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Neuroendocrine regulation of reproduction in Atlantic cod (*Gadus morhua*): Evidence of *Eya3* as an integrator of photoperiodic cues and nutritional regulation to initiate sexual maturation.

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Abstract

Evidence from mammals and aves alludes to a possibly conserved seasonal photoperiod induced neuroendocrine cascade which stimulates subsequent sexual maturation however our understanding of this mechanism in teleosts is lacking. Unlike all teleosts studied to date, the Atlantic cod (*Gadus morhua*) is a short day breeder with the reduction in day-length from the summer solstice stimulating gametogenesis. Cod specific orthologues of *eya3*, *tshβ* and *dio2* were identified and their expression was monitored in the brain and pituitary of cod held under either stimulated or inhibited photoperiod conditions. While no differential expression was apparent in brain *dio2* & *tshβ* and pituitary *tshβ*, there was significant temporal variation in expression of pituitary *eya3* under the SNP treatment, with expression level elevating in association with active gametogenesis. Under the LL treatment, sexual maturation was inhibited and there was a corresponding suppression of *eya3* expression. In a second study the impact of size/energetic status on the initiation of sexual maturation was investigated. In the feed restricted population maturation was significantly suppressed (5% sexually mature) compared to the ab libitum fed stock (95% sexually mature) with there being a concomitant significant suppression in pituitary *eya3* expression. Overall, these results suggest that pituitary *eya3* has the potential to act as an integrator of both environmental and energetic regulation of sexual maturation of cod. Being the first account of *eya3* induction in a short day breeding teleost, the conserved association with stimulation of reproduction and not seasonal state indicates that the upstream drivers which initiate the pathway differ among vertebrates according to their breeding strategies, but the pathway itself and its role in the reproductive cascade appears to be conserved across the vertebrate clade.

Keywords: Atlantic cod; *eya3*; *dio2*, *tshβ*, reproduction; photoperiod; seasonality.

Introduction

Initiation of sexual maturation and subsequent gametogenesis in teleosts is under the neuroendocrine control of the brain-pituitary-gonad (BPG) axis (Zohar et al. 2010). Although the BPG axis has been well described in vertebrates, the external factors and associated mechanisms which initiate gametogenesis and regulate reproduction remain to be clearly defined in fish (Migaud et al. 2010). In temperate regions, many species utilise seasonal changes in the environment to entrain reproduction and maximise offspring survival. While a number of environmental cues have been shown to influence the timing of recruitment into gametogenesis and subsequent spawning (Wright and Trippel 2009), photoperiod has long been acknowledged as the main proximate environmental cue for initiating and entraining seasonal reproduction (Sumpter 1990, Migaud et al. 2010). In fact, the power of photoperiod to entrain reproduction can be evidenced through the numerous studies which show that maturation can be artificially regulated and even inhibited through photoperiod manipulation in a range of species (Imsland et al. 2003, Begtashi et al. 2004, Davie et al. 2007a, Felip et al. 2008, Carrillo et al. 2010, Taranger et al. 2010). However, the performance of photoperiod manipulation in commercial settings can be variable (Taranger et al. 2006) in part due to technical failings in the systems used (Cowan et al. 2011) but is also due, in part, to our lack of understanding of the photoneuroendocrine systems (PNES) which governs how fish perceive their environment to then entrain maturation (Migaud et al. 2010).

Like many temperate species, Atlantic cod (*Gadus morhua*) is a capital breeder exhibiting group-synchronous spawning in batches over several weeks in the winter, spring and early summer (Kjesbu and Kryvi 1989, Wright 2013). Classified as short-day breeders, the reduction in day length following summer solstice initiates gametogenesis which continues through the autumn and winter months (Davie et al. 2007c). Concomitantly it

has been observed that there is increased expression of the gonadotropin, *follicle stimulating hormone* (*fshβ*), in male (de Almeida et al. 2011) and female (Cowan et al. 2012) cod from September onwards, marking the initiation of the BPG cascade. Importantly, if cod are maintained under continuous illumination from the summer solstice, to mask the reduction in photoperiod, gametogenesis and the associated autumnal gonadotropin surge are completely inhibited (Cowan et al. 2012). This would suggest that between the reduction of photoperiod from the summer solstice in June, and the gonadotropin gene expression surge seen from September onwards, lies an as yet unidentified signalling pathway linking the proximate photoperiod cue to the initiation of the BPG cascade in cod.

One such pathway has emerged over the last decade linking sexual development and photoperiod in seasonal vertebrates. There is increasing evidence to suggest that the pathway itself appears to be conserved across the vertebrate clade with examples emerging in mammalian, avian and most recently some teleost models (Follett 2015). This pathway begins with transduction of the light signal into the pituitary. In mammals, this light signal is relayed exclusively by melatonin, while avian models exhibit direct innervation through deep brain photoreceptors (Dardente et al. 2014, Follett 2015). This light signal up-regulates expression of eyes-absent homolog 3 (*eya3*) in the pars tuberalis (PT) of the pituitary. As a transcriptional coactivator, Eya3, modulates *thyroid stimulating hormone* (*tshβ*) expression, up-regulating Tshβ production in the PT. Tshβ is then transported into the medio-basal hypothalamus (MBH), up-regulating *type 2 iodothyronine deiodinase* (*dio2*) and supressing *type 3 iodothyronine deiodinase* (*dio3*) expression creating a reciprocal switching mechanism which modulates thyroid hormone production. The Eya3-Tshβ-Dio2/3 pathway thus provides a clear seasonal signal to the BPG axis, with the potential to consolidate the PNES and BPG axes (Dardente et al.

2014). Indeed, photoperiod manipulation studies in aves have identified both *eya3* and *tshβ* in the first wave response to the short day (SD) to long day (LD) switch, followed by an up-regulation of *dio2* and down-regulation of *dio3*, preceding a surge in gonadotropin secretion, indicating stimulation of the BPG (Yoshimura et al. 2003, Nakao et al. 2008b, Ono et al. 2009a, 2009b). Furthermore, intracerebroventricular administration of Tsh to quail held under continuous SD up-regulates *dio2* expression, stimulating the reproductive axis, while administration of Tshβ antibodies to quail exposed to continuous LD elicited the opposite response (Nakao et al. 2008a). Studies in quail particularly have highlighted how rapidly this pathway can be induced ultimately resulting in an up-regulation of luteinising hormone (LH) within 22 hours of the photoperiod switch (Nakao et al. 2008a). Similar responses have been shown in mammals (Revel et al. 2006, Barrett et al. 2007).

While the pathway appears to be conserved across the vertebrate clade, there are some differences in the upstream drivers and the resultant down-stream affects. In the case of long-day breeding birds and mammals, including quail (Nakao et al. 2008a, Ono et al. 2009b), mice (Ono et al. 2008, Masumoto et al. 2010), hamsters (Watanabe et al. 2004, Yasuo et al. 2010) and rats (Yasuo et al. 2007, Ross et al. 2011), which initiate their reproductive cycle under an increasing photoperiod, the Eya3-Tshβ-Dio2/3 pathway is positively stimulated following an increasing photoperiod or LD light signal. However, for short-day breeding sheep and Saanen goats, the pathway is less straightforward. In sheep it would appear that the pathway is initiated by a LD light signal, as is the case for long-day breeders (Dupré et al. 2010, Sáenz de Miera et al. 2013) which is possibly a reflection of the key stimulatory role of LD on sheep reproductive cycle irrespective of their SD breeding behaviour (Dardente 2012). For Saanen goats however, a LD stimulus

inhibits Dio2 production, suggestive of a reversal of the pathways role to reflect the seasonal entrainment of reproduction (Yasuo et al. 2006).

Studies of this pathway in teleosts are lacking and they are limited to species which initiate gametogenesis in response to a LD stimulus. Nakane et al. (2013) first reported in masu salmon (*Oncorhynchus masou masou* B.) that a stimulatory LD photoperiod upregulated *tshβ* and *dio2* expression in the saccus vasculosus. Moreover, removal of this organ appeared to inhibit maturation, which would strongly suggest a role in mediating photic perception and the subsequent regulation of the BPG axis (Nakane et al. 2013). In the three-spined stickleback (*Gasterosteus aculeatus* L.), *tshβ* expression again echoes the mammalian model, being upregulated under LD stimulus, and was strongly linked to the maturation response (O'Brien et al. 2012). These studies both support an overall conservation of the pathway in vertebrates, but are a long way from truly defining it and linking the PNES and BPG axes in fish, highlighting a need for additional research. In order to expand on our current understanding of the mechanisms controlling seasonal physiology in vertebrates and more specifically driving maturation in fish, the current study aimed to identify the Atlantic cod paralogues for key targets within the proposed pathway (*eya3*, *tshβ* and *dio2*) and then explore, in two separate trials, the response to environmental (photoperiod) and nutritional regulation of sexual maturation. The purpose of these studies was to investigate if a pathway similar to that seen in other vertebrate models also exists in Atlantic cod, developing our understanding of the PNES-BPG network and potentially highlighting early biomarkers for maturation commitment in Atlantic cod.

Materials & Methods

Fish Husbandry & Sample Collection

Photoperiod control of maturation study

This study was conducted at Machrihanish Marine Environmental Research Laboratory (55.44 º N, 5.44 º W). Immature mixed-sex Atlantic cod (Approx. 14 Months post hatch, avg. weight = 411 ± 5.59g) were randomly allocated between two fully covered light-proof tanks. Fish (n= 374) were acclimated for 5 weeks prior to initiation of the trial and throughout the study fish were fed to satiation in a commercial cod diet (BioMar, Grangemouth, UK) throughout the ambient daylight period. A baseline sample of 13 fish (6 male, 7 female) was collected in July, following which the experimental treatments commenced. Two treatments were set up; 1) a simulated natural photoperiod (SNP) to stimulate maturation, and 2) a constant light treatment (LL) applied from July to inhibit maturation. Fish were sampled monthly over the course of 13 months from July to the subsequent August, with up to 12 fish being euthanized in accordance with the Animals (Scientific Procedures) Act 1986 (ASPA). Alongside individual biometric data (total weight and length), whole brains and pituitaries were dissected from each sampled fish and individually snap frozen, within 1 minute of dissection, over liquid nitrogen vapour and stored at – 70 ºC. All sampling was completed between 10:00 and 12:00 on the day of collection. Furthermore, gonadal samples were dissected and preserved in 10% neutral buffered formalin, prior to histological classification of ovarian development into one of seven developmental stages (for full details see Cowan et al. 2011). For further details on the husbandry, experimental setup and sampling protocols used see Cowan et al. (2012).

Nutritional regulation of maturation study

This study was conducted at Marine Science Scotland, Marine Laboratory facility in Aberdeen (57.06 º N, 2.04 º W). Immature under-yearling (0-group) cod were collected between August and March from the wild, on the east coast of Scotland, offshore from Stonehaven and transferred to the Marine Laboratory prior to the study starting. Fish were held under ambient photoperiod conditions and a constant temperature of 9°C and were fed *ad libitum* with a pelleted feed (Vitalis, 5mm; Skretting UK). On the 9th April 165 individuals (mean weigh 40 ± 1.6g) were anaesthetised (MS222, Pharmaq, Fordingbridge, UK) and PIT tagged (Trovan Ltd, Hull UK). Following recovery from the tagging, the population was split by size and in to two conditions, a high ration treatment (*ad libitum* feeding) (wet weight = 56.15 ± 2.13 g, Total length = 19.19 ± 0.21 cm, n = 81) and a low ration treatment (feed restricted to a daily ration of 2.25% body weight increasing to 3% body weight over the course of the trial) (Wet weight = 34.32 ± 0.99 g, total length = 16.62 ± 0.16 cm, n = 84). Each treatment group was further split into duplicate rearing units where environmental conditions were retained as before. Fish were monitored every 21 days where all individuals wet weight and total length were recorded which allowed for the adjustment of feeding rations in the low ration population. On the 12th September, 23rd October and 21st November, 20 individuals from each treatment were randomly selected and euthanised in accordance with the Animals (Scientific Procedures) Act 1986 (ASPA). Individual weight and length was recorded before fish were dissected to remove the liver which was weighed to calculate hepatosomatic index (HSI) (liver weight/somatic weight *100) and gonads were removed and weighed to calculate gonadosomatic index (GSI) (gonad weight/somatic weight *100) before gonad samples were preserved in 10% neutral buffered formalin for later histological analysis. Gonadal development was qualified by histological analysis in accordance with the descriptions in Bucholtz et al (2007), Almeida et al. (2008) and

188 Kjesbu and Kryvi (1989). In addition, pituitaries from the 21st of November sampling
189 were snap frozen over liquid nitrogen and subsequently stored at -80°C for expression
190 analysis (*n* = 12 per treatment, 6M:6F).
191
192 *Ethical Statement*
193 All experimental work was performed in accordance with the Animals (Scientific
194 Procedures) Act 1986 (ASPA).
195
196 RNA Extraction & cDNA Synthesis
197 For both studies the same total RNA extraction and complementary DNA (cDNA)
198 synthesis methodology was used. Briefly, total RNA was extracted from the pituitary and
199 whole brain samples for each fish by thawing in 1 ml TRIzol® reagent (Invitrogen, UK)
200 per 100 mg of tissue. Samples were then homogenized over ice, and RNA was extracted
201 from the solution following the manufacturers' protocol, and eluted in 15 µl or 50 µl of
202 MilliQ water for pituitary or brain samples respectively. RNA yield was checked using a
203 ND 100 Nanodrop spectrophotometer (Labtech Int., East Sussex, and UK). cDNA was
204 generated using a High-Capacity cDNA Reverse Transcriptase kit (Applied Biosystems,
205 USA) as follows: 1µg of total RNA in volume of 10µl MilliQ water was added to a master
206 mix containing 2 µl of RT buffer, 0.8 µl dNTPs mix, 2 µl RT Random Primer, 1 µl
207 Reverse Transcriptase and 4.2 µl of MilliQ water, to create a final reaction volume of 20
208 µl. Thermocycling conditions were 25 ºC for 10 mins, 37 ºC for 120 mins, and 85 ºC for
209 5 mins. Samples were then diluted with MilliQ water to a final volume of 200 µl (1:10)
210 and stored at -20 ºC until required.
211
212 Primer Design & Molecular Cloning

213 Partial cDNA sequences for each target gene (*Eya3*, *Tshβ* & *Dio2*) were generated using
214 primers designed with primer-BLAST software
215 (http://www.ncbi.nlm.nih.gov/tools/primer-blast). One primer pair was designed for each
216 gene of interest based on gene sequences identified within the Atlantic cod genome
217 (gadMor1, January 2010 assembly) (Table 1). For each gene, an *in silico* analysis was
218 performed for quality assurance of the chosen sequences whereby the cod specific
219 transcripts were compared against previously described vertebrate sequences to compare
220 sequence identity and putative conserved domains using BLAST and ClustalW analysis
221 while MEGA6® (Tamura et al. 2013) was used to deduce and bootstrap phylogenetic
222 trees using the maximum likelihood method (Felsenstein 1981). The house-keeping gene,
223 e*longation factor 1 alpha* (*ef1α*), was selected as the reference gene for normalisation of
224 the quantitative PCR (QPCR) data. One primer pair for this gene was also designed,
225 following the same process described above.
226 Each primer pair was verified by Polymerase Chain Reaction (PCR). These PCR
227 reactions were performed using; 1µl reaction buffer, 0.8 µl of forward and reverse primers
228 (10 pmol µl-1), 0.45 µl MgCl2, 0.25 µl dNTPs, 0.04 µl Klear Taq DNA polymerase
229 (KBioSciences, UK), 5.66 µl MilliQ and 1 µl of synthesised cDNA. The thermal cycling
230 program consisted of a 15 min initial denaturation at 95 ºC, followed by 35 cycles of 95
231 ºC for 30 s, X ºC for 30 s, and 72 ºC for 60 s, with a final extension at 72 ºC for 4 mins
232 where the annealing temperature, denoted X, varied with each specific primer pair (Table
233 1). PCR products were then checked on a 1 % agarose gel to verify size and the presence
234 of a single product, before being extracted and purified from the gel using a NucleoSpin®
235 Gel and PCR Clean-up kit (MACHEREY-NAGEL, Germany). The purified PCR
236 products were then cloned into a PGEM®-T easy vector (PGEM®-T easy vector systems,
237 Promega, UK). Plasmid insertion was checked by restriction digest, as well as sequencing

238 (GATC Biotech, London, UK). Upon confirmation of correct product insert, a serial
239 dilution of linearised plasmid sample was used for the subsequent QPCR assay.
240
241 Quantitative PCR
242 cDNA for each sample was synthesised from RNA using the methods described above.
243 To rationalise analysis that was performed, gene expression analysis was focused on *eya3*
244 and *tshβ* in the pituitary and *tshβ* and *dio2* in the whole brain (excluding pituitary) to be
245 reflective of the sites of expression of these targets reactive to seasonal photoperiod
246 stimulation reported in the literature. *ef1α* was used in all cases as a housekeeping
247 reference gene and expression was measured in both brain and pituitary samples.
248 In order to validate QPCR assays, a serial dilution of linearised plasmid containing the
249 ligated target gene fragment for each gene was tested, from which three dilutions were
250 taken forward as standards to enable absolute quantification of mRNA levels. Each
251 QPCR plate was prepared, including in duplicate, three chosen standards for that
252 particular gene, one non-template control (MilliQ water), and four internal control
253 samples consisting of four random cDNA samples from the target tissue which were
254 included in every plate to control for inter-assay variation.
255 The QPCR assay was performed using 0.7 µl of both forward and reverse primer (10
256 pmol ul-1), with one fortieth of the original cDNA reaction, 10 µl of ABsolute™ QPCR
257 SYBR-green master mix (Thermo Scientific, St. Leon-Rot, Germany), and 3.6 µl of
258 MilliQ water to a final reaction volume of 20 µl. The reactions were run on an Eppendorf
259 Mastercycler® ep realplex thermocycler using the following programme; 95 ºC for 15
260 mins, 45 cycles of 95 ºC for 15 s, X ºC (annealing temperature of primers, see Table 1)
261 for 15 s, 72 ºC for 30 s, followed by a temperature ramp from 65 ºC to 95 ºC with
262 fluorescence being measured every 0.5 ºC to create a melt curve. The copy numbers of

each gene were automatically calculated by the realplex software by comparison to the standard curve created by the serial plasmid dilutions which following correction was then expressed as the absolute copy number per µg of total RNA. QPCR efficiency was calculated for each plate run, with efficiency being greater that 90 % in all cases. A melt curve was also created to verify the specificity of the primers used, and as an indication of assay contamination.

Data Analysis

For the photoperiod study, temporal and treatment variations in target gene expression were analysed using a general linear model with time, treatment and time×treatment interaction considered with the genders being analysed separately with the assumptions of normality and homogeneity of variance being achieved through log. transformation. For the nutritional study to account for differences in initial size and repeated measures the changes in length, weight and Fulton's condition factor (K), over the course of the study were analysed using a linear mixed-effects model, with time considered as a categorical variable and with individual fish included as a random effect. The fish random effect was assumed to vary with time according to an antedependence structure of order 2. Assumptions of normality and homogeneity of variance were achieved through log. transformation. Maturity was analysed using a binomial GLM, with logit link to account for the binomial nature of this response. Sex and treatment were treated as factors, with time as a continuous variable. A significance level of $p < 0.05$ was applied in all analyses. All models were implemented in R3.2.2 using mgcv, MASS and nlme libraries (model scripts are included within the Supplementary Material and Methods).

RESULTS

Atlantic cod *eya3*, *tshβ* and *dio2* partial cDNA sequence and phylogenetic analysis

BLAST analysis of the cod genome (gadMor1 assembly) identified predicted gene sequences for each of the chosen targets. For *eya3*, there is a single gene annotated (ENSDMOG00000012887) which consists of a partial coding sequence (cds) length of 1446bp with higher similarity to other teleost *eya3* sequences (64-84% identity) than to other vertebrates (*circa* 40-60% identity mammalian and avian Eya3 sequences) (Supplemental figure 1). The 482 amino acid (aa) deduced partial protein fragment included the conserved C-terminal "EYA" domain as well as three putative haloacid dehalogenase (HAD) motifs (Supplemental figure 2).

There are two *tshβ* paralogs annotated within the cod genome; *tshβa* (ENSGMOG00000017064) located on GeneScaffold 2156, and (ENSGMOG00000011010) on GeneScaffold 2185 which shall be referred to here as *tshββ*. Phylogenetic analysis (Supplemental figure 3) and synteny analysis (Alignment created in the Genomicus online resource, *data not shown*) confirmed *tshβa* to be the ancestral vertebrate orthologue thus it was this transcript that was taken forward for expression analysis. The 387 bp partial cds sequence translated into a deduced aa sequence of 129 aa with moderate similarity to other teleosts (55-65% identity) which was lower still with other vertebrates (*circa* 40% identity). However, all 12 cysteine residues of the conserved cysteine-knot domain common to all glycoprotein hormone family β subunits were identifiable (Supplemental figure 4).

With regards to *dio2*, a partial cds sequence, 228bp long (ENSGMOG00000020274) was identified from the cod genome which translated into a deduced aa sequence of 76 aa. While only a small partial fragment, the predicted aa showed high similarity with teleost and mammalian *dio2* sequences (65-75% identity). Phylogenetic analysis (Supplemental

figure 5) grouped the partial sequence within teleost *dio2* gene clade. The deduced aa sequence contained a portion of the iodothyronine deiodinase domain (Supplemental figure 6).

Photoperiod control of maturation study

Brain expression of dio2 and tshβ

Within the period July to December there was no significant interaction of time and photoperiod treatment on whole brain *dio2* expression for male (P = 0.235) or female (P = 0.258) Atlantic cod (Figure 1). Similarly, there were no significant differences in the temporal expression between July and December of *tshβ* in the brain between treatments, for males (P = 0.789) or females (P = 0.454) potentially due to the high variability observed in individual expression level (Figure 2). Due to the lack of significant temporal variation with respect to treatment no further samples were analysed.

Pituitary expression of eya3 and tshβ

Expression of *tshβ* in the pituitary did not significantly differ between treatments over time between July and December for either males (P = 0.867) or females (P = 0.141) (Figure 3). *eya3* exhibited significant differences in temporal expression between treatments for both males (P < 0.001) and females (P < 0.001), for this reason the sample set was extended to a full annual cycle from July to the subsequent August. Cod held under the SNP stimulus exhibited an increase in pituitary *eya3* expression from September, with significantly elevated expression in November, December, February and March for females, and in October, November, December, February and March for males with respect to LL treated animals (Figure 4). Expression levels then returned to being comparable with LL treated fish from April to August. In contrast, LL individuals showed

no significant temporal variation in expression. While monthly mean *eya3* levels showed a similar trend to that observed in gonadosomatic index (Figure 4) there was no significant correlation between the parameters. However, when stage of gonadal development was considered there was a clear association between *eya3* expression level and histological stage of development in ovaries (Figure 5) with levels being significantly elevated in individuals that were undergoing active gametogenesis in comparison to those which were either immature or regressed.

Nutritional regulation of maturation study

Over the course of the study there was no difference in growth performance between the two sexes within a given treatment. The high ration treatment had an overall 46% increase in growth rate over the study period compared to the low ration treatment (calculated as thermal growth coefficient, *data not presented*). Therefore, by the studies end the relative difference in mean weight between treatments had increased from 73% to 182% (Table 2). Fultons condition factor was not affected by treatment ($p = 0.19$), being maintained at 0.9 ± 0.14 over the course of the study, however relative liver size (HSI) was significantly lower in the lower ration treatment at all sampling points compared to the high ration treatment (Table 2). Fish were classified as mature or immature through histological analysis of the gonads. Males were classified maturing if spermatids were present, while females were classified maturing when cortical alveoli were observed. By the completion of the study 95% of fish from the high ration treatment were maturing while only 5% (one fish) were mature within the low ration treatment (Table 2). There was no significant difference in pituitary *eya3* expression levels between males and females and so samples were pooled by treatment. Analysis of the final terminal sample revealed that expression

was significantly elevated in the high ration group (Figure 6) with mean expression levels being 1.6 times higher than in the low ration group.

DISCUSSION

Entrainment of seasonal physiology and in particular reproduction, is a fundamentally basic mechanism evident in most temperate vertebrates. It is well known that seasonal maturation cycles are achieved through precise coordination of gonadal recruitment and development with the prevailing environmental signals gated by permissive physiological states. The mechanism by which these various signals are integrated centrally and thereafter used to entrain the neuroendocrine pathways and subsequently gonadal development remains elusive. There have been a number of studies collectively suggesting that, at its core, there is a conserved neuroendocrine pathway that ultimately results in the stimulation of thyroid hormones to initiate the BPG cascade which has the potential to act as such a master regulator (Follett 2015, Helfer et al. 2018, Shinomiya & Yoshimura 2018). Though evidence of this functional pathway in teleosts is limited, studies performed to date suggest an up-regulation of the proposed pathway in response to a stimulatory (LD) photoperiod (O'Brien et al. 2012, Nakane et al. 2013). The current study is the first to examine this pathway in a teleost species which initiates gametogenesis following a SD stimulus (Davie et al. 2007c). Results show a clear seasonal mRNA expression profile of the transcriptional coactivator Eya3 that initiates the pathway, being upregulated by a stimulatory SD photoperiod. The data also suggests that *eya3* expression could represent a candidate biomarker for the "master regulator" of maturation commitment given that this upregulation can be suppressed through either a photoperiod manipulation or through dietary restriction; interventions which both independently can suppress the initiation of maturation.

In silico analysis of the cod genome clearly identified cod specific partial cDNA sequences for *eya3*, *tshβ* and *dio2*. In all cases the deduced aa sequence shared high identity and conserved functional domains which confirm these transcripts as being cod

387 specific orthologues . Although Lorgen et al. (2015) have identified two *dio2* paralogs
388 (*dio2a* and *dio2b*) in Atlantic salmon, phylogenetic analysis indicates that salmon *dio2a*
389 and *dio2b* diverged during the salmon specific whole-genome duplication, and therefore
390 the sub-functionalisation theory they discuss represents an adaptation of the salmonid
391 family alone. As in the current study, only one *dio2* gene has yet been identified among
392 other teleost species. Maugars et al. (2014) demonstrated *tshβ* duplication in a wide range
393 of teleost species including Atlantic cod, as confirmed in the current study. The current
394 analysis rationalised the target choice based on synteny analysis and confirmation of
395 functional domains to conclude that *tshβa* was the most conserved *tshβ* paralog among
396 teleosts which was in agreement with O'Brien et al. (2012). However, as these data
397 suggest a teleost specific duplication of *tshβ*, functional studies of these paralogues are
398 required to explore any possible sub functionalisation of their role that may have
399 occurred. To that end, recent results emerging from salmon, following the completion of
400 the current work, suggest that the t*shβb* could play a more direct role in seasonal
401 regulation in physiology which clearly warrants wider investigation across teleosts
402 (Fleming et al. 2019). Overall, while further work should be undertaken to sequence the
403 full length of each target gene, the high level of identity observed for *eya3*, *tshβa* and
404 *dio2* would suggest evolutionary conservation, reflective of an important functional role
405 for these genes in cod.

406 The working model of the neuroendocrine pathway sees the light signal being relayed to
407 the pituitary where *eya3* expression upregulates Tshβ production which subsequently
408 stimulates *dio2* expression in the third ventricle of the brain (Follett 2015). Of the three
409 genes analysed in the current study, *eya3* alone exhibited a significant seasonal response.
410 This was observed as a surge in expression from August until March in the SNP
411 treatment, exhibiting a maximal 17-fold difference in February for females, and a 19-fold

412 difference in November for males, before returning to basal levels as day length
413 increased. By comparison, the LL treatment showed no significant elevation in
414 expression for the duration of the study. It is clear from these results that expression of
415 the transcriptional coactivator *eya3*, follows a distinct seasonal cycle, and furthermore
416 this pattern can be inhibited through photoperiod manipulation by masking the seasonal
417 change in day length. Neither pituitary *tshβ*, nor whole brain *dio2* or *tshβ* showed clear
418 evidence of differential temporal expression with respect to the photoperiod treatments.
419 It should be acknowledged that there was notable individual variance in expression level
420 of *tshβ* in particular, the drivers of which are unclear. A confounding factor may be due
421 to the fact that whole-brain homogenates were used to look at expression of both *dio2*
422 and *tshβ* in the current study. In mammals and birds, *tshβ* is known to exhibit localised
423 expression within the ependymal cells of the PT under LD stimulus (Nakao et al. 2008a,
424 Yasuo et al. 2010). Although fish do not possess a PT, previous studies in masu salmon
425 have isolated photoperiod induced *Dio2* expression to a region known as the *saccus*
426 *vasculosus* (Nakane et al. 2013). The same study also found significant differences in
427 Dio2 and Tsh protein levels in this region between salmon exposed to LD and SD
428 stimulus, but no concurrent differences at the mRNA level, suggesting that post-
429 transcriptional mechanisms may be more important in regulating patterns in expression
430 for these genes and modulating the cascade of events which follow.
431 The response of *eya3* to SD stimulus, shown in the present study, has never before been
432 described in other vertebrate models. In long-day breeders such as hamsters, rats, mice
433 and quail, upregulation of Eya3 occurs in response to a LD stimulus and typically plays
434 a permissive role in the initiation of the reproductive cycle. Long-day breeders which
435 experience the transition from SD to LD exhibit an initial increase in Eya3, which
436 stimulates Tshβ production. This in turn induces the expression of *dio2*, which is

responsible for converting the prehormone thyroxine T4 into its bioactive form triiodothyronine T3. Increased expression of T3 appears to regulate GnRH production, thus initiating the reproductive cycle (Yoshimura 2004, Barrett et al. 2007, Hanon et al. 2008, Yasuo and Yoshimura 2009). In Soay sheep, the same expression cascade is observed, with stimulation of *eya3* expression following a LD stimulus as despite breeding in reducing daylengths of the autumn, the Soay sheep rely on this LD signal to initiate the reproductive cycle during the following winter (Dardente 2012, Hazlerigg et al. 2018). For Saanen goats however, the LD signal suppresses *dio2* expression in the brain, in direct contrast to the expression profile in sheep. Initiation of the pathway in goats appears to follow a SD stimulus, suggesting a switch in the initiation of the pathway from LD to SD between these closely related species (Yasuo et al. 2006). Saanen goats therefore represent the only other comparable example of SD stimulated gametogenesis among all vertebrate examples studied to date. Although that study focused on *dio2* expression, it showed positive stimulation of the pathway following a SD stimulus. It is clear that in the current study, *Eya3* expression is initiated by a SD cue in Atlantic cod, and provides a clear signal of seasonally altering photoperiod. We would therefore conclude that the upstream drivers which initiate the *eya3-tshβ-dio2* pathway differ among vertebrates according to their breeding strategies, but the pathway itself and its role in the reproductive cascade appears to be conserved across the vertebrate clade.

In relation to other fish species, this study is the first investigation of *eya3*, *tshβ* or *dio2* expression in a teleost species which shows stimulation of the initiation of the gametogenesis pathway under a SD stimulus. Though masu salmon have been suggested to be short-day breeders (e.g. Nakane et al. 2013), masu salmon utilise a LD cue to initiate gametogenesis (Takashima & Yamada, 1984) with the decision to mature being taken prior to the summer solstice as day length is increasing (Amano et al. 1993, 1995) like

462 other salmonids (Migaud et al. 2010) and furthermore, much like Soay sheep (Hazlerigg
463 et al 2018). LD induced *tshβ* expression in the *saccus vasculosus* of the masu salmon is
464 thought to act as the main upstream driver governing the induction of season-associated
465 Dio2, and the resultant stimulation of the BPG axis (Nakane et al. 2013). Similarly, in
466 long-day breeding sticklebacks, acute induction of *tshβ* in conjunction with *gnrh* and *lhβ*
467 under a LD stimulus, support the idea of a conserved pathway linking the PNES and BPG
468 axes in species where long-day photoperiods stimulates the initiation of the reproductive
469 cycle (O'Brien et al. 2012). As neither study has considered *eya3* expression, it is difficult
470 to draw general conclusions about the conservation of this pathway in fish. Individually
471 *eya3*, *tshβ* and *dio2* have each been identified as important messengers of the seasonal
472 photoperiod in teleosts, but it is clear that further work is required to fully understand
473 if/how these genes interact.
474 The nutritional regulation of maturation study clarified that size is indeed a major factor
475 regulating maturation commitment in Atlantic cod. There was a significant difference in
476 total weight between treatments at the end of the experiment. However, it is more likely
477 that weight around the autumn equinox, when cod are likely to assess their energetic
478 status and initiate reproduction, represents the threshold for growth/energy theorised by
479 Bromage et al. (2001). The smallest fish to subsequently mature measured just 136 g in
480 mid-September, and on average, maturing fish measured 281 g (± 15 g) at this time. By
481 comparison, immature fish had a mean weight of 122 g (± 9.6 g) with the largest immature
482 fish weighing 185 g during this period. This suggests a minimum threshold weight of ~
483 130 g must be reached prior to the switch from long day to short days around the autumn
484 equinox, and that all fish that reach a weight above ~190 g are likely to be recruited into
485 maturation. Thus a size range of 130 – 190 g is theorised to be the critical determining
486 threshold range for growth/energy acquisition in cod. This lower weight is similar to that

found in a previous experimental study of cod growth and maturity, although there were also population specific weight differences in the maturation threshold (Harrald et al., 2010). As body weight, energy storage and growth are generally correlated it is possible that the rate of change in weight or lipid storage in the liver may be the actual energetic signal for maturation commitment (Wright, 2007).

The analysis of *Eya3* expression in this study is, to the authors knowledge, the first study to provide evidence for growth/energy related regulation of this gene, linking photoperiod entrainment with the growth and reproductive axes. As this gene is strongly associated with the photoperiodic signal, previous work has utilised photoperiodic manipulations to assess the function of this gene in relaying the photoperiodic message to the BPG axis (Ono et al. 2009b, Masumoto et al. 2010, Hazelrigg et al. 2018). However, such studies have not attempted to assess the potential role of size or energetic state and would be ill equipped to do so given the confounding maturation response to photoperiod. Given the ability to suppress maturation through diet in cod, this study provided a perfect opportunity to assess the possible interaction between the somatotropic axis and this gene under the same photoperiodic conditions. The significant suppression of maturation and *eya3* expression in response to nutritional restriction, indicates that the growth axis (via a sized linked indicator) may indeed regulate *eya3* production, providing a mechanism for the integration of environmental and endogenous drivers of maturation in cod. This work is still in its infancy, and though this preliminary study greatly improves our understanding of the mechanisms shaping the maturation response of fish that are SD breeders such as cod, further work is needed.

As is the case for many temperate fish species, initiation of gametogenesis in cod is limited to a specific "window of opportunity", which is defined by a specific environmental signal which must coincide with a permissive physiological state to allow

commitment to reproduce the following season (Thorpe et al. 1990, Bromage et al. 2001). Previous experiments in cod indicate this window spans between the summer solstice and mid-autumn (Davie et al. 2007b) when the beginning of gametogenesis is measureable by the increased expression of gonadotropins (Cowan et al. 2012). Precise definitions of this window represents a gap in our current understanding of the maturation cycle in cod. The current study has identified *eya3* as a component of a potential master regulator integrating information concerning both the permissive physiological state (~190g wet weight) as well as the proximate photoperiodic cue. This narrows down our search for the specific photoperiod cue which initiates gametogenesis to when day length declines to around 12 hrs. Increasing *fshβ* expression and GSI values appear to coincide with the up-regulation of *eya3* observed (Cowan et al. 2012), indicating that the seasonal response initiated by *eya3* may occur over a very short time span as demonstrated in mammals and aves (Nakao et al. 2008a, Masumoto et al. 2010, Dupré et al. 2010). Future work should investigate the photoperiodic induction of *eya3* expression in tighter temporal resolution than has been achieved to date.

The aim of this study was to investigate if the *eya3-tshβ-dio2* pathway, which is known to consolidate the PNES and BPG axis in other seasonal vertebrates, is conserved in Atlantic cod. *eya3* was found to clearly show potential as an integrator of both environmental and energetic regulation of sexual maturation of the species. This is also the first account of *eya3* induction in a teleost which initiates gametogenesis following a SD stimulus suggesting an overall conservation of the functional pathway in vertebrates. By inference this work therefore alludes to the presence of an as yet unidentified process that allows for differential responsiveness to seasonal photic stimulation upstream of *eya3* which requires further investigation.

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739 **Table 1**. Primer name, sequence, predicted amplicon size, annealing temperature and Ensembl transcript ID for each gene of interest.

Name	Sequence	Size (bp)	Annealing temperature (°C)	Ensembl Transcript ID
*eya3*F	5'-TCCCTGCTGCTGATCCAGTCC-3'	109	61.5	ENSGMOT00000014154
*eya3*R	5'-AGACCTCTCCCAGGCCGTAGA-3'			
*tshβ*F	5'-AGCGAGGGCAGCTCTCTGTTC-3'	116	61.5	ENSGMOT00000018765
*tshβ*R	5'-GTACACGTGGGCCTGTTGCTG-3'			
*dio2*F	5'-GTTCCTCGCGCTGTACGACTC-3'	130	61.5	ENSGMOT00000022279
*dio2*R	5'-CCAGATGGAGCGCATCCCC-3'			
*ef1α*F	5'-TGAACCACCCTGGCACCATCT-3'	84	60	ENSGMOT00000013187
*ef1α*R	5'-GCTCGTTGAACTTGCAGGCGA-3'			

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742

743 **Table 2:** Biometric data separated by gender and treatment from the study initiation to the maturation assessment window for the second study
744 population.

745

Treatment	Date	Wet weight (g)	Total Length (cm)	HSI (%)	GSI (%)	Proportion mature (%)	N
Female	12th September	253.8 ± 17.7	29.9 ± 0.7	10.32 ± 0.27	0.27 ± 0.01	0	11
High ration	23rd October	407.1 ± 36.5	34.6 ± 0.9	11.16 ± 0.29	0.39 ± 0.03	66.7%	9
	21st November	514.5 ± 37.4	35.8 ± 0.8	10.56 ± 0.40	0.55 ± 0.05	100%	9
Female	12th September	126.5 ± 9.7	23.5 ± 0.9	3.66 ± 0.25	0.25 ± 0.02	0	10
Low ration	23rd October	172.9 ± 12.6	28.3 ± 0.7	3.04 ± 0.33	0.29 ± 0.03	0	10
	21st November	178.5 ± 16.4	27.1 ± 1.0	2.90 ± 0.28	0.33 ± 0.04	0	11
Male	12th September	244.6 ± 24.2	29.4 ± 0.6	9.70 ± 0.28	0.08 ± 0.01	0	9
High ration	23rd October	332.5 ± 34.1	31.9 ± 0.9	10.38 ± 0.39	0.23 ± 0.02	27.3%	11
	21st November	454.3 ± 41.6	34.5 ± 0.9	11.28 ± 0.39	1.03 ± 0.18	92.3%	13
Male	12th September	115.9 ± 12.4	23.6 ± 1.0	2.75 ± 0.24	0.04 ± 0.01	0	10
Low ration	23rd October	148.7 ± 8.0	26.5 ± 0.6	2.77 ± 0.26	0.08 ± 0.01	0	10
	21st November	179.8 ± 13.8	26.9 ± 1.0	3.45 ± 0.30	0.20 ± 0.03	11.1%	9

746

Figure 1. Absolute mRNA expression levels of *Dio2* in the brain of **A**) female and **B**) male Atlantic cod exposed to simulated natural photoperiod (SNP) or constant light (LL) from July to December. Data presented as mean ± SE

Figure 2. Absolute mRNA expression levels of *Tsh*β in the brain of **A**) female and **B**) male Atlantic cod exposed to simulated natural photoperiod (SNP) or constant light (LL) from July to December. Data presented as mean ± SE.

Figure 3. Absolute mRNA expression levels of *Tsh*β in the pituitary of **A**) female and **B**) male Atlantic cod exposed to simulated natural photoperiod (SNP) or constant light (LL) from July to December. Data presented as mean ± SE.

Figure 4. Absolute mRNA expression levels of *Eya3* in the pituitary of A) female and B) male Atlantic cod in comparison to C) gonadosomatic index (GSI) in fish exposed to simulated natural photoperiod (SNP) or constant light (LL) from July to August. * denotes a significant difference between treatments in a given month. Data presented as mean ± SE.

Figure 5. Absolute mRNA expression levels of *Eya3* in the pituitary of female Atlantic cod under the SNP lighting treatment, classed according to stage of maturity (Imm: Immature (GSI = 0.9 ± 0.1%), CA: Cortical alveoli (GSI = 2.0 ± 0.2%), EV: early vitellogenesis (GSI = 4.0 ± 0.5%), LV: late vitellogenesis (GSI = 10.1 ± 1.8%), SPW: Spawning (GSI = 19.7 ± 4.1%), SPT: spent (GSI = 2.2 ± 0.2%), REG: Regressing (GSI = 3.0 ± 0.6%). Significant differences in *Eya3* expression between developmental stages are denoted by lowercase lettering. Data presented as mean ± SE (*n*= 6-14 per developmental stage).

Figure 6. Absolute mRNA expression levels of pituitary *Eya3* in sexually mature Atlantic cod which had been fed *ad libitum* (high ration) or immature Atlantic cod which were reared on a restricted ration (2.25% - 3% body weight, Low ration) for 7 months prior to assessment. Superscripts denote a significant difference between treatments. Data presented as mean ± SE (*n*=12)

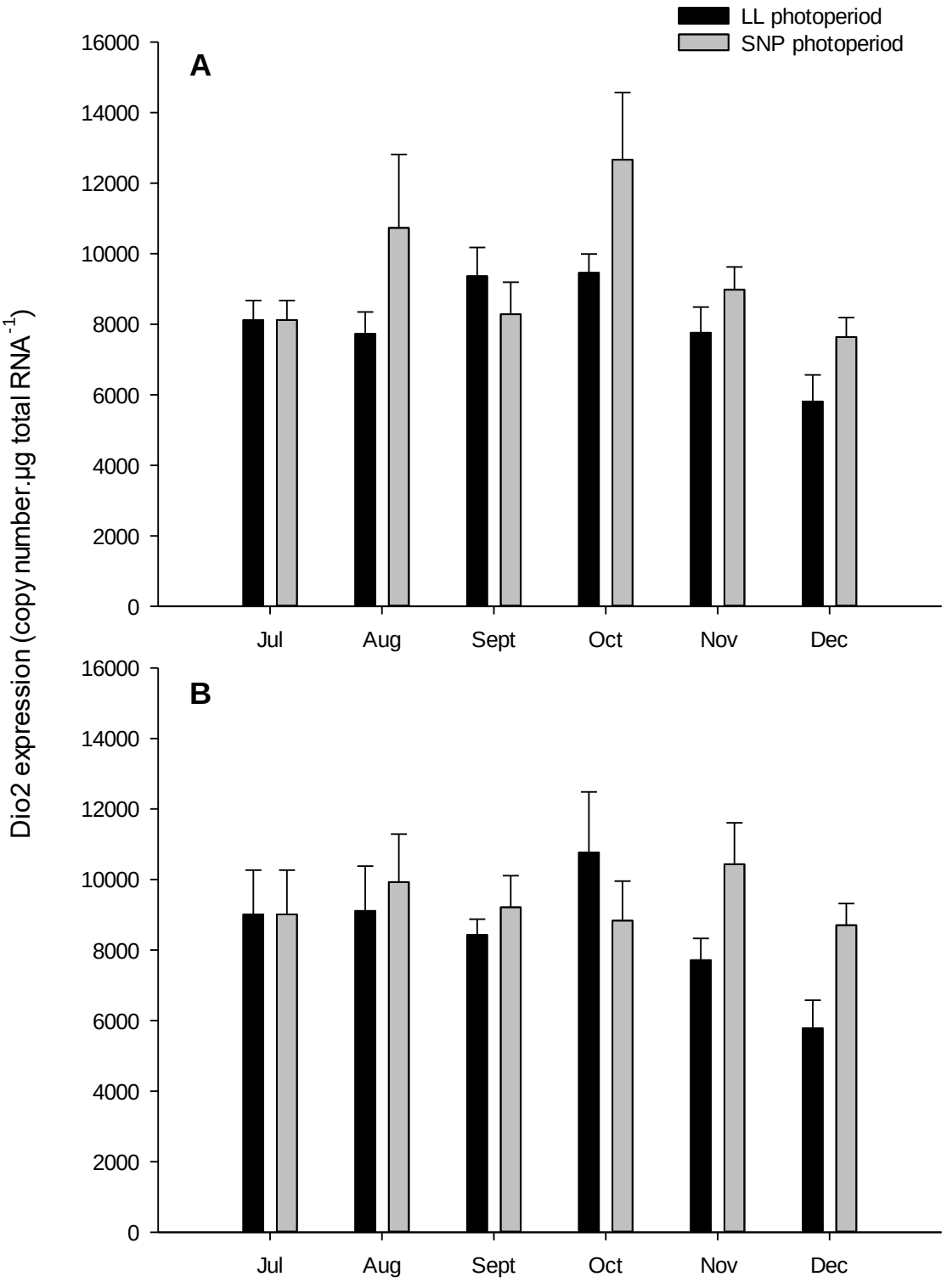
LL photoperiod
SNP photoperiod
A
B
Dio2 expression (copy number.µg total RNA-1)
16000
14000
12000
10000
8000
6000
4000
2000
0
Jul
Aug
Sept
Oct
Nov
Dec

Figure 1

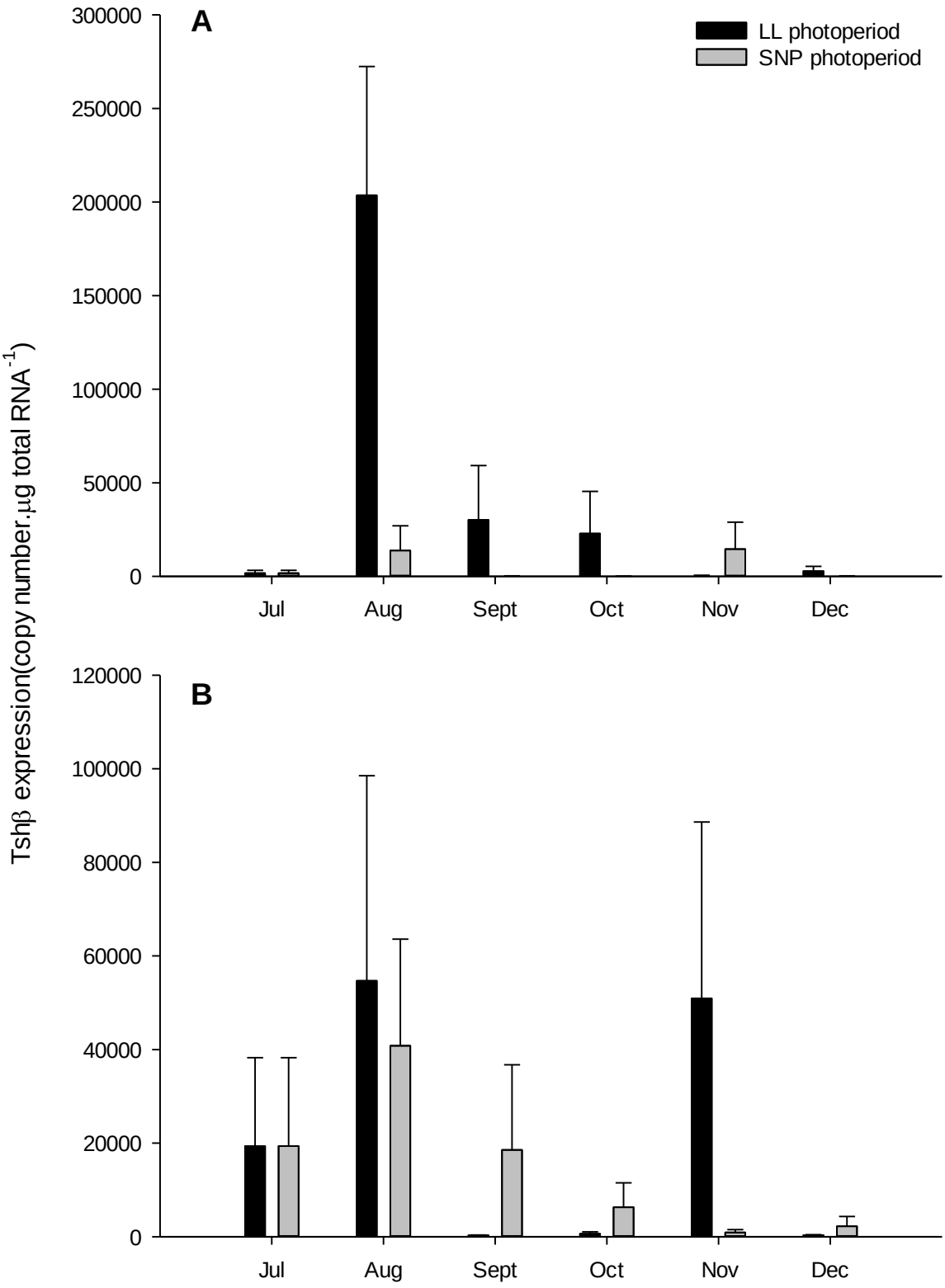
A
LL photoperiod
SNP photoperiod
300000
250000
200000
150000
100000
50000
0
Jul
Aug
Sept
Oct
Nov
Dec
B
120000
100000
80000
60000
40000
20000
0
Jul
Aug
Sept
Oct
Nov
Dec
Tshβ expression(copy number.μg total RNA-1)

Figure 2

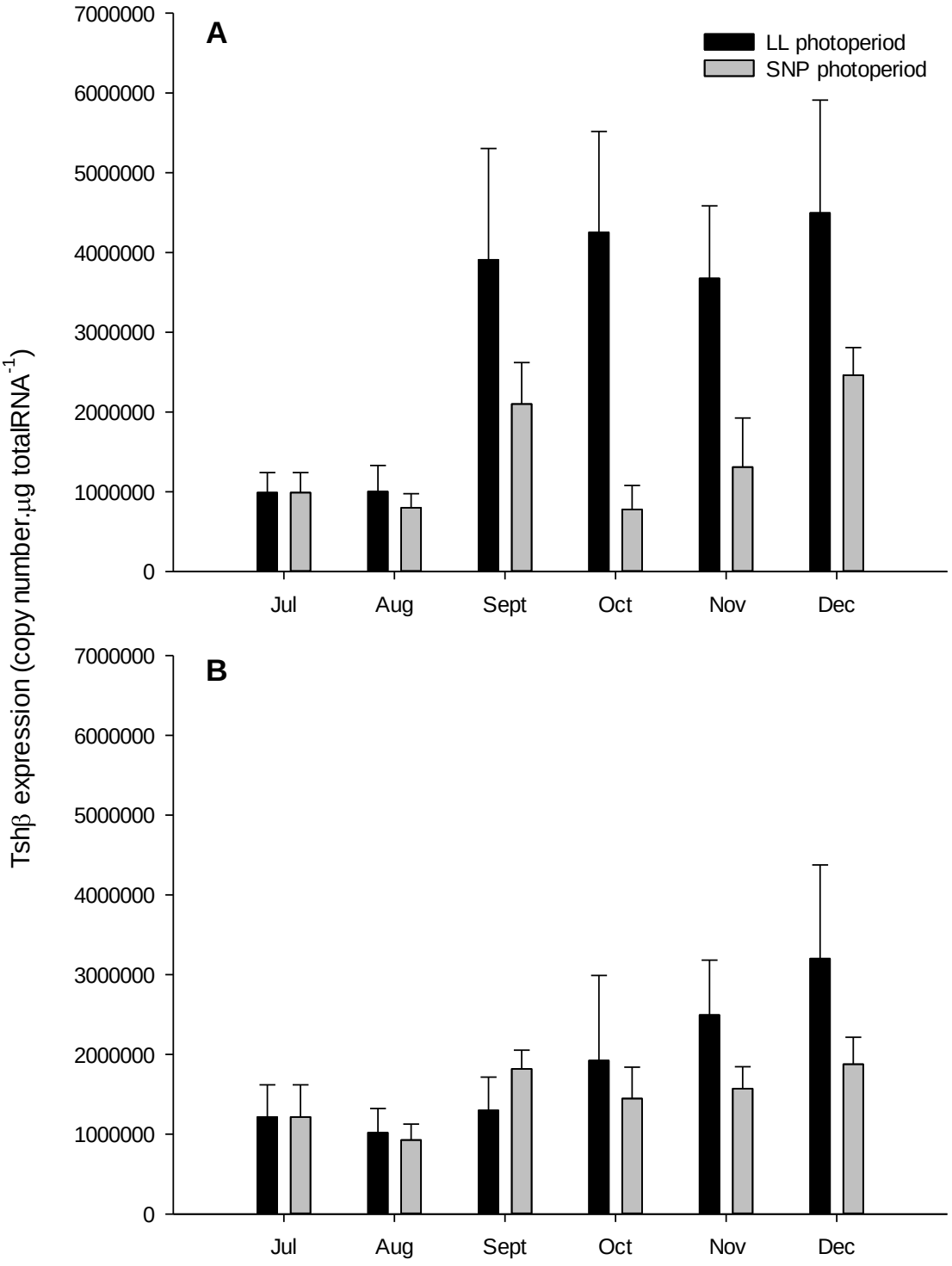
A
B
LL photoperiod
SNP photoperiod
Tshβ expression (copy number,µg totalRNA-1)
7000000
6000000
5000000
4000000
3000000
2000000
1000000
0
Jul
Aug
Sept
Oct
Nov
Dec

Figure 3

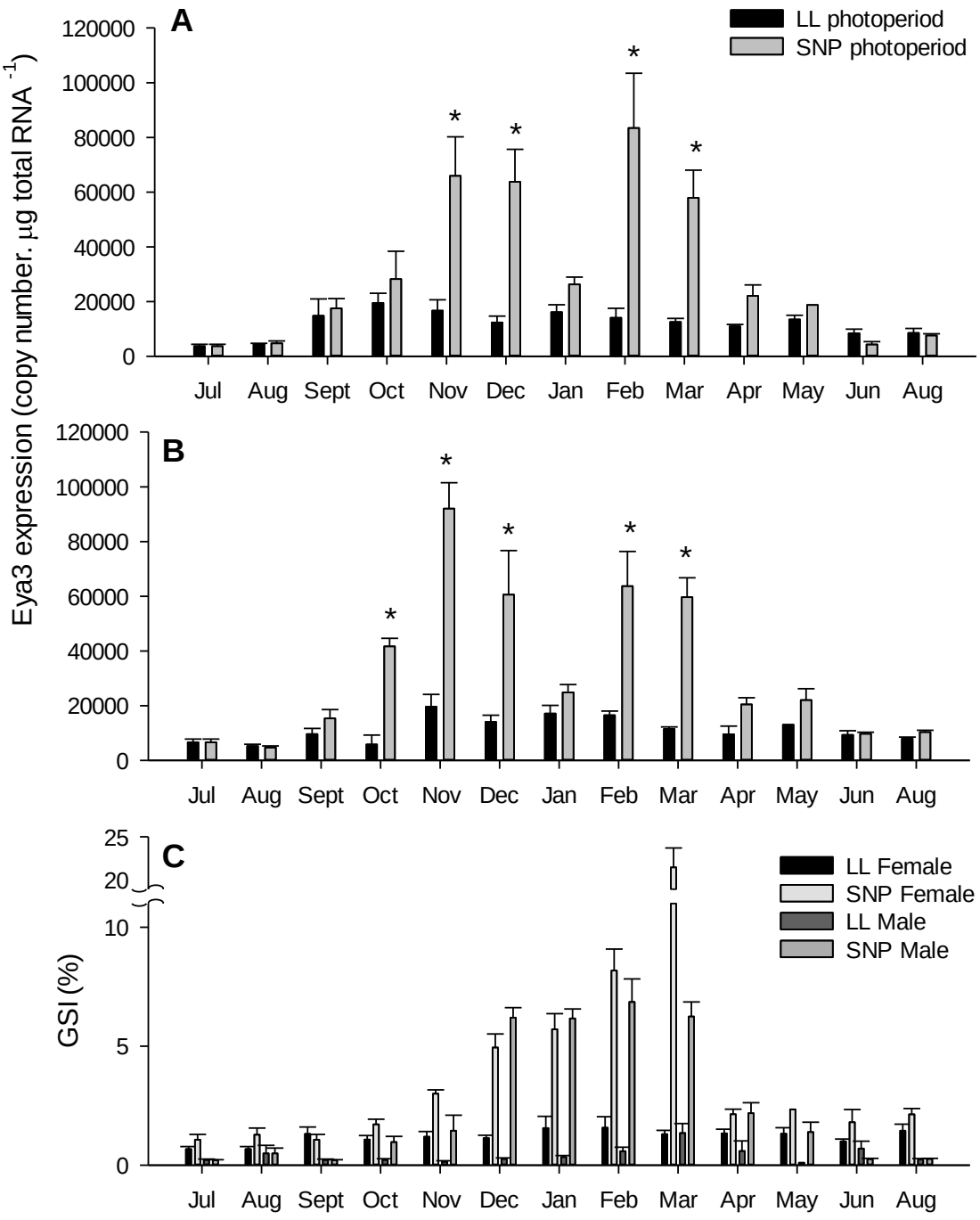
A
LL photoperiod
SNP photoperiod
Eya3 expression (copy number. µg total RNA -1)
120000
100000
80000
60000
40000
20000
0
Jul
Aug
Sept
Oct
Nov
Dec
Jan
Feb
Mar
Apr
May
Jun
Aug
B
C
LL Female
SNP Female
LL Male
SNP Male
GSI (%)
25
20
10
5
0

Figure 4

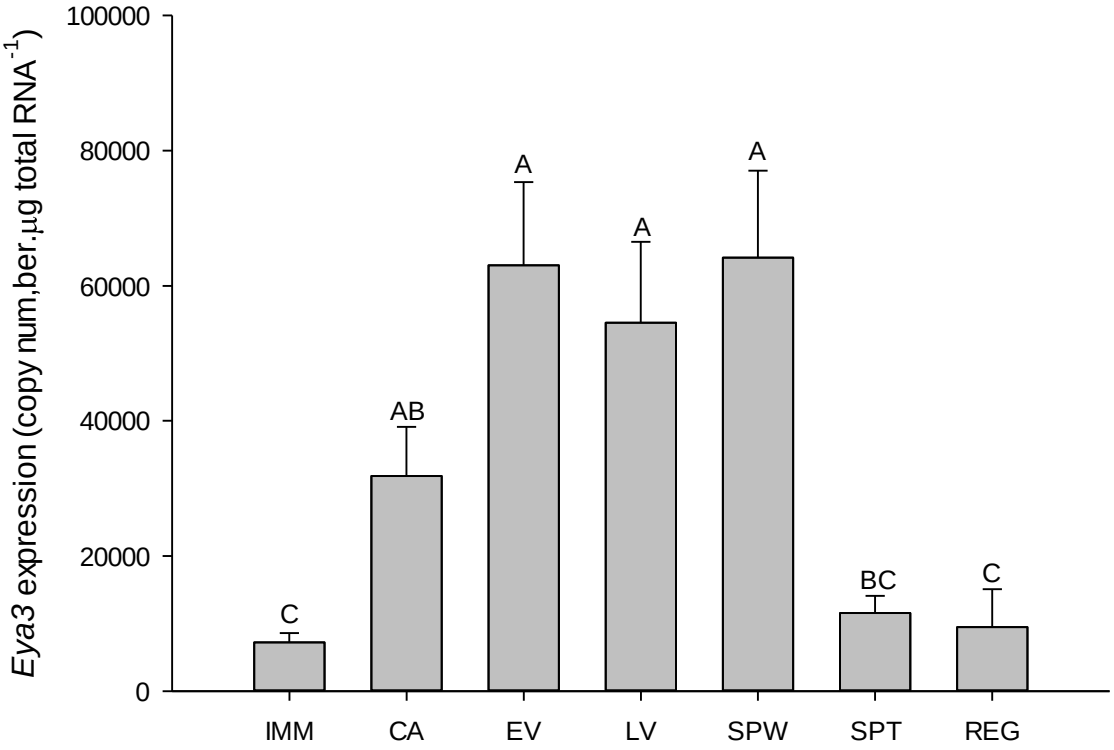
100000
80000
60000
40000
20000
0
Eya3 expression (copy num,ber.µg total RNA-1)
C
AB
A
A
A
BC
C
IMM
CA
EV
LV
SPW
SPT
REG

Figure 5

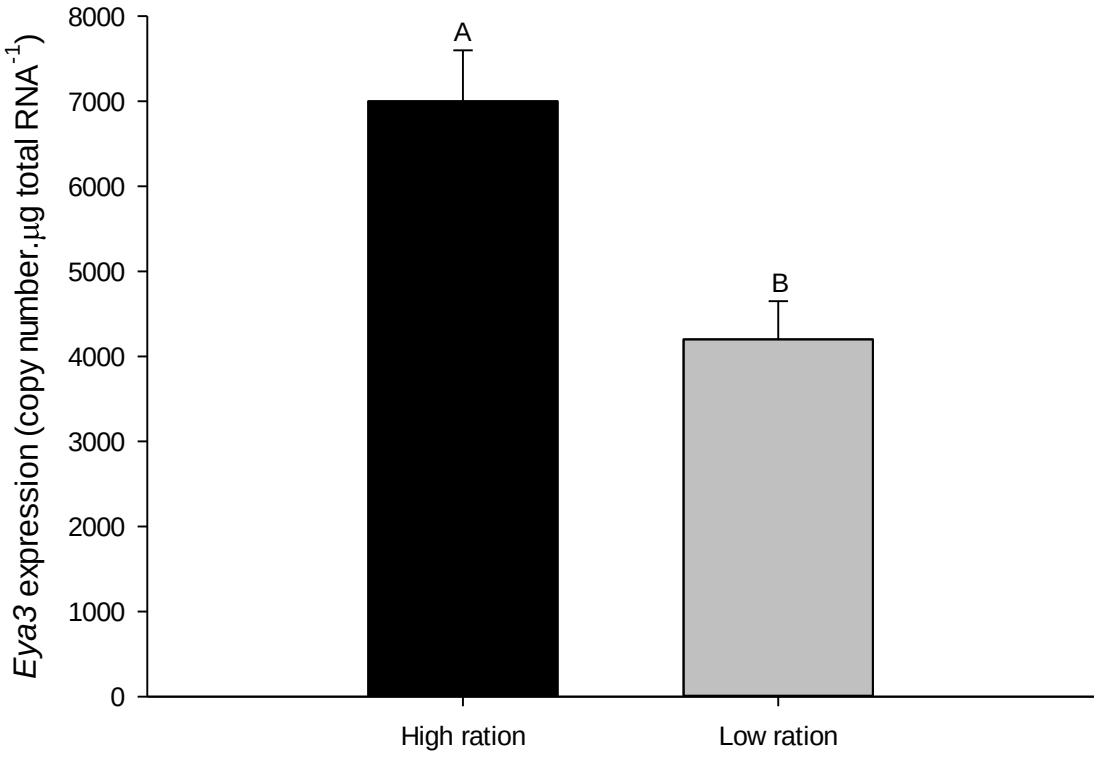
8000
7000
6000
5000
4000
3000
2000
1000
0
Eya3 expression (copy number.µg total RNA-1)
A
B
High ration
Low ration

Figure 6