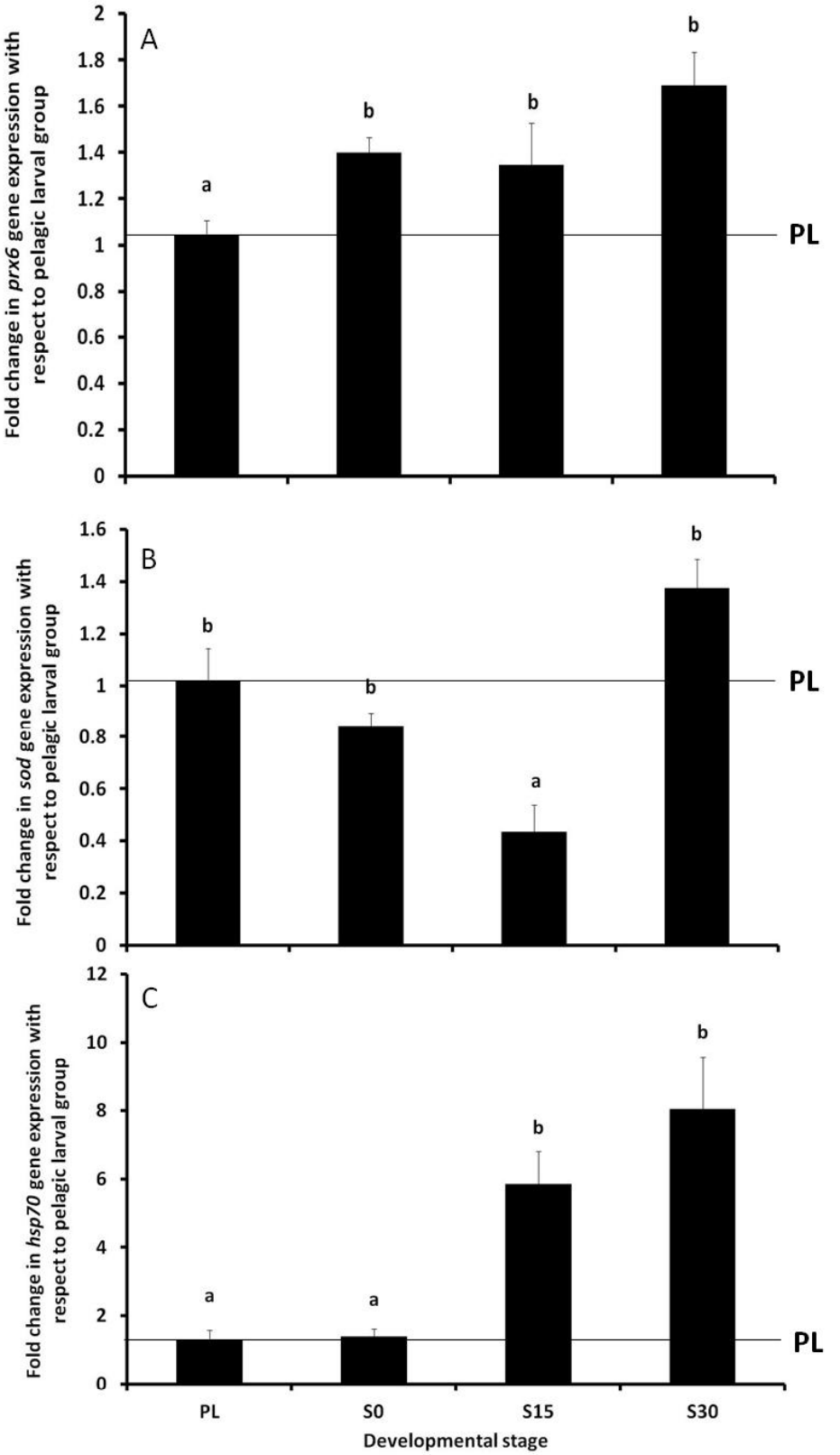

Fold change in gh gene expression with respect to pelagic larval group
20
18
16
14
12
10
8
6
4
2
0
a
b
b
c
PL
PL
S0
S15
S30
Developmental stage

Figure 3


A
Fold change in g6pd gene expression with respect to pelagic larval group
a
c
b
bc
PL
B
Fold change in bal gene expression with respect to pelagic larval group
a
a
ab
b
PL
C
Fold change in pk gene expression with respect to pelagic larval group
b
ab
ab
a
PL
D
Fold change in cox gene expression with respect to pelagic larval group
a
a
ab
b
PL
PL
S0
S15
S30
Developmental stage

7

Figure 4


A
Fold change in prx6 gene expression with respect to pelagic larval group
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
a
b
b
b
PL
B
Fold change in sod gene expression with respect to pelagic larval group
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
b
b
a
b
PL
C
Fold change in hsp70 gene expression with respect to pelagic larval group
12
10
8
6
4
2
0
a
a
b
b
PL
PL
S0
S15
S30
Developmental stage