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

4

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19 **Abstract**

20 Evidence from mammals and aves alludes to a possibly conserved seasonal photoperiod
21 induced neuroendocrine cascade which stimulates subsequent sexual maturation however
22 our understanding of this mechanism in teleosts is lacking. Unlike all teleosts studied to
23 date, the Atlantic cod (*Gadus morhua*) is a short day breeder with the reduction in day-
24 length from the summer solstice stimulating gametogenesis. Cod specific orthologues of
25 *eEya3*, *tFshβ* and *dDio2* were identified and their expression was monitored in the brain
26 and pituitary of cod held under either stimulated or inhibited photoperiod conditions.
27 While no differential expression was apparent in brain *dDio2* & *tFshβ* and pituitary *tFshβ*,
28 there was significant temporal variation in expression of pituitary *eEya3* under the SNP
29 treatment, with expression level elevating in association with active gametogenesis.
30 Under the LL treatment, sexual maturation was inhibited and there was a corresponding
31 suppression of *eEya3* expression. In a second study the impact of size/energetic status on
32 the initiation of sexual maturation was investigated. In the feed restricted population
33 maturation was significantly suppressed (5% sexually mature) compared to the ab libitum
34 fed stock (95% sexually mature) with there being a concomitant significant suppression
35 in pituitary *eEya3* expression. Overall, these results suggest that pituitary *eEya3* has the
36 potential to act as an integrator of both environmental and energetic regulation of sexual
37 maturation of cod. Being the first account of *Eeya3* induction in a short day breeding
38 teleost, the conserved association with stimulation of reproduction and not seasonal state
39 indicates that the upstream drivers which initiate the pathway differ among vertebrates
40 according to their breeding strategies, but the pathway itself and its role in the
41 reproductive cascade appears to be conserved across the vertebrate clade.

42

43 Keywords: Atlantic cod; *Eeya3*; *Ddio2*, *Ttshβ*, reproduction; photoperiod; seasonality.

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44 **Introduction**

45 Initiation of sexual maturation and subsequent gametogenesis in teleosts is under the
46 neuroendocrine control of the brain-pituitary-gonad (BPG) axis (Zohar et al. 2010).
47 Although the BPG axis has been well described in vertebrates, the external factors and
48 associated mechanisms which initiate gametogenesis and regulate reproduction remain
49 to be clearly defined in fish (Migaud et al. 2010). In temperate regions, many species
50 utilise seasonal changes in the environment to entrain reproduction and maximise
51 offspring survival. While a number of environmental cues have been shown to influence
52 the timing of recruitment into gametogenesis and subsequent spawning (Wright and
53 Trippel 2009), photoperiod has long been acknowledged as the main proximate
54 environmental cue for initiating and entraining seasonal reproduction (Sumpter 1990,
55 Migaud et al. 2010). In fact, the power of photoperiod to entrain reproduction can be
56 evidenced through the numerous studies which show that maturation can be artificially
57 regulated and even inhibited through photoperiod manipulation in a range of species
58 (Imsland et al. 2003, Begtashi et al. 2004, Davie et al. 2007a, Felip et al. 2008, Carrillo
59 et al. 2010, Taranger et al. 2010). However, the performance of photoperiod manipulation
60 in commercial settings can be variable (Taranger et al. 2006) in part due to technical
61 failings in the systems used (Cowan et al. 2011) but is also due, in part, to our lack of
62 understanding of the photoneuroendocrine systems (PNES) which governs how fish
63 perceive their environment to then entrain maturation (Migaud et al. 2010).
64 Like many temperate species, Atlantic cod (*Gadus morhua*) is a capital breeder exhibiting
65 group-synchronous spawning in batches over several weeks in the winter, spring and
66 early summer (Kjesbu and Kryvi 1989, Wright 2013). Classified as short-day breeders,
67 the reduction in day length following summer solstice initiates gametogenesis which
68 continues through the autumn and winter months (Davie et al. 2007c). Concomitantly it

69 has been observed that there is increased expression of the gonadotropin, *follicle*
70 *stimulating hormone* (*fshβ*), in male (de Almeida et al. 2011) and female (Cowan et al.
71 2012) cod from September onwards, marking the initiation of the BPG cascade.
72 Importantly, if cod are maintained under continuous illumination from the summer
73 solstice, to mask the reduction in photoperiod, gametogenesis and the associated
74 autumnal gonadotropin surge are completely inhibited (Cowan et al. 2012). This would
75 suggest that between the reduction of photoperiod from the summer solstice in June, and
76 the gonadotropin gene expression surge seen from September onwards, lies an as yet
77 unidentified signalling pathway linking the proximate photoperiod cue to the initiation of
78 the BPG cascade in cod.

79 One such pathway has emerged over the last decade linking sexual development and
80 photoperiod in seasonal vertebrates. There is increasing evidence to suggest that the
81 pathway itself appears to be conserved across the vertebrate clade with examples
82 emerging in mammalian, avian and most recently some teleost models (Follett 2015).
83 This pathway begins with transduction of the light signal into the pituitary. In mammals,
84 this light signal is relayed exclusively by melatonin, while avian models exhibit direct
85 innervation through deep brain photoreceptors (Dardente et al. 2014, Follett 2015). This
86 light signal up-regulates expression of eyes-absent homolog 3 (*Eya3*) in the pars
87 tuberalis (PT) of the pituitary. As a transcriptional coactivator, Eya3, modulates *thyroid*
88 *stimulating hormone* (*tFshβ*) expression, up-regulating Tshβ production in the PT. Tshβ
89 is then transported into the medio-basal hypothalamus (MBH), up-regulating *type 2*
90 *iodothyronine deiodinase* (*dDio2*) and suppressing *type 3 iodothyronine deiodinase*
91 (*dDio3*) expression creating a reciprocal switching mechanism which modulates thyroid
92 hormone production. The Eya3-Tshβ-Dio2/3 pathway thus provides a clear seasonal
93 signal to the BPG axis, with the potential to consolidate the PNES and BPG axes

94 (Dardente et al. 2014). Indeed, photoperiod manipulation studies in aves have identified
95 both *Eya3* and *Tsh β* in the first wave response to the short day (SD) to long day (LD)
96 switch, followed by an up-regulation of *Ddio2* and down-regulation of *Ddio3*, preceding
97 a surge in gonadotropin secretion, indicating stimulation of the BPG (Yoshimura et al.
98 2003, Nakao et al. 2008b, Ono et al. 2009a, 2009b). Furthermore, intracerebroventricular
99 administration of Tsh to quail held under continuous SD up-regulates *Ddio2* expression,
100 stimulating the reproductive axis, while administration of Tsh β antibodies to quail
101 exposed to continuous LD elicited the opposite response (Nakao et al. 2008a). Studies in
102 quail particularly have highlighted how rapidly this pathway can be induced ultimately
103 resulting in an up-regulation of luteinising hormone (LH) within 22 hours of the
104 photoperiod switch (Nakao et al. 2008a). Similar responses have been shown in mammals
105 (Revel et al. 2006, Barrett et al. 2007).

106 While the pathway appears to be conserved across the vertebrate clade, there are some
107 differences in the upstream drivers and the resultant down-stream affects. In the case of
108 long-day breeding birds and mammals, including quail (Nakao et al. 2008a, Ono et al.
109 2009b), mice (Ono et al. 2008, Masumoto et al. 2010), hamsters (Watanabe et al. 2004,
110 Yasuo et al. 2010) and rats (Yasuo et al. 2007, Ross et al. 2011), which initiate their
111 reproductive cycle under an increasing photoperiod, the *Eya3*-*Tsh β* -*Dio2/3* pathway is
112 positively stimulated following an increasing photoperiod or LD light signal. However,
113 for short-day breeding sheep and Saanen goats, the pathway is less straightforward. In
114 sheep it would appear that the pathway is initiated by a LD light signal, as is the case for
115 long-day breeders (Dupré et al. 2010, Sáenz de Miera et al. 2013) which is possibly a
116 reflection of the key stimulatory role of LD on sheep reproductive cycle irrespective of
117 their SD breeding behaviour (Dardente 2012). For Saanen goats however, a LD stimulus

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

120 Studies of this pathway in teleosts are lacking and they are limited to species which
121 initiate gametogenesis in response to a LD stimulus. Nakane et al. (2013) first reported
122 in masu salmon (*Oncorhynchus masou masou* B.) that a stimulatory LD photoperiod
123 upregulated *Fishβ* and *dDio2* expression in the saccus vasculosus. Moreover, removal of
124 this organ appeared to inhibit maturation, which would strongly suggest a role in
125 mediating photic perception and the subsequent regulation of the BPG axis (Nakane et
126 al. 2013). In the three-spined stickleback (*Gasterosteus aculeatus* L.), *tFshβ* expression
127 again echoes the mammalian model, being upregulated under LD stimulus, and was
128 strongly linked to the maturation response (O'Brien et al. 2012). These studies both
129 support an overall conservation of the pathway in vertebrates, but are a long way from
130 truly defining it and linking the PNES and BPG axes in fish, highlighting a need for
131 additional research. In order to expand on our current understanding of the mechanisms
132 controlling seasonal physiology in vertebrates and more specifically driving maturation
133 in fish, the current study aimed to identify the Atlantic cod paralogues for key targets
134 within the proposed pathway (*Eeya3*, *Fishβ* and *Ddio2*) and then explore, in two separate
135 trials, the response to environmental (photoperiod) and nutritional regulation of sexual
136 maturation. The purpose of these studies was to investigate if a pathway similar to that
137 seen in other vertebrate models also exists in Atlantic cod, developing our understanding
138 of the PNES-BPG network and potentially highlighting early biomarkers for maturation
139 commitment in Atlantic cod.

140 **Materials & Methods**

141 Fish Husbandry & Sample Collection

142 *Photoperiod control of maturation study*

143 This study was conducted at Machrihanish Marine Environmental Research Laboratory
144 (55.44 ° N, 5.44 ° W). Immature mixed-sex Atlantic cod (Approx. 14 Months post hatch,
145 avg. weight = 411 ± 5.59g) were randomly allocated between two fully covered light-
146 proof tanks. Fish (n= 374) were acclimated for 5 weeks prior to initiation of the trial and
147 throughout the study fish were fed to satiation in a commercial cd diet (BioMar,
148 Grangemouth, UK) throughout the ambient daylight period. A baseline sample of 13 fish
149 (6 male, 7 female) was collected in July, following which the experimental treatments
150 commenced. Two treatments were set up; 1) a simulated natural photoperiod (SNP) to
151 stimulate maturation, and 2) a constant light treatment (LL) applied from July to inhibit
152 maturation. Fish were sampled monthly over the course of 13 months from July to the
153 subsequent August, with up to 12 fish being euthanized in accordance with the Animals
154 (Scientific Procedures) Act 1986 (ASPA). Alongside individual biometric data (total
155 weight and length), whole brains and pituitaries were dissected from each sampled fish
156 and individually snap frozen, within 1 minute of dissection, over liquid nitrogen vapour
157 and stored at – 70 °C. All sampling was completed between 10:00 and 12:00 on the day
158 of collection. Furthermore, gonadal samples were dissected and preserved in 10% neutral
159 buffered formalin, prior to histological classification of ovarian development into one of
160 seven developmental stages (for full details see Cowan et al. 2011). For further details on
161 the husbandry, experimental setup and sampling protocols used see Cowan et al. (2012).

162

163 *Nutritional regulation of maturation study*

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

189 [Kjesbu and Kryvi \(1989\)](#). In addition, pituitaries from the 21st of November sampling
190 were snap frozen over liquid nitrogen and subsequently stored at -80°C for expression
191 analysis ($n = 12$ per treatment, 6M:6F).

192

193 *Ethical Statement*

194 All experimental work was performed in accordance with the Animals (Scientific
195 Procedures) Act 1986 (ASPA).

196

197 RNA Extraction & cDNA Synthesis

198 For both studies the same total RNA extraction and complementary DNA (cDNA)
199 synthesis methodology was used. Briefly, total RNA was extracted from the pituitary and
200 whole brain samples for each fish by thawing in 1 ml TRIzol® reagent (Invitrogen, UK)
201 per 100 mg of tissue. Samples were then homogenized over ice, and RNA was extracted
202 from the solution following the manufacturers' protocol, and eluted in 15 µl or 50 µl of
203 MilliQ water for pituitary or brain samples respectively. RNA yield was checked using a
204 ND 100 Nanodrop spectrophotometer (Labtech Int., East Sussex, and UK). cDNA was
205 generated using a High-Capacity cDNA Reverse Transcriptase kit (Applied Biosystems,
206 USA) as follows: 1 µg of total RNA in volume of 10 µl MilliQ water was added to a master
207 mix containing 2 µl of RT buffer, 0.8 µl dNTPs mix, 2 µl RT Random Primer, 1 µl
208 Reverse Transcriptase and 4.2 µl of MilliQ water, to create a final reaction volume of 20
209 µl. Thermocycling conditions were 25 °C for 10 mins, 37 °C for 120 mins, and 85 °C for
210 5 mins. Samples were then diluted with MilliQ water to a final volume of 200 µl (1:10)
211 and stored at -20 °C until required.

212

213 Primer Design & Molecular Cloning

214 Partial cDNA sequences for each target gene (*Eya3*, *Tshβ* & *Dio2*) were generated using
215 primers designed with primer-BLAST software
216 (<http://www.ncbi.nlm.nih.gov/tools/primer-blast>). One primer pair was designed for each
217 gene of interest based on gene sequences identified within the Atlantic cod genome
218 (*gadMor1*, January 2010 assembly) (Table 1). For each gene, an *in silico* analysis was
219 performed for quality assurance of the chosen sequences whereby the cod specific
220 transcripts were compared against previously described vertebrate sequences to compare
221 sequence identity and putative conserved domains using BLAST and ClustalW analysis
222 while MEGA6® (Tamura et al. 2013) was used to deduce and bootstrap phylogenetic
223 trees using the maximum likelihood method (Felsenstein 1981). The house-keeping gene,
224 *Elongation factor 1 alpha* (*Eef1a*), was selected as the reference gene for normalisation
225 of the quantitative PCR (QPCR) data. One primer pair for this gene was also designed,
226 following the same process described above.

227 Each primer pair was verified by Polymerase Chain Reaction (PCR). These PCR
228 reactions were performed using; 1µl reaction buffer, 0.8 µl of forward and reverse primers
229 (10 pmol µl⁻¹), 0.45 µl MgCl₂, 0.25 µl dNTPs, 0.04 µl Klear Taq DNA polymerase
230 (KBioSciences, UK), 5.66 µl MilliQ and 1 µl of synthesised cDNA. The thermal cycling
231 program consisted of a 15 min initial denaturation at 95 °C, followed by 35 cycles of 95
232 °C for 30 s, X °C for 30 s, and 72 °C for 60 s, with a final extension at 72 °C for 4 mins
233 where the annealing temperature, denoted X, varied with each specific primer pair (Table
234 1). PCR products were then checked on a 1 % agarose gel to verify size and the presence
235 of a single product, before being extracted and purified from the gel using a NucleoSpin®
236 Gel and PCR Clean-up kit (MACHEREY-NAGEL, Germany). The purified PCR
237 products were then cloned into a PGEM®-T easy vector (PGEM®-T easy vector systems,
238 Promega, UK). Plasmid insertion was checked by restriction digest, as well as sequencing

239 (GATC Biotech, London, UK). Upon confirmation of correct product insert, a serial
240 dilution of linearised plasmid sample was used for the subsequent QPCR assay.

241

242 Quantitative PCR

243 cDNA for each sample was synthesised from RNA using the methods described above.

244 To rationalise analysis that was performed, gene expression analysis was focused on

245 *eEya3* and *Ftshβ* in the pituitary and *Ftshβ* and *dDio2* in the whole brain (excluding
246 pituitary) to be reflective of the sites of expression of these targets reactive to seasonal

247 photoperiod stimulation reported in the literature. *eEfla* was used in all cases as a
248 housekeeping reference gene and expression was measured in both brain and pituitary
249 samples.

250 In order to validate QPCR assays, a serial dilution of linearised plasmid containing the
251 ligated target gene fragment for each gene was tested, from which three dilutions were
252 taken forward as standards to enable absolute quantification of mRNA levels. Each
253 QPCR plate was prepared, including in duplicate, three chosen standards for that
254 particular gene, one non-template control (MilliQ water), and four internal control
255 samples consisting of four random cDNA samples from the target tissue which were
256 included in every plate to control for inter-assay variation.

257 The QPCR assay was performed using 0.7 µl of both forward and reverse primer (10
258 pmol ul⁻¹), with one fortieth of the original cDNA reaction, 10 µl of ABSolute™ QPCR
259 SYBR-green master mix (Thermo Scientific, St. Leon-Rot, Germany), and 3.6 µl of
260 MilliQ water to a final reaction volume of 20 µl. The reactions were run on an Eppendorf
261 Mastercycler® ep realplex thermocycler using the following programme; 95 °C for 15
262 mins, 45 cycles of 95 °C for 15 s, X °C (annealing temperature of primers, see Table 1)
263 for 15 s, 72 °C for 30 s, followed by a temperature ramp from 65 °C to 95 °C with

264 fluorescence being measured every 0.5 °C to create a melt curve. The copy numbers of
265 each gene were automatically calculated by the realplex software by comparison to the
266 standard curve created by the serial plasmid dilutions which following correction was
267 then expressed as the absolute copy number per µg of total RNA. QPCR efficiency was
268 calculated for each plate run, with efficiency being greater than 90 % in all cases. A melt
269 curve was also created to verify the specificity of the primers used, and as an indication
270 of assay contamination.

271

272 Data Analysis

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

288 **RESULTS**

289 Atlantic cod *eEya3*, *Ftshβ* and *Ddio2* partial cDNA sequence and phylogenetic analysis
290 BLAST analysis of the cod genome (gadMor1 assembly) identified predicted gene
291 sequences for each of the chosen targets. For *Eya3*, there is a single gene annotated
292 (ENSDMOG00000012887) which consists of a partial coding sequence (cds) length of
293 1446bp with higher homology-similarity to other teleost *Eya3* sequences (64-84%
294 identity) and a reduced homology with than to other vertebrates (*circa* 40-60% identity
295 mammalian and avian *Eya3* sequences) (Supplemental figure 1). The 482 amino acid (aa)
296 deduced partial protein fragment included the conserved C-terminal “EYA” domain as
297 well as three putative haloacid dehalogenase (HAD) motifs (Supplemental figure 2).

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

309 With regards to *dDio2*, a partial cds sequence, 228bp long (ENSGMOG00000020274)
310 was identified from the cod genome which translated into a deduced aa sequence of 76
311 aa. While only a small partial fragment, the predicted aa showed high homology
312 similarity with teleost and mammalian *Ddio2* sequences (65-75% identity). Phylogenetic

313 analysis (Supplemental figure 5) grouped the partial sequence within teleost *Ddio2* gene
314 clade. The deduced aa sequence contained a portion of the iodothyronine deiodinase
315 domain (Supplemental figure 6).

316

317 Photoperiod control of maturation study

318 *Brain expression of *Ddio2* and *Tshβ**

319 Within the period July to December there was no significant interaction of time and
320 photoperiod treatment on whole brain *Ddio2* expression for male (P = 0.235) or female
321 (P = 0.258) Atlantic cod (Figure 1). Similarly, there were no significant differences in the
322 temporal expression between July and December of *Tshβ* in the brain between
323 treatments, for males (P = 0.789) or females (P = 0.454) potentially due to the high
324 variability observed in individual expression level (Figure 2). Due to the lack of
325 significant temporal variation with respect to treatment no further samples were analysed.

326

327 *Pituitary expression of *eEya3* and *Tshβ**

328 Expression of *Tshβ* in the pituitary did not significantly differ between treatments over
329 time between July and December for either males (P = 0.867) or females (P = 0.141)
330 (Figure 3). *Eya3* exhibited significant differences in temporal expression between
331 treatments for both males (P < 0.001) and females (P < 0.001), for this reason the sample
332 set was extended to a full annual cycle from July to the subsequent August. Cod held
333 under the SNP stimulus exhibited an increase in pituitary *eEya3* expression from
334 September, with significantly elevated expression in November, December, February and
335 March for females, and in October, November, December, February and March for males
336 with respect to LL treated animals (Figure 4). Expression levels then returned to being
337 comparable with LL treated fish from April to August. In contrast, LL individuals showed

338 no significant temporal variation in expression. While monthly mean *Eeya3* levels
339 showed a similar trend to that observed in gonadosomatic index (Figure 4) there was no
340 significant correlation between the parameters. However, when stage of gonadal
341 development was considered there was a clear association between *Eeya3* expression
342 level and histological stage of development in ovaries (Figure 5) with levels being
343 significantly elevated in individuals that were undergoing active gametogenesis in
344 comparison to those which were either immature or regressed.

345

346 Nutritional regulation of maturation study

347 Over the course of the study there was no difference in growth performance between the
348 two sexes within a given treatment. The high ration treatment had an overall 46% increase
349 in growth rate over the study period compared to the low ration treatment (calculated as
350 thermal growth coefficient, *data not presented*). Therefore, by the studies end the relative
351 difference in mean weight between treatments had increased from 73% to 182% (Table
352 2). Fultons condition factor was not affected by treatment ($p = 0.19$), being maintained at
353 0.9 ± 0.14 over the course of the study, however relative liver size (HSI) was significantly
354 lower in the lower ration treatment at all sampling points compared to the high ration
355 treatment (Table 2). Fish were classified as mature or immature through histological
356 analysis of the gonads. Males were classified maturing if spermatids were present, while
357 females were classified maturing when cortical alveoli were observed. By the completion
358 of the study 95% of fish from the high ration treatment were maturing while only 5%
359 (one fish) were mature within the low ration treatment (Table 2). There was no significant
360 difference in pituitary *eya3* expression levels between males and females and so samples
361 were pooled by treatment. Analysis of the final terminal sample revealed that expression

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

364 **DISCUSSION**

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

386 *In silico* analysis of the cod genome clearly identified cod specific partial cDNA
387 sequences for *Eya3*, *Ftshβ* and *Ddio2*. In all cases the deduced aa sequence shared high
388 identity and conserved functional domains which confirm these transcripts as being cod

389 specific orthologues . Although Lorgen et al. (2015) have identified two *dDio2* paralogs
390 (*Ddio2a* and *Ddio2b*) in Atlantic salmon, phylogenetic analysis indicates that salmon
391 *Ddio2a* and *Ddio2b* diverged during the salmon specific whole-genome duplication, and
392 therefore the sub-functionalisation theory they discuss represents an adaptation of the
393 salmonid family alone. As in the current study, only one *dDio2* gene has yet been
394 identified among other teleost species. Maugars et al. (2014) demonstrated *Tshβ*
395 duplication in a wide range of teleost species including Atlantic cod, as confirmed in the
396 current study. The current analysis rationalised the target choice based on synteny
397 analysis and confirmation of functional domains to conclude that *Tshβa* was the most
398 conserved *Tshβ* paralog among teleosts which was in agreement with O'Brien et al.
399 (2012). However, as these data suggest a teleost specific duplication of *Tshβ*, functional
400 studies of these paralogues are required to explore any possible sub functionalisation of
401 their role that may have occurred. To that end, recent results emerging from salmon,
402 following the completion of the current work, suggest that the *tshβb* could play a more
403 direct role in seasonal regulation in physiology which clearly warrants wider
404 investigation across teleosts (Fleming et al. 2019). Overall, while further work should be
405 undertaken to sequence the full length of each target gene, the high level of identity
406 observed for *Eya3*, *Tshβa* and *dDio2* would suggest evolutionary conservation,
407 reflective of an important functional role for these genes in cod.

408 The working model of the neuroendocrine pathway sees the light signal being relayed to
409 the pituitary where *Eya3* expression upregulates Tshβ production which subsequently
410 stimulates *Ddio2* expression in the third ventricle of the brain (Follett 2015). Of the three
411 genes analysed in the current study, *eEya3* alone exhibited a significant seasonal
412 response. This was observed as a surge in expression from August until March in the SNP
413 treatment, exhibiting a maximal 17-fold difference in February for females, and a 19-fold

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

439 responsible for converting the prehormone thyroxine T4 into its bioactive form
440 triiodothyronine T3. Increased expression of T3 appears to regulate GnRH production,
441 thus initiating the reproductive cycle (Yoshimura 2004, Barrett et al. 2007, Hanon et al.
442 2008, Yasuo and Yoshimura 2009). In Soay sheep, the same expression cascade is
443 observed, with stimulation of *Eya3* expression following a LD stimulus as despite
444 breeding in reducing daylengths of the autumn, the Soay sheep rely on this LD signal to
445 initiate the reproductive cycle during the following winter (Dardente 2012, Hazlerigg et
446 al. 2018). For Saanen goats however, the LD signal suppresses *Ddio2* expression in the
447 brain, in direct contrast to the expression profile in sheep. Initiation of the pathway in
448 goats appears to follow a SD stimulus, suggesting a switch in the initiation of the pathway
449 from LD to SD between these closely related species (Yasuo et al. 2006). Saanen goats
450 therefore represent the only other comparable example of SD stimulated gametogenesis
451 among all vertebrate examples studied to date. Although that study focused on *Ddio2*
452 expression, it showed positive stimulation of the pathway following a SD stimulus. It is
453 clear that in the current study, *Eya3* expression is initiated by a SD cue in Atlantic cod,
454 and provides a clear signal of seasonally altering photoperiod. We would therefore
455 conclude that the upstream drivers which initiate the *Eya3-Ttshβ-Ddio2* pathway differ
456 among vertebrates according to their breeding strategies, but the pathway itself and its
457 role in the reproductive cascade appears to be conserved across the vertebrate clade.

458 In relation to other fish species, this study is the first investigation of *Eya3*, *Ttshβ* or
459 *Ddio2* expression in a teleost species which shows stimulation of the initiation of the
460 gametogenesis pathway under a SD stimulus. Though masu salmon have been suggested
461 to be short-day breeders (e.g. Nakane et al. 2013), masu salmon utilise a LD cue to initiate
462 gametogenesis (Takashima & Yamada, 1984) with the decision to mature being taken
463 prior to the summer solstice as day length is increasing (Amano et al. 1993, 1995) like

464 other salmonids (Migaud et al. 2010) and furthermore, much like Soay sheep (Hazlerigg
465 et al 2018). LD induced *Tsh β* expression in the *saccus vasculosus* of the masu salmon is
466 thought to act as the main upstream driver governing the induction of season-associated
467 Dio2, and the resultant stimulation of the BPG axis (Nakane et al. 2013). Similarly, in
468 long-day breeding sticklebacks, acute induction of *Tsh β* in conjunction with *gGnrhRH*
469 and *Llh β* under a LD stimulus, support the idea of a conserved pathway linking the PNES
470 and BPG axes in species where long-day photoperiods stimulates the initiation of the
471 reproductive cycle (O'Brien et al. 2012). As neither study has considered *Eey3*
472 expression, it is difficult to draw general conclusions about the conservation of this
473 pathway in fish. Individually *Eey3*, *Tsh β* and *dDio2* have each been identified as
474 important messengers of the seasonal photoperiod in teleosts, but it is clear that further
475 work is required to fully understand if/how these genes interact.

476 The nutritional regulation of maturation study clarified that size is indeed a major factor
477 regulating maturation commitment in Atlantic cod. There was a significant difference in
478 total weight between treatments at the end of the experiment. However, it is more likely
479 that weight around the autumn equinox, when cod are likely to assess their energetic
480 status and initiate reproduction, represents the threshold for growth/energy theorised by
481 Bromage et al. (2001). The smallest fish to subsequently mature measured just 136 g in
482 mid-September, and on average, maturing fish measured 281 g (\pm 15 g) at this time. By
483 comparison, immature fish had a mean weight of 122 g (\pm 9.6 g) with the largest immature
484 fish weighing 185 g during this period. This suggests a minimum threshold weight of ~
485 130 g must be reached prior to the switch from long day to short days around the autumn
486 equinox, and that all fish that reach a weight above ~190 g are likely to be recruited into
487 maturation. Thus a size range of 130 – 190 g is theorised to be the critical determining
488 threshold range for growth/energy acquisition in cod. This lower weight is similar to that

489 found in a previous experimental study of cod growth and maturity, although there were
490 also population specific weight differences in the maturation threshold (Harrald et al.,
491 2010). As body weight, energy storage and growth are generally correlated it is possible
492 that the rate of change in weight or lipid storage in the liver may be the actual energetic
493 signal for maturation commitment (Wright, 2007).

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

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

514 commitment to reproduce the following season (Thorpe et al. 1990, Bromage et al. 2001).
515 Previous experiments in cod indicate this window spans between the summer solstice and
516 mid-autumn (Davie et al. 2007b) when the beginning of gametogenesis is measurable
517 by the increased expression of gonadotropins (Cowan et al. 2012). Precise definitions of
518 this window represents a gap in our current understanding of the maturation cycle in cod.
519 The current study has identified *Eeya3* as a component of a potential master regulator
520 integrating information concerning both the permissive physiological state (~190g wet
521 weight) as well as the proximate photoperiodic cue. This narrows down our search for
522 the specific photoperiod cue which initiates gametogenesis to when day length declines
523 to around 12 hrs. Increasing *Fshβ* expression and GSI values appear to coincide with the
524 up-regulation of *Eeya3* observed (Cowan et al. 2012), indicating that the seasonal
525 response initiated by *Eeya3* may occur over a very short time span as demonstrated in
526 mammals and aves (Nakao et al. 2008a, Masumoto et al. 2010, Dupré et al. 2010). Future
527 work should investigate the photoperiodic induction of *Eeya3* expression in tighter
528 temporal resolution than has been achieved to date.

529 The aim of this study was to investigate if the *Eeya3-tTshβ-dDio2* pathway, which is
530 known to consolidate the PNES and BPG axis in other seasonal vertebrates, is conserved
531 in Atlantic cod. *Eeya3* was found to clearly show potential as an integrator of both
532 environmental and energetic regulation of sexual maturation of the species. This is also
533 the first account of *Eeya3* induction in a teleost which initiates gametogenesis following
534 a SD stimulus suggesting an overall conservation of the functional pathway in
535 vertebrates. By inference this work therefore alludes to the presence of an as yet
536 unidentified process that allows for differential responsiveness to seasonal photic
537 stimulation upstream of *Eeya3* which requires further investigation.

538

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741 **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
<i>Eya3</i> F	5'-TCCCTGCTGCTGATCCAGTCC-3'	109	61.5	ENSGMOT00000014154
<i>Eya3</i> R	5'-AGACCTCTCCCAGGCCGTAGA-3'			
<i>Tshβ</i> F	5'-AGCGAGGGCAGCTCTCTGTTC-3'	116	61.5	ENSGMOT00000018765
<i>Tshβ</i> R	5'-GTACACGTGGGCCTGTTGCTG-3'			
<i>Ddio2</i> F	5'-GTTCTCGCGCTGTACGACTC-3'	130	61.5	ENSGMOT00000022279
<i>Ddio2</i> R	5'-CCAGATGGAGCGCATCCCC-3'			
<i>Eef1α</i> F	5'-TGAACCACCCTGGCACCATCT-3'	84	60	ENSGMOT00000013187
<i>Eef1α</i> R	5'-GCTCGTTGAACTGCAGGCCGA-3'			

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744

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

747

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

748

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 \pm 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 \pm 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 \pm 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 \pm 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 = $0.9 \pm 0.1\%$), CA: Cortical alveoli (GSI = $2.0 \pm 0.2\%$), EV: early vitellogenesis (GSI = $4.0 \pm 0.5\%$), LV: late vitellogenesis (GSI = $10.1 \pm 1.8\%$), SPW: Spawning (GSI = $19.7 \pm 4.1\%$), SPT: spent (GSI = $2.2 \pm 0.2\%$), REG: Regressing (GSI = $3.0 \pm 0.6\%$). Significant differences in *Eya3* expression between developmental stages are denoted by lowercase lettering. Data presented as mean \pm 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 \pm SE ($n=12$)

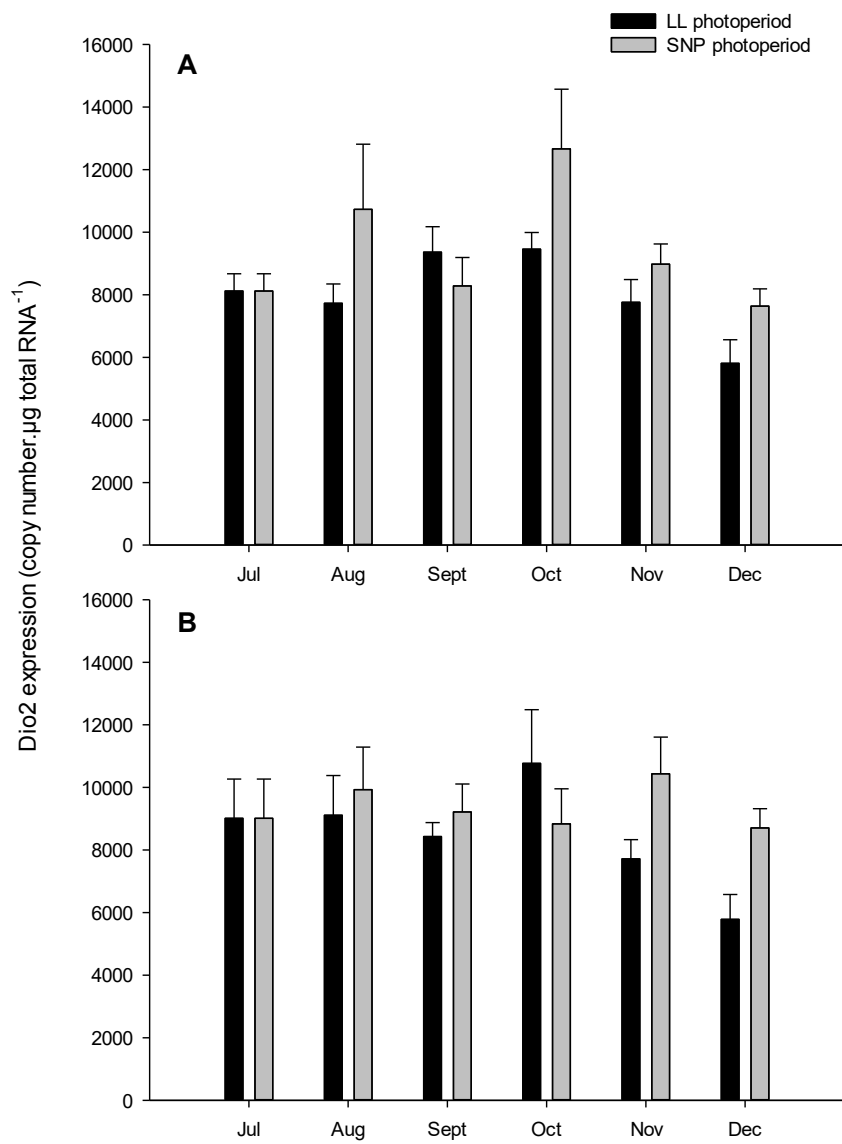


Figure 1

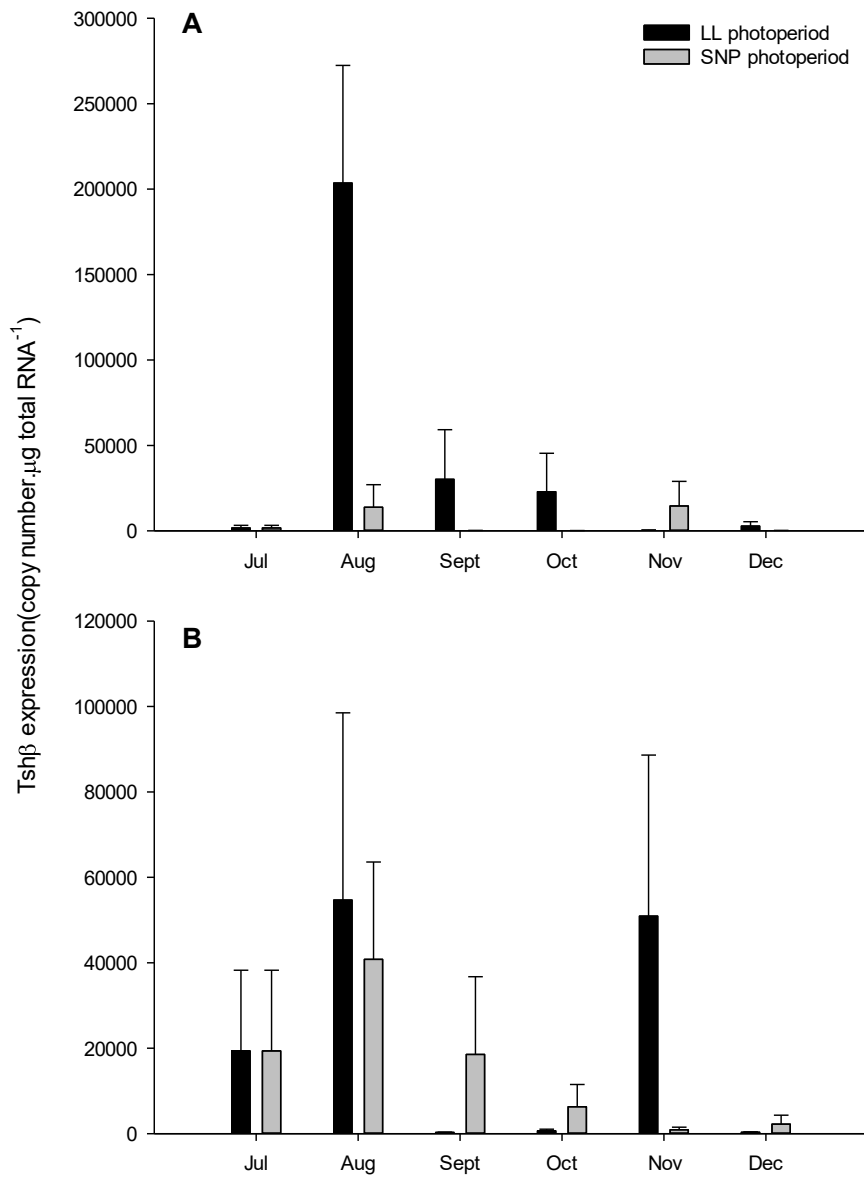


Figure 2

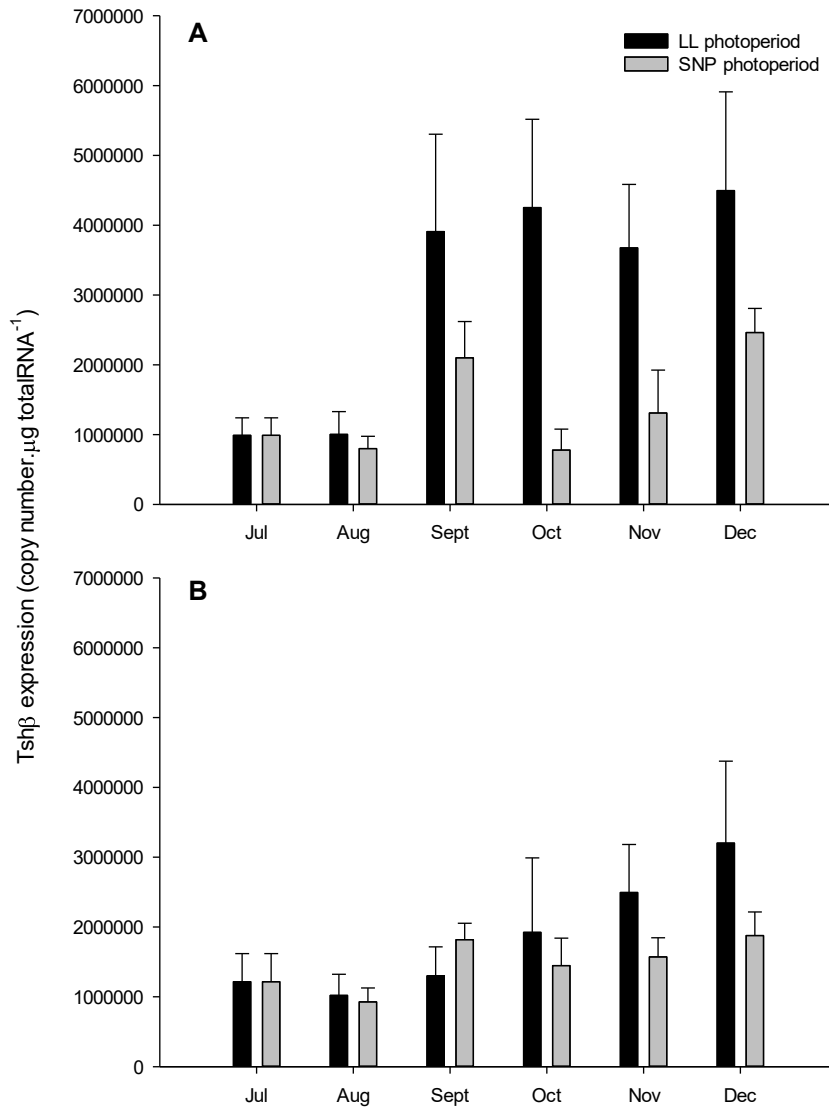


Figure 3

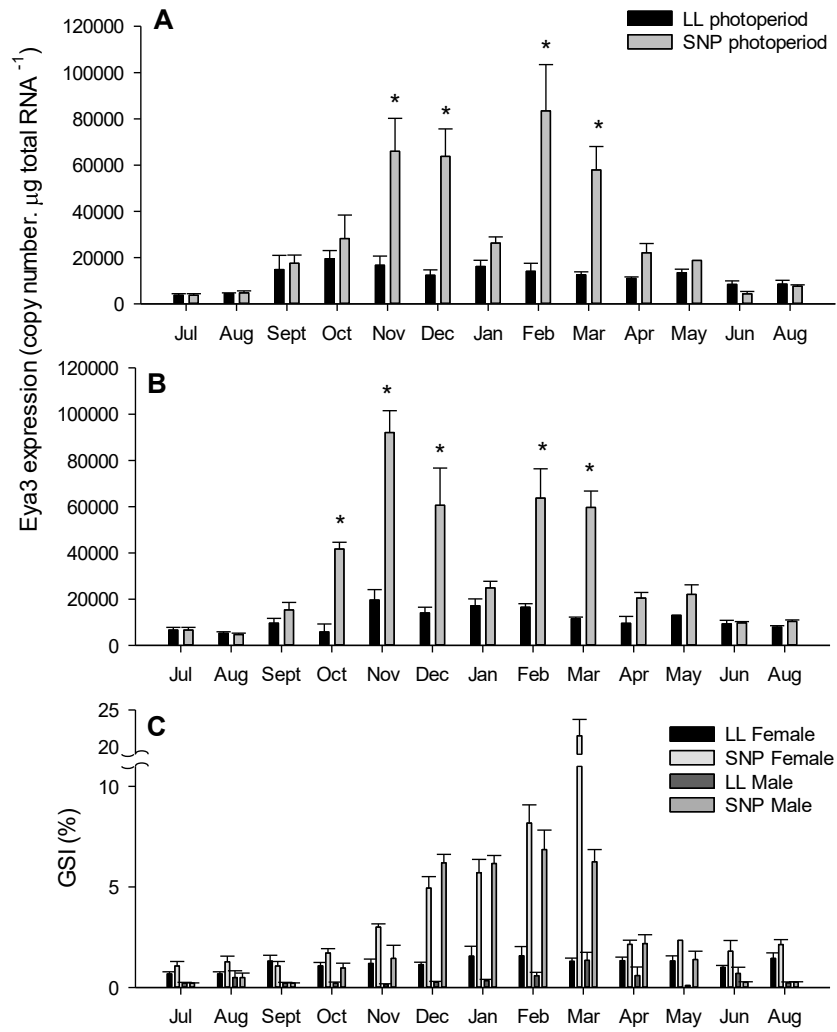


Figure 4

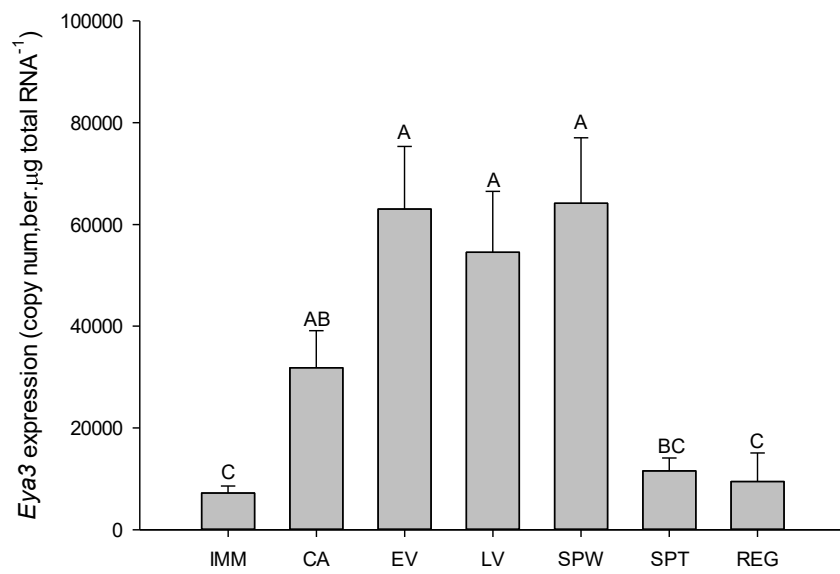


Figure 5

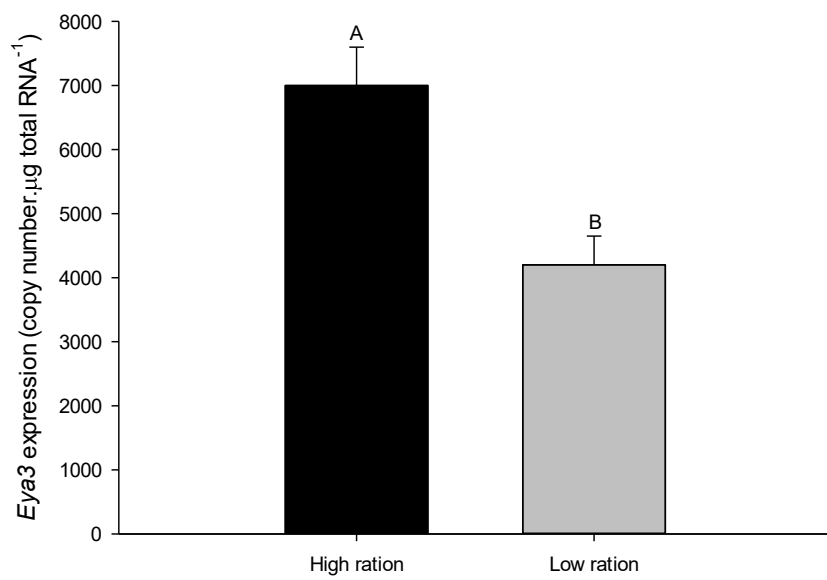


Figure 6

Reviewer #1: This manuscript describes the cDNA cloning and characteristics of Eya3, Tshb, and Dio2 in the brain of Atlantic cod, and their expression profiles under SNP and LL conditions and under food-controlled conditions. The authors found that Eya3 expressions of Eya3 in the pituitary increased during the reproductive season, although no changes were observed in Tshb in the pituitary as well as Dio2 and Tshb in the brain. In addition, the authors also showed that high food induced the expression of Eya3. The results clearly suggest that the pathway (Eya3-Tshb-Dio2) exists in the Atlantic cod, which is a short-day spawner, and it has an impact on the nutritional network as well. This seems to be the first report showing the Eya3-Tshb-Dio2 pathway is activated under SD conditions in fish. The data obtained in the present study sound scientific and contribute to reproductive physiology in relation to environmental regulation (photoperiod). There are several comments and suggestions to improve this manuscript.

The authors would like to thank the reviewer for the acknowledgement of the value of the work

Firstly, the authors measured Tshb and Dio2 in the "whole" brain and showed little difference in these genes during the reproductive cycle. The authors mentioned that little difference is due to the high variability. I want to know why the authors did not separate the brain into the several parts and measure the gene expressions. Alternatively, in-situ hybridization suggests high expression region of these genes in the brain.

The authors did not separate the brains as in our opinion there is no truly consistent approach to macroscopic dissection of the brains for expression analysis. As the reviewers rightly point out, in-situ localisation would provide such spatial definition however such a technique was outside the capacity of the current work.

Secondly, the expression of Eya3 under high and low ration indicates that IGF is highly expressed in the brain through conversion of T4 to T3. Measurement of igf1 expression in the liver may emphasize the present data.

This is an interesting opinion and worthy of further investigation however we are not capable of completing such analysis in the current work.

Minor comments

The authors mentioned "For further details on the husbandry, experimental setup and sampling protocols used see Cowan et al. (2012)." However, brief descriptions of experimental protocol help deep understandings of this study. There is no description in GSI for this experiment.

Description of the calculation of GSI is included within the current manuscript (L184-185)

Line 142: Food?

Details have been added (L146-147)

Line 143: Please provide the data of light-proof tanks and culture conditions.

Line 144: Please provide light conditions in the present study.

Full details of the husbandry arrangements as well as the lighting arrangements including light system, light field intensity etc are explained in the sister publication Cowan et al. 2012. In the interest of brevity this was not repeated in the current work rather we cross reference to the manuscript that is published within the same journal.

Line 143: Anesthetization?

Line 165: What kind of anesthesia used in the present study?

Anaesthetic details have been added (L169-170)

Line 179: HSI should be described.

HSI has been described. (L183)

Line 310: If the separated brain is used in this analysis, different expression patterns may occur.

As explained above we do not believe there is a consistently accurate manner to subsection a whole brain hence our approach for whole brain analysis to assure consistent interpretation between samples and treatments.

Line 346: The authors used cortical alveoli as a sign of maturation. I think that vitellogenic oocytes are better sign of maturation. How about males?

As we state in the results Males were classified maturing if spermatids were present (L354), while females were classified maturing when cortical alveoli were observed (L355). Cortical Alveoli staging was selected as, for gadoids, it is an earlier indicator for maturation commitment than vitellogenesis itself (Tobin, Wright and Sullivan (2010), Bucholtz et al (2007)).

Line 348: I think that if nutrition parameters (condition factor and others) differ between sex, the authors may do it.

Apologies but we are not clear what the question is thus we have not edited the manuscript.

Figure 2: Are there any reasons why Tshb is high in Aug?

The authors do not have an explanation for the drivers of this. The lack of strong seasonal expression and the high individual variability (which is one of the contributing factors to the Aug elevation in Fig 2) is explored in the discussion.

Figure 4: Are there any reasons why Eya3 is low in Jan?

The authors do not have an explanation for the drivers of this.

In the manuscript "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" the authors use the short day breeding teleost Atlantic cod to explore how photoperiod and energy status are integrated through the pathway *Eya3*-Tsh-Dio2. The three orthologs were identified including two versions of the *tsh β* gene. The authors chose the vertebrate ortholog *tsh β a* in their further studies together with *eya3* and *dio2*. However, only *eya3* appears to be regulated by photoperiod and energy status in Atlantic cod. This is surprising as *Eya3* is known to up-regulate Tsh production and subsequently upregulate Dio2.

The manuscript is well written, exploring an interesting topic and using a highly interesting animal model. The results from the current experiments show contradictory results compare to other species. Both *tsh β a* and *dio2* seems to be unaffected by the experimental treatments. Despite the promising observations made by the authors I have some concerns:

Of major concern is that the authors only investigated gene expression of the *tsh β a*. Recent studies (e.g Fleming et al., Sci Rep 9, 4561 (2019) and Irachi et al., Mol. Cell. Endocrinol 519, 1 (2021)) have demonstrated that in teleost the paralog *tsh β b* could play an important role in the key seasonal events. In particular, although not significant, the *tsh β a* seems almost to be opposite of *eya3* in LL compared to snp.

We would like to thank the reviewers for highlighting how this is an ever changing field as more evidence is brought forward. And to that end that is why we seek publication of the current work to add it in to the dialogue within the community to help catalyse more focused work going forward. We have added reference to Fleming et al 2019 to the discussion to demonstrate that the Tsh β a/b functionalization within teleosts requires further research (L402)

It would be interesting to see how the ratio between T4 and T3 changes during maturation and to compare LL and snp. Did the authors collect plasma that could allow such a study?

Unfortunately, plasma is not available to measure in this specific study

As for the *eya3* it would be more convincing if there were any experiments that show colocalization with either paralog of *tsh β* .

Co-localistaion of expresion was an aspiration of the PhD project that this study was part of, however it was not possible to complete such work within the students studies. We agree this would be a useful point of clarification for future work.

Minor adjustments and suggestions

In the method section the authors need to provide more details on how the photoperiod was set. In particular, when the authors use artificial light, they should provide what kind of light and with colour spectrum and intensity. How was the density of the fish and how was the light distribution in the tanks?

Full details of the husbandry arrangements including stocking density (<10Kg.m³) as well as the lighting arrangements including light system, light field intensity etc are explained in the sister publication Cowan et al. 2012. In the interest of brevity this was not repeated in the current work rather we cross reference to the manuscript that is published within the same journal.

In the discussion, the authors argue that the variation in *tsh β* expression could be that they used whole whole-brain homogenates. This is a bit confusing as there is also great variation in pituitary *tsh β* .

*The purpose of the discussion is to acknowledge that there is great individual variation in the expression of *tsh β* and we are not clear as to why that is the case. It could lie in the fact that the analysis is based on whole tissue and not localised expression or it could be the fact that *tsh β* is not in fact expressive of seasonal state. Both points are now covered with the addition of the Fleming 2019 reference now.*

I'm a bit confused as to why the authors used a reference gene (*ef1a*) when the gene expression data was transformed to absolute quantification. Thus, this needs some clarification.

Absolute quantification permits a user to communicate expression in terms of the abundance of mRNA fragments in terms of the equivalent absolute copy numbers, as opposed to referring to relative fold change in abundance. However there remains a need for a reference gene which accommodates/corrects for unintentional technical bias within the experiment which may be caused by various aspects e.g. cDNA synthesis, RNA extraction efficiency etc. The inclusion of a housekeeping correction is routine in our laboratories and I would argue is routine for most users that perform absolute quantification. Therefore we have not provided further correction to the manuscript.

The table is a bit confusing in regard to GSI. Could the authors make it more clear and include GSI of the maturing fish?

The authors are assuming this is in reference to Table 2? In which case we are not clear as to how this can be meaningfully clarified further. The GSI data is that of the fish studied ($n = 9 - 13$). Maturation status was confirmed for each individual based on histological analysis (L344-346). At this stage in their development, physical size differences in gonads do not really exist hence the need to define maturation based on histology. For example in the October 23rd samples for the High ration treatment (when there is the greatest differentiation of mature vs immature within the study samples) the mean GSI for mature vs immature males is 0.23 vs 0.23 % and for females it is 0.4 vs 0.3 %. Therefore, to contain the size of the table we have presented the sample mean as a whole. If the editor wishes us to add these extra columns we could, but we feel it would bring greater confusion rather than greater clarity.

It would be helpful if histological sections of the gonads of immature, maturing and mature fish was provided.

For clarity we have included the reference to the standard authority on histological classification which includes high quality imagery and explanations Bucholtz et al (2007), Almeida et al. (2008) and Kjesbu and Kryvi (1989). (L187-188)

In line 268: "the changes in length, weight and K". What is K?

Clarification "Fultons condition factor (K)" has been added.(L276)

A deeper explanation of the data analysis should be provided in supplemental including the code used for linear mixed affect model.

The code for the LME has now been added to the supplementary data file. (L285)

In line 282, the authors write a about degrees of homology (high homology). I suggest that this is change as homology is not divided into degrees.

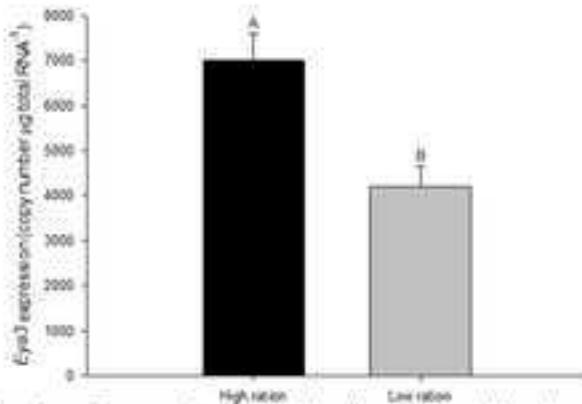
Homology has been replaced with similarity

Normally, in teleost genes are written in italic with small letters (*eya3*). Proteins are written with the first letter capitalized (Eya3).

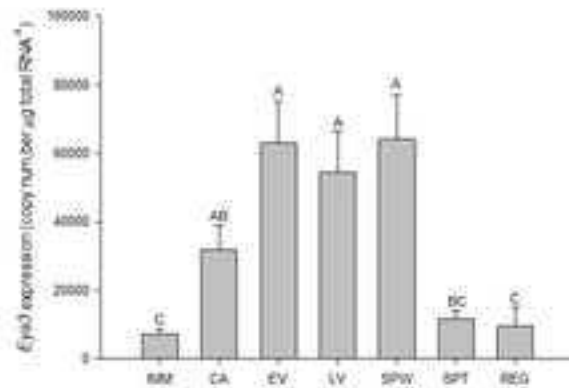
This has been corrected through the manuscript.

Highlights

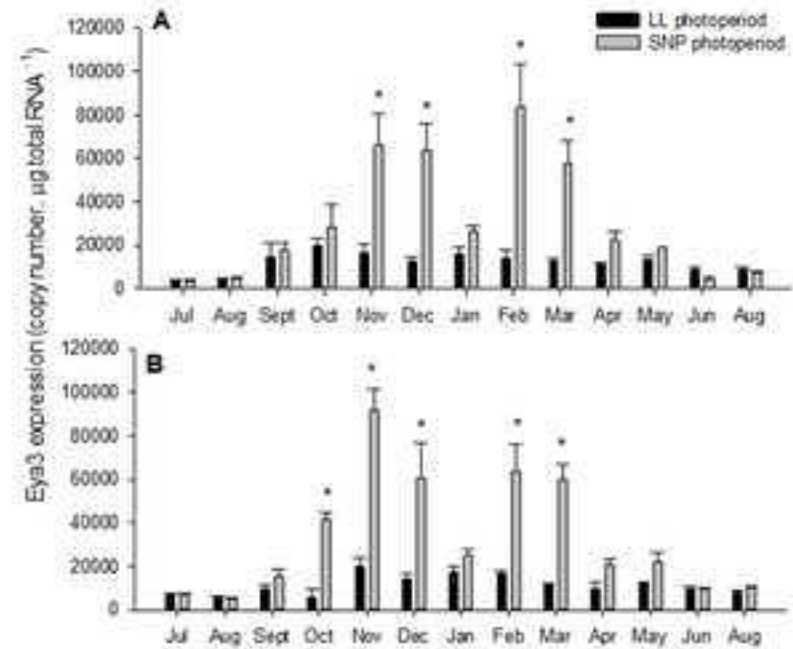
- Evidence of conservation of the PNES across the vertebrate clade.
- *Eya3* exhibits a strong seasonal expression pattern which is photoperiod sensitive
- First evidence of nutritional regulation of *Eya3* expression associated with maturation



Suppression of *eya3* expression in nutritionally restricted Atlantic cod, suggest that pituitary *eya3* has the potential to act as an integrator of both environmental and energetic regulation of sexual maturation of cod



Eya3 expression within the pituitary of Atlantic cod aligns with active gonadogenesis



Eya3 expression within the brain of the Atlantic cod demonstrates a seasonal expression pattern and is sensitive to photoperiod inhibition. 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.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

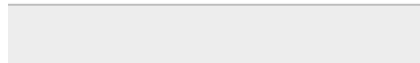
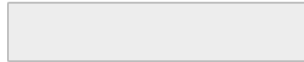
The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:



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Supplementary Material

[Supplemental figures Doyle et al CBPA_revised.pdf](#)



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

4

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16

17

18 **Abstract**

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

41

42 **Keywords:** Atlantic cod; *eya3*; *dio2*, *tsh β* , reproduction; photoperiod; seasonality.

43 **Introduction**

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

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

68 has been observed that there is increased expression of the gonadotropin, *follicle*
69 *stimulating hormone (fsh β)*, in male (de Almeida et al. 2011) and female (Cowan et al.
70 2012) cod from September onwards, marking the initiation of the BPG cascade.
71 Importantly, if cod are maintained under continuous illumination from the summer
72 solstice, to mask the reduction in photoperiod, gametogenesis and the associated
73 autumnal gonadotropin surge are completely inhibited (Cowan et al. 2012). This would
74 suggest that between the reduction of photoperiod from the summer solstice in June, and
75 the gonadotropin gene expression surge seen from September onwards, lies an as yet
76 unidentified signalling pathway linking the proximate photoperiod cue to the initiation of
77 the BPG cascade in cod.

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

93 2014). Indeed, photoperiod manipulation studies in aves have identified both *eya3* and
94 *tsh β* in the first wave response to the short day (SD) to long day (LD) switch, followed
95 by an up-regulation of *dio2* and down-regulation of *dio3*, preceding a surge in
96 gonadotropin secretion, indicating stimulation of the BPG (Yoshimura et al. 2003, Nakao
97 et al. 2008b, Ono et al. 2009a, 2009b). Furthermore, intracerebroventricular
98 administration of Tsh to quail held under continuous SD up-regulates *dio2* expression,
99 stimulating the reproductive axis, while administration of Tsh β antibodies to quail
100 exposed to continuous LD elicited the opposite response (Nakao et al. 2008a). Studies in
101 quail particularly have highlighted how rapidly this pathway can be induced ultimately
102 resulting in an up-regulation of luteinising hormone (LH) within 22 hours of the
103 photoperiod switch (Nakao et al. 2008a). Similar responses have been shown in mammals
104 (Revel et al. 2006, Barrett et al. 2007).

105 While the pathway appears to be conserved across the vertebrate clade, there are some
106 differences in the upstream drivers and the resultant down-stream affects. In the case of
107 long-day breeding birds and mammals, including quail (Nakao et al. 2008a, Ono et al.
108 2009b), mice (Ono et al. 2008, Masumoto et al. 2010), hamsters (Watanabe et al. 2004,
109 Yasuo et al. 2010) and rats (Yasuo et al. 2007, Ross et al. 2011), which initiate their
110 reproductive cycle under an increasing photoperiod, the *Eya3*-*Tsh β* -*Dio2/3* pathway is
111 positively stimulated following an increasing photoperiod or LD light signal. However,
112 for short-day breeding sheep and Saanen goats, the pathway is less straightforward. In
113 sheep it would appear that the pathway is initiated by a LD light signal, as is the case for
114 long-day breeders (Dupré et al. 2010, Sáenz de Miera et al. 2013) which is possibly a
115 reflection of the key stimulatory role of LD on sheep reproductive cycle irrespective of
116 their SD breeding behaviour (Dardente 2012). For Saanen goats however, a LD stimulus

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

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

139 **Materials & Methods**

140 Fish Husbandry & Sample Collection

141 *Photoperiod control of maturation study*

142 This study was conducted at Machrihanish Marine Environmental Research Laboratory
143 (55.44 ° N, 5.44 ° W). Immature mixed-sex Atlantic cod (Approx. 14 Months post hatch,
144 avg. weight = 411 ± 5.59g) were randomly allocated between two fully covered light-
145 proof tanks. Fish (n= 374) were acclimated for 5 weeks prior to initiation of the trial and
146 throughout the study fish were fed to satiation in a commercial cod diet (BioMar,
147 Grangemouth, UK) throughout the ambient daylight period. A baseline sample of 13 fish
148 (6 male, 7 female) was collected in July, following which the experimental treatments
149 commenced. Two treatments were set up; 1) a simulated natural photoperiod (SNP) to
150 stimulate maturation, and 2) a constant light treatment (LL) applied from July to inhibit
151 maturation. Fish were sampled monthly over the course of 13 months from July to the
152 subsequent August, with up to 12 fish being euthanized in accordance with the Animals
153 (Scientific Procedures) Act 1986 (ASPA). Alongside individual biometric data (total
154 weight and length), whole brains and pituitaries were dissected from each sampled fish
155 and individually snap frozen, within 1 minute of dissection, over liquid nitrogen vapour
156 and stored at – 70 °C. All sampling was completed between 10:00 and 12:00 on the day
157 of collection. Furthermore, gonadal samples were dissected and preserved in 10% neutral
158 buffered formalin, prior to histological classification of ovarian development into one of
159 seven developmental stages (for full details see Cowan et al. 2011). For further details on
160 the husbandry, experimental setup and sampling protocols used see Cowan et al. (2012).

161

162 *Nutritional regulation of maturation study*

163 This study was conducted at Marine Science Scotland, Marine Laboratory facility in
164 Aberdeen (57.06 ° N, 2.04 ° W). Immature under-yearling (0-group) cod were collected
165 between August and March from the wild, on the east coast of Scotland, offshore from
166 Stonehaven and transferred to the Marine Laboratory prior to the study starting. Fish were
167 held under ambient photoperiod conditions and a constant temperature of 9°C and were
168 fed *ad libitum* with a pelleted feed (Vitalis, 5mm; Skretting UK). On the 9th April 165
169 individuals (mean weigh 40 ± 1.6g) were anaesthetised (MS222, Pharmaq,
170 Fordingbridge, UK) and PIT tagged (Trovan Ltd, Hull UK). Following recovery from the
171 tagging, the population was split by size and in to two conditions, a high ration treatment
172 (*ad libitum* feeding) (wet weight = 56.15 ± 2.13 g, Total length = 19.19 ± 0.21 cm, *n* =
173 81) and a low ration treatment (feed restricted to a daily ration of 2.25% body weight
174 increasing to 3% body weight over the course of the trial) (Wet weight = 34.32 ± 0.99 g,
175 total length = 16.62 ± 0.16 cm, *n* = 84). Each treatment group was further split into
176 duplicate rearing units where environmental conditions were retained as before. Fish
177 were monitored every 21 days where all individuals wet weight and total length were
178 recorded which allowed for the adjustment of feeding rations in the low ration population.
179 On the 12th September, 23rd October and 21st November, 20 individuals from each
180 treatment were randomly selected and euthanised in accordance with the Animals
181 (Scientific Procedures) Act 1986 (ASPA). Individual weight and length was recorded
182 before fish were dissected to remove the liver which was weighed to calculate
183 hepatosomatic index (HSI) (liver weight/somatic weight *100) and gonads were removed
184 and weighed to calculate gonadosomatic index (GSI) (gonad weight/somatic weight
185 *100) before gonad samples were preserved in 10% neutral buffered formalin for later
186 histological analysis. Gonadal development was qualified by histological analysis in
187 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: 1 µg of total RNA in volume of 10 µl 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 °C for 10 mins, 37 °C for 120 mins, and 85 °C 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 °C until required.

211

212 Primer Design & Molecular Cloning

213 Partial cDNA sequences for each target gene (*Eya3*, *Tsh β* & *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 *elongation factor 1 alpha (ef1 α)*, 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; 1 μ l reaction buffer, 0.8 μ l of forward and reverse primers
228 (10 pmol μ l⁻¹), 0.45 μ l MgCl₂, 0.25 μ l dNTPs, 0.04 μ l Klear Taq DNA polymerase
229 (KBioSciences, UK), 5.66 μ l MilliQ and 1 μ l of synthesised cDNA. The thermal cycling
230 program consisted of a 15 min initial denaturation at 95 °C, followed by 35 cycles of 95
231 °C for 30 s, X °C for 30 s, and 72 °C for 60 s, with a final extension at 72 °C 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 *tsh β* in the pituitary and *tsh β* 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. *efl α* 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 μ l of both forward and reverse primer (10

256 pmol μ l⁻¹), with one fortieth of the original cDNA reaction, 10 μ l of ABsolute™ QPCR

257 SYBR-green master mix (Thermo Scientific, St. Leon-Rot, Germany), and 3.6 μ l of

258 MilliQ water to a final reaction volume of 20 μ l. The reactions were run on an Eppendorf

259 Mastercycler® ep realplex thermocycler using the following programme; 95 °C for 15

260 mins, 45 cycles of 95 °C for 15 s, X °C (annealing temperature of primers, see Table 1)

261 for 15 s, 72 °C for 30 s, followed by a temperature ramp from 65 °C to 95 °C with

262 fluorescence being measured every 0.5 °C to create a melt curve. The copy numbers of

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

269

270 Data Analysis

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

286 **RESULTS**

287 Atlantic cod *eya3*, *tsh β* and *dio2* partial cDNA sequence and phylogenetic analysis

288 BLAST analysis of the cod genome (gadMor1 assembly) identified predicted gene
289 sequences for each of the chosen targets. For *eya3*, there is a single gene annotated
290 (ENSDMOG00000012887) which consists of a partial coding sequence (cds) length of
291 1446bp with higher similarity to other teleost *eya3* sequences (64-84% identity) than to
292 other vertebrates (*circa* 40-60% identity mammalian and avian Eya3 sequences)
293 (Supplemental figure 1). The 482 amino acid (aa) deduced partial protein fragment
294 included the conserved C-terminal “EYA” domain as well as three putative haloacid
295 dehalogenase (HAD) motifs (Supplemental figure 2).

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

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

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

314

315 Photoperiod control of maturation study

316 *Brain expression of *dio2* and *tsh β**

317 Within the period July to December there was no significant interaction of time and
318 photoperiod treatment on whole brain *dio2* expression for male ($P = 0.235$) or female (P
319 $= 0.258$) Atlantic cod (Figure 1). Similarly, there were no significant differences in the
320 temporal expression between July and December of *tsh β* in the brain between treatments,
321 for males ($P = 0.789$) or females ($P = 0.454$) potentially due to the high variability
322 observed in individual expression level (Figure 2). Due to the lack of significant temporal
323 variation with respect to treatment no further samples were analysed.

324

325 *Pituitary expression of *eya3* and *tsh β**

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

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

343

344 Nutritional regulation of maturation study

345 Over the course of the study there was no difference in growth performance between the
346 two sexes within a given treatment. The high ration treatment had an overall 46% increase
347 in growth rate over the study period compared to the low ration treatment (calculated as
348 thermal growth coefficient, *data not presented*). Therefore, by the studies end the relative
349 difference in mean weight between treatments had increased from 73% to 182% (Table
350 2). Fultons condition factor was not affected by treatment ($p = 0.19$), being maintained at
351 0.9 ± 0.14 over the course of the study, however relative liver size (HSI) was significantly
352 lower in the lower ration treatment at all sampling points compared to the high ration
353 treatment (Table 2). Fish were classified as mature or immature through histological
354 analysis of the gonads. Males were classified maturing if spermatids were present, while
355 females were classified maturing when cortical alveoli were observed. By the completion
356 of the study 95% of fish from the high ration treatment were maturing while only 5%
357 (one fish) were mature within the low ration treatment (Table 2). There was no significant
358 difference in pituitary *eya3* expression levels between males and females and so samples
359 were pooled by treatment. Analysis of the final terminal sample revealed that expression

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

362 **DISCUSSION**

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

384 *In silico* analysis of the cod genome clearly identified cod specific partial cDNA
385 sequences for *eya3*, *tsh β* and *dio2*. In all cases the deduced aa sequence shared high
386 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 *tsh β* 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 *tsh β a* was the most conserved *tsh β* 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 *tsh β* , 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 *tsh β b* 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*, *tsh β a* 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 Tsh β 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 *tsh β* , nor whole brain *dio2* or *tsh β* 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 *tsh β* 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 *tsh β* in the current study. In mammals and birds, *tsh β* 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 *Tsh β* production. This in turn induces the expression of *dio2*, which is

437 responsible for converting the prehormone thyroxine T4 into its bioactive form
438 triiodothyronine T3. Increased expression of T3 appears to regulate GnRH production,
439 thus initiating the reproductive cycle (Yoshimura 2004, Barrett et al. 2007, Hanon et al.
440 2008, Yasuo and Yoshimura 2009). In Soay sheep, the same expression cascade is
441 observed, with stimulation of *eya3* expression following a LD stimulus as despite
442 breeding in reducing daylengths of the autumn, the Soay sheep rely on this LD signal to
443 initiate the reproductive cycle during the following winter (Dardente 2012, Hazlerigg et
444 al. 2018). For Saanen goats however, the LD signal suppresses *dio2* expression in the
445 brain, in direct contrast to the expression profile in sheep. Initiation of the pathway in
446 goats appears to follow a SD stimulus, suggesting a switch in the initiation of the pathway
447 from LD to SD between these closely related species (Yasuo et al. 2006). Saanen goats
448 therefore represent the only other comparable example of SD stimulated gametogenesis
449 among all vertebrate examples studied to date. Although that study focused on *dio2*
450 expression, it showed positive stimulation of the pathway following a SD stimulus. It is
451 clear that in the current study, *Eya3* expression is initiated by a SD cue in Atlantic cod,
452 and provides a clear signal of seasonally altering photoperiod. We would therefore
453 conclude that the upstream drivers which initiate the *eya3-tsh β -dio2* pathway differ
454 among vertebrates according to their breeding strategies, but the pathway itself and its
455 role in the reproductive cascade appears to be conserved across the vertebrate clade.

456 In relation to other fish species, this study is the first investigation of *eya3*, *tsh β* or *dio2*
457 expression in a teleost species which shows stimulation of the initiation of the
458 gametogenesis pathway under a SD stimulus. Though masu salmon have been suggested
459 to be short-day breeders (e.g. Nakane et al. 2013), masu salmon utilise a LD cue to initiate
460 gametogenesis (Takashima & Yamada, 1984) with the decision to mature being taken
461 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 *tsh β* 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 *tsh β* in conjunction with *gnrh* and *lh β*
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*, *tsh β* 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 (\pm 15 g) at this time. By
481 comparison, immature fish had a mean weight of 122 g (\pm 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

487 found in a previous experimental study of cod growth and maturity, although there were
488 also population specific weight differences in the maturation threshold (Harrald et al.,
489 2010). As body weight, energy storage and growth are generally correlated it is possible
490 that the rate of change in weight or lipid storage in the liver may be the actual energetic
491 signal for maturation commitment (Wright, 2007).

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

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

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

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

536

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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
<i>eya3F</i>	5'-TCCCTGCTGCTGATCCAGTCC-3'	109	61.5	ENSGMOT00000014154
<i>eya3R</i>	5'-AGACCTCTCCCAGGCCGTAGA-3'			
<i>tshβF</i>	5'-AGCGAGGGCAGCTCTCTGTTC-3'	116	61.5	ENSGMOT00000018765
<i>tshβR</i>	5'-GTACACGTGGGCCTGTTGCTG-3'			
<i>dio2F</i>	5'-GTTCCCTCGCGCTGTACGACTC-3'	130	61.5	ENSGMOT00000022279
<i>dio2R</i>	5'-CCAGATGGAGCGCATCCCC-3'			
<i>eflαF</i>	5'-TGAACCACCCTGGCACCATCT-3'	84	60	ENSGMOT00000013187
<i>eflαR</i>	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 High ration	12 th September	253.8 ± 17.7	29.9 ± 0.7	10.32 ± 0.27	0.27 ± 0.01	0	11
	23 rd October	407.1 ± 36.5	34.6 ± 0.9	11.16 ± 0.29	0.39 ± 0.03	66.7%	9
	21 st November	514.5 ± 37.4	35.8 ± 0.8	10.56 ± 0.40	0.55 ± 0.05	100%	9
Female Low ration	12 th September	126.5 ± 9.7	23.5 ± 0.9	3.66 ± 0.25	0.25 ± 0.02	0	10
	23 rd October	172.9 ± 12.6	28.3 ± 0.7	3.04 ± 0.33	0.29 ± 0.03	0	10
	21 st November	178.5 ± 16.4	27.1 ± 1.0	2.90 ± 0.28	0.33 ± 0.04	0	11
Male High ration	12 th September	244.6 ± 24.2	29.4 ± 0.6	9.70 ± 0.28	0.08 ± 0.01	0	9
	23 rd October	332.5 ± 34.1	31.9 ± 0.9	10.38 ± 0.39	0.23 ± 0.02	27.3%	11
	21 st November	454.3 ± 41.6	34.5 ± 0.9	11.28 ± 0.39	1.03 ± 0.18	92.3%	13
Male Low ration	12 th September	115.9 ± 12.4	23.6 ± 1.0	2.75 ± 0.24	0.04 ± 0.01	0	10
	23 rd October	148.7 ± 8.0	26.5 ± 0.6	2.77 ± 0.26	0.08 ± 0.01	0	10
	21 st 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 \pm 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 \pm 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 \pm 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 \pm 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 = $0.9 \pm 0.1\%$), CA: Cortical alveoli (GSI = $2.0 \pm 0.2\%$), EV: early vitellogenesis (GSI = $4.0 \pm 0.5\%$), LV: late vitellogenesis (GSI = $10.1 \pm 1.8\%$), SPW: Spawning (GSI = $19.7 \pm 4.1\%$), SPT: spent (GSI = $2.2 \pm 0.2\%$), REG: Regressing (GSI = $3.0 \pm 0.6\%$). Significant differences in *Eya3* expression between developmental stages are denoted by lowercase lettering. Data presented as mean \pm 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 \pm SE ($n=12$)

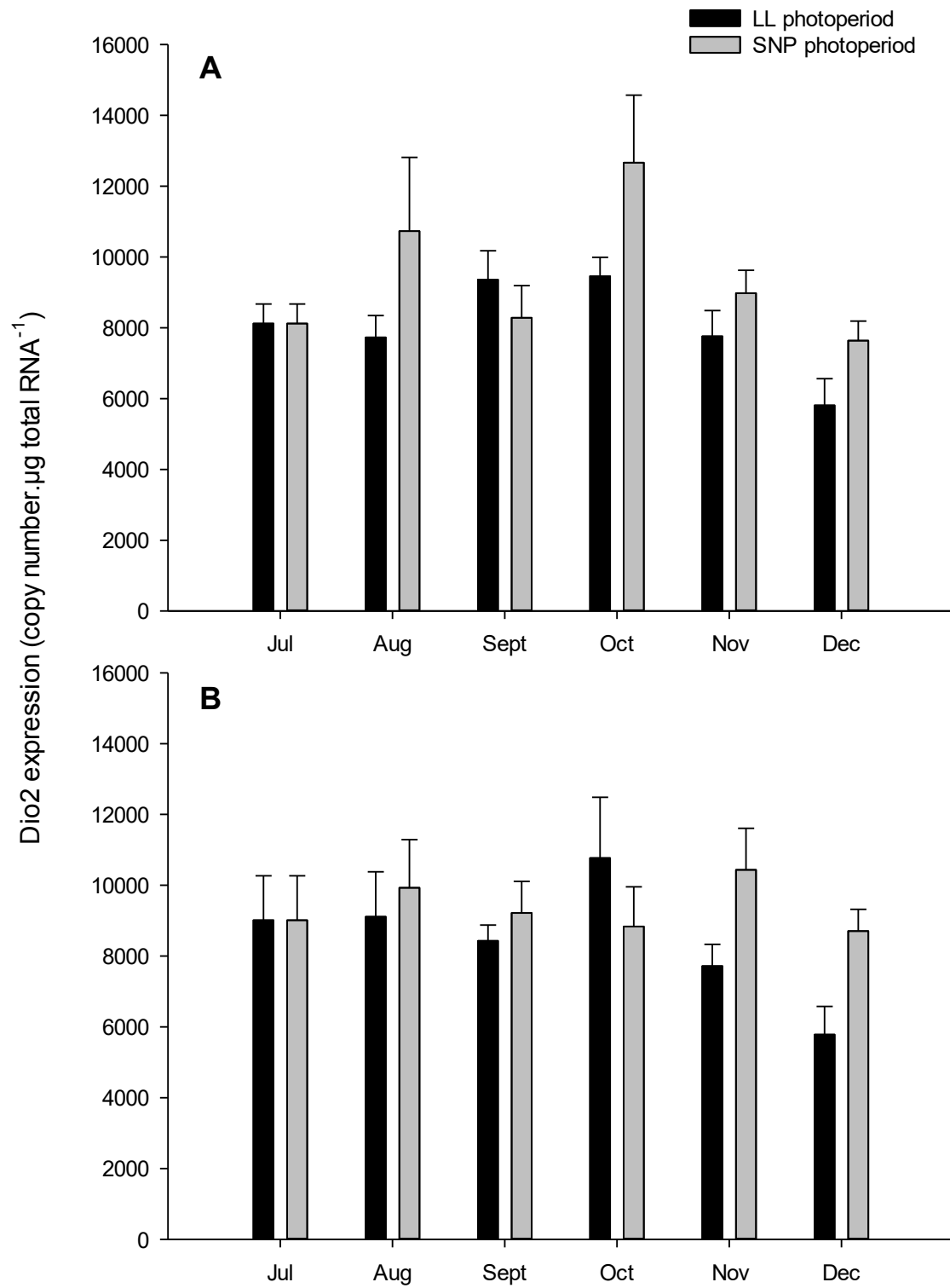


Figure 1

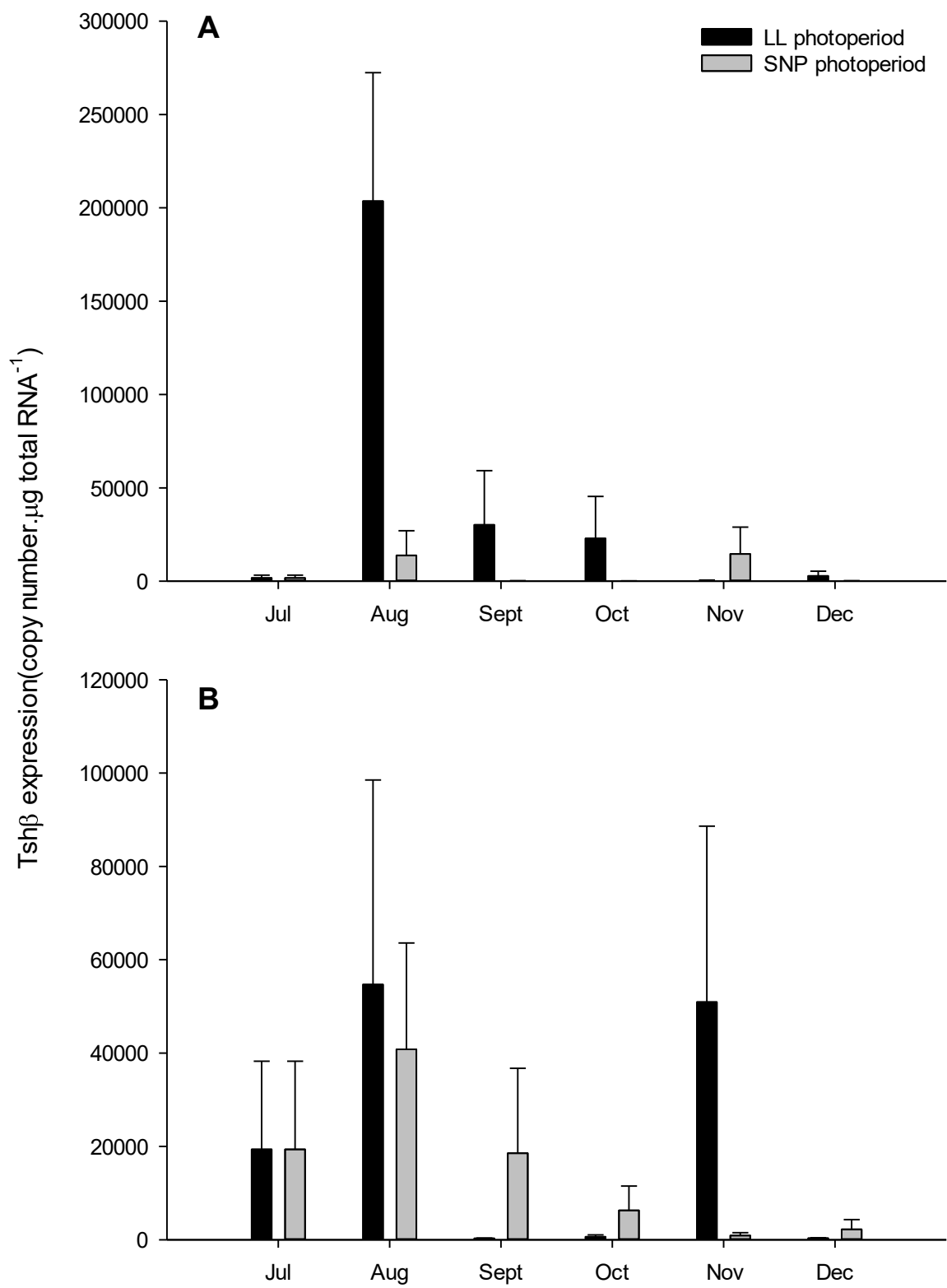


Figure 2

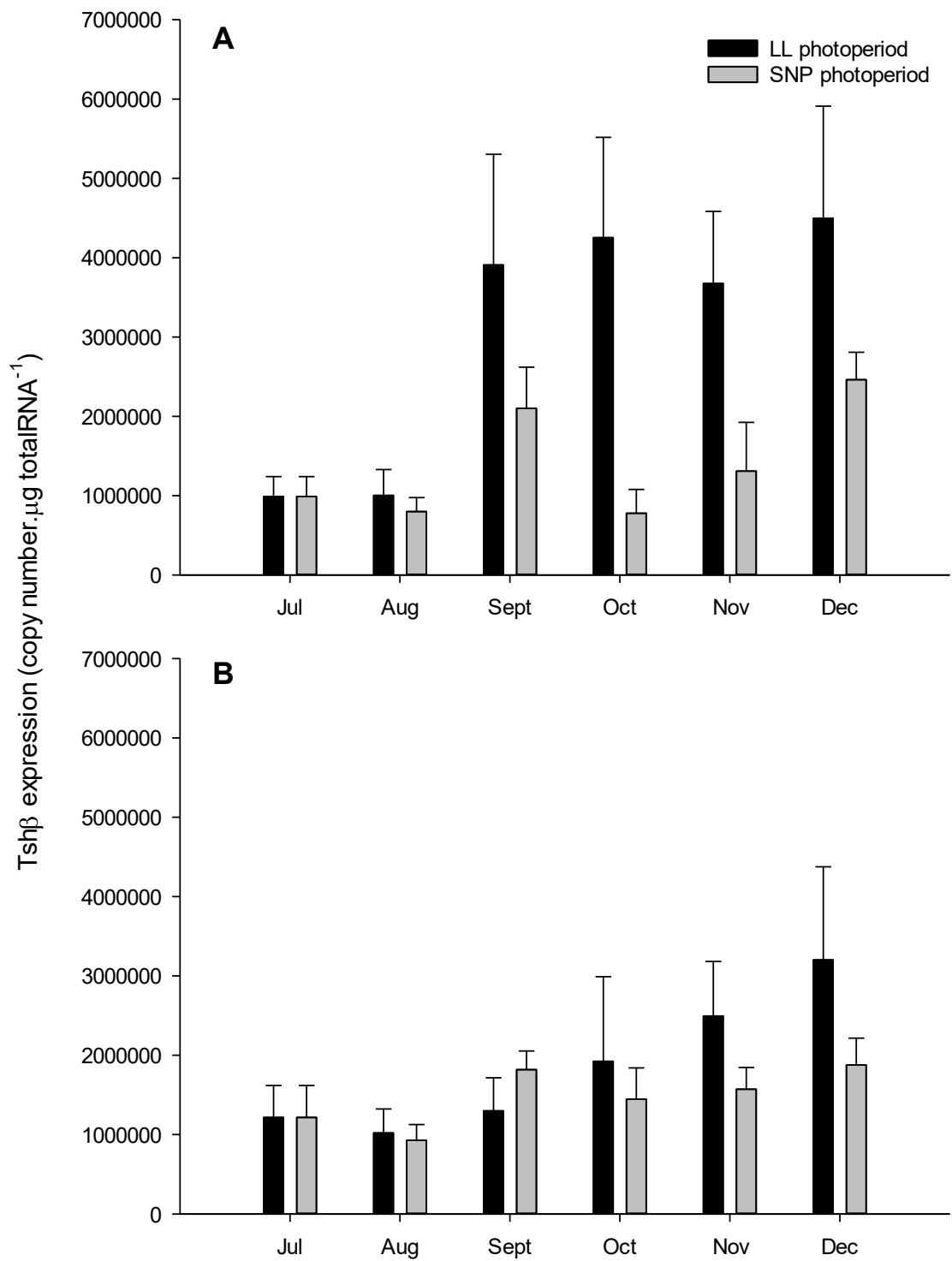


Figure 3

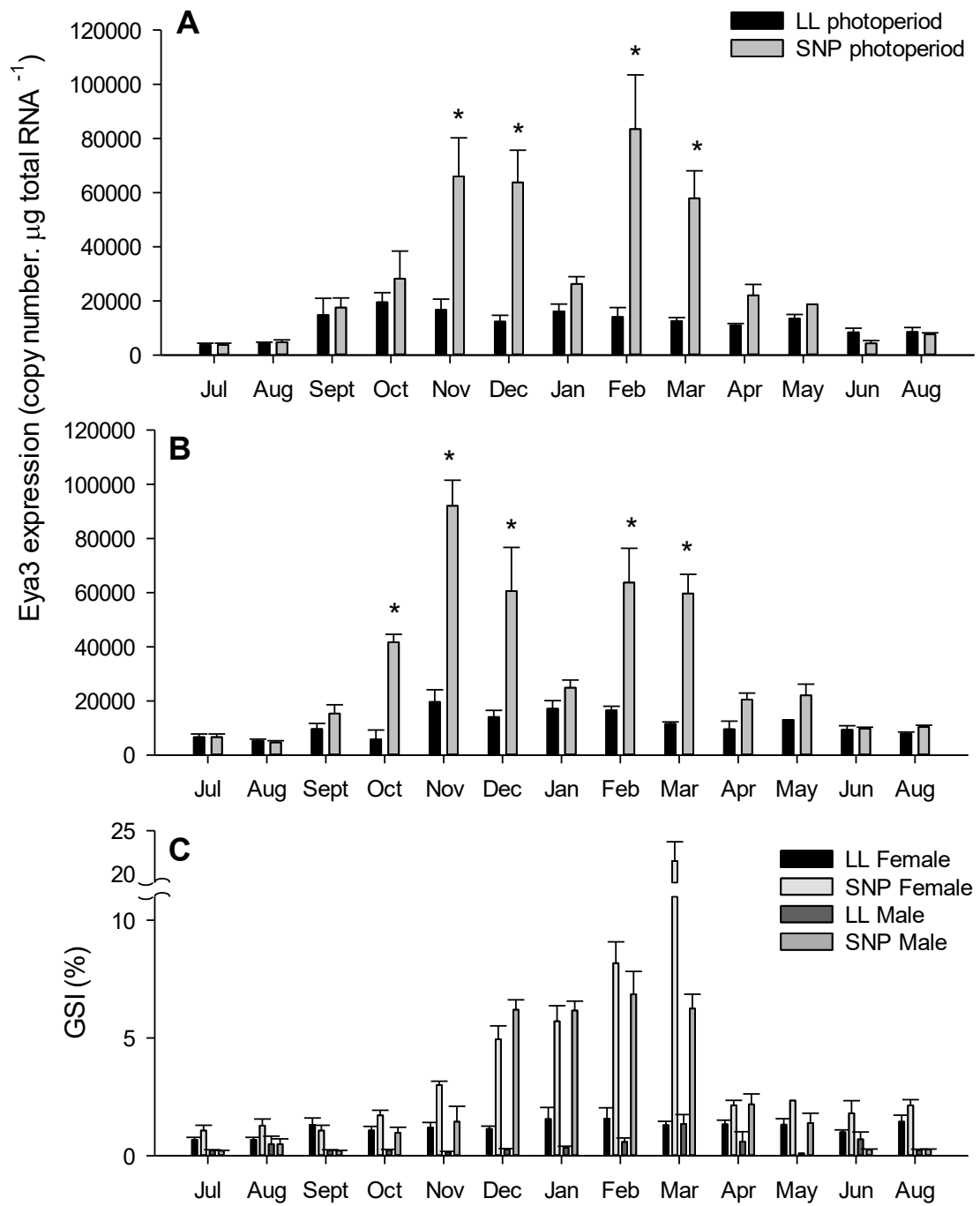


Figure 4

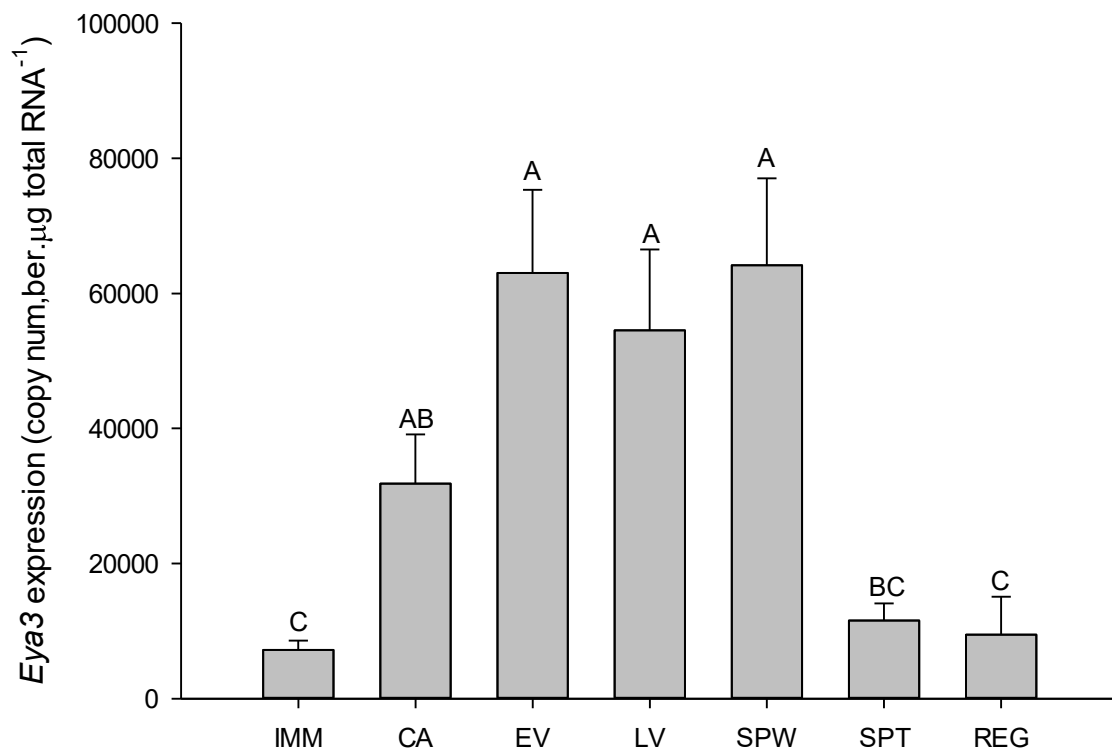


Figure 5

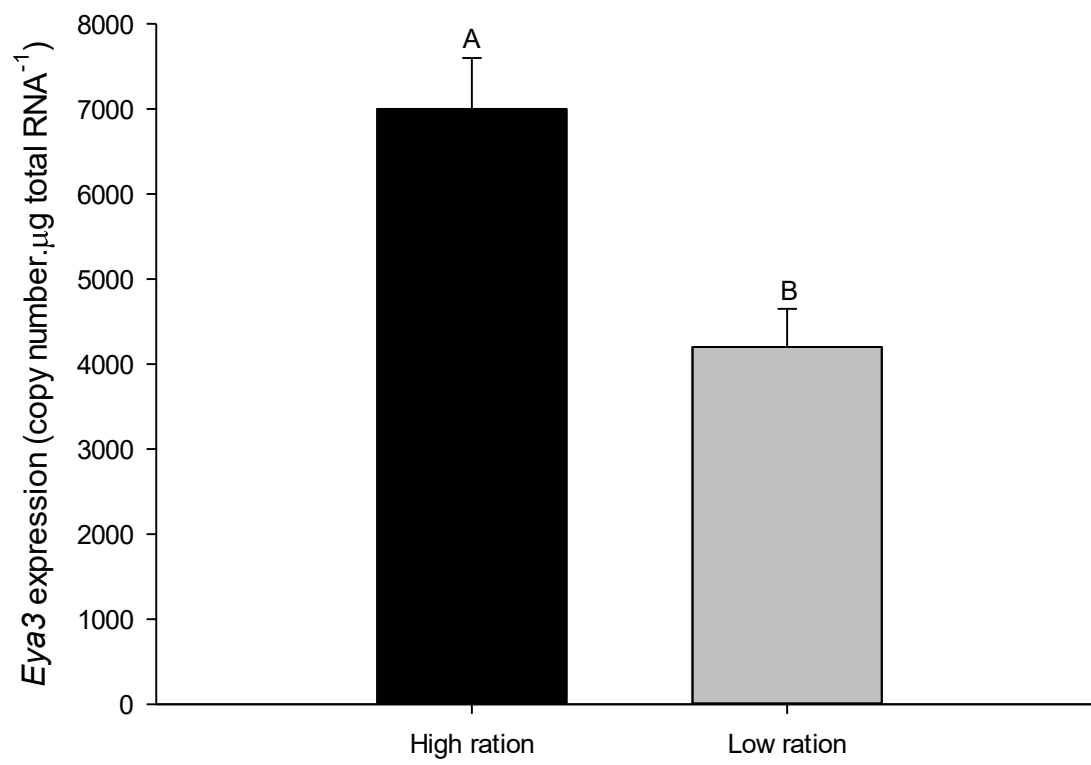


Figure 6