

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

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**Supplementary Material**

## Supplementary Materials and Methods

### Data Analysis

For the nutritional study to account for differences in initial size and repeated measures the changes in length, weight and Fulton's condition factor (K), over the course of the study were analysed using a linear mixed-effects model, with time considered as a categorical variable and with individual fish included as a random effect. The fish random effect was assumed to vary with time according to an antedependence structure of order 2. All models were implemented in R3.2.2 using mgcv, MASS and nlme libraries utilising the following scripts:

```
LWSGR<-read.csv(".csv")  
  
library(nlme)  
  
LT.1<-lme(Length~Time*Treatment, data=LWSGR, random=~Time|Fish)  
  
summary(LT.1)  
  
LT.2<-lme(Weight~Time*Treatment, data=LWSGR, random=~Time|Fish)  
  
summary(LT.2)
```

### **OR**

```
library(lme4)  
  
MEM2013<-read.csv("MixedEffects_R_Formatted.csv")  
  
print(MEM2013)  
  
LowToHigh<-(MEM2013$Fish[MEM2013$Treatment=='1'])  
  
LowToHigh1<-  
(MEM2013$Length[MEM2013$Treatment=='1']~MEM2013$Time[MEM2013$Treatment=='1']|MEM2  
013$Fish[MEM2013$Treatment=='1'])  
  
HighToLow2<-  
(MEM2013$Length[MEM2013$Treatment=='2']~MEM2013$Time[MEM2013$Treatment=='2']|MEM2  
013$Fish[MEM2013$Treatment=='2'])  
  
par(mfrow=c(11,6))  
  
print(plot((MEM2013$Length[MEM2013$Treatment=='1'],MEM2013$Time[MEM2013$Treatment=='1  
]|MEM2013$Fish[MEM2013$Treatment=='1']), main='Low to High Treatment', xlab='Sampling  
Period', ylab='Length', ylim=1.2*range(MEM2013$Length),layout=c(11,6))  
  
print(plot(HighToLow1, main='High to Low Treatment', xlab='Sampling Period', ylab='Length',  
ylim=1.2*range(LowToHigh1$Length, HighToLow1$Length),layout=c(11,6), aspect=1.0,  
position=c(0,0,1,0.5), more=T)
```

**Supplemental figure 1.** Phylogenetic tree analysis of the vertebrate *Eya3* gene. The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model (Tamura and Nei 1993). The tree with the highest log likelihood (-12589.9852) is shown. Initial tree(s) for the heuristic search were obtained by applying the Neighbor-Joining method to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 15 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 1204 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (Tamura et al. 2013). Accession numbers: Nile tilapia *Oreochromis niloticus* XM\_003447243.3, three-spined stickleback *Gasterosteus aculeatus* ENSGACT00000003106, fugu *Takifugu rubripes* ENSTRUT00000029672, Japanese medaka *Oryzias latipes* ENSORLT00000011779, tongue sole *Cyanoglossus semilaevis* XM\_008331068, Atlantic cod *Gadus morhua* ENSGMOT00000014154, cave fish *Astyanax mexicanus* ENSAMXT00000017928, zebrafish *Danio rerio* (*Eya3*; ENSDART00000049840, *Eya2*; ENSDART00000011652, *Eya1*; ENSDART00000136443), spotted gar *Lepisosteus oculatus* ENSLOCT00000005087, red jungle fowl *Gallus gallus* ENSGALT00000001127, rat *Rattus norvegicus* ENSRNOT00000056170, Guinea pig *Cavia porcellus* ENSCPOT00000012631, sheep *Ovis aries* NM\_001161733

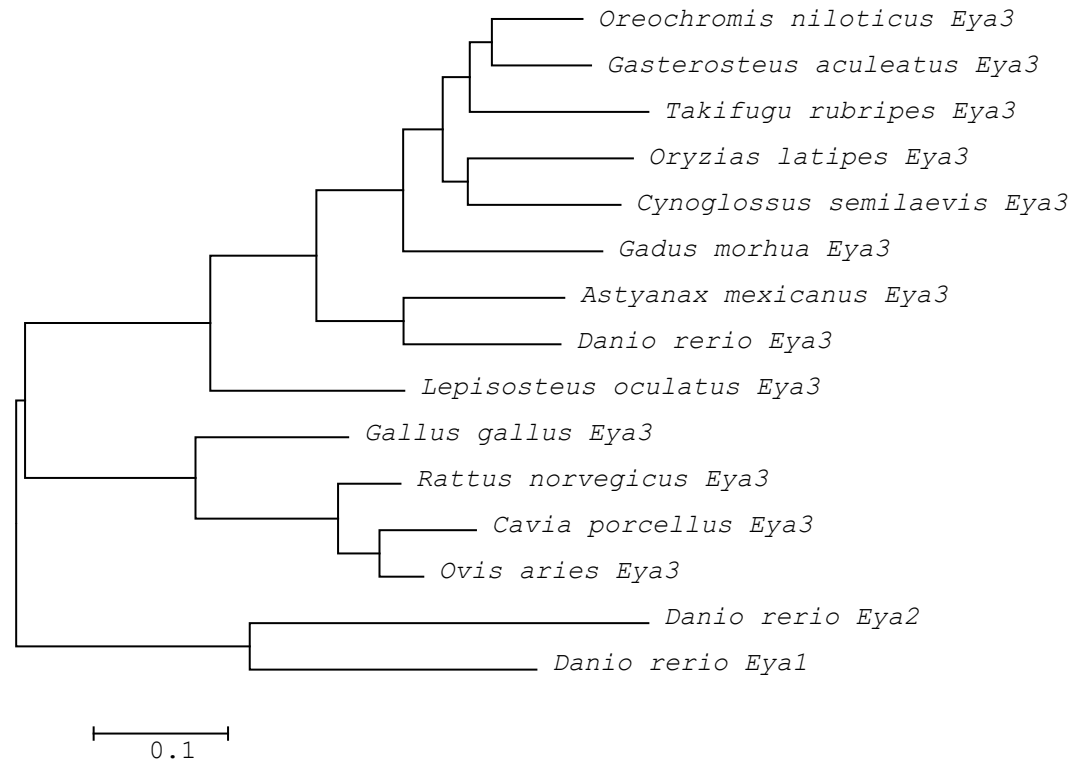
**Supplemental figure 2.** Alignment of the deduced aa sequence of Atlantic cod *Gadus morhua* *Eya3* transcript (Accession number: ENSGMOT00000014154) compared to other vertebrate EYA3 with zebrafish *Danio rerio* EYA1 as an outgroup. Conserved amino acid residues are shaded. The EYA domain is highlighted in blue and the putative HAD-like motifs as defined by (Rayapureddi et al. 2003) are outlined.

**Supplemental figure 3** Phylogenetic tree analysis of the vertebrate *Tsh $\beta$*  gene. The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model (Tamura and Nei 1993). The tree with the highest log likelihood (-2589.7105) is shown. Initial tree(s) for the heuristic search were obtained by applying the Neighbor-Joining method to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Evolutionary analyses were conducted in MEGA6 (Tamura et al. 2013). Accession numbers: red drum *Sciaenops ocellatus* GU144513, seabass *Dicentrarchus labrax* KJ095101, fugu *Takifugu rubripes* ENSTRUT00000046825, three-spined stickleback *Gasterosteus aculeatus* (*Tsh $\beta$ a*; ENSGACG00000005276, *Tsh $\beta$ b*; ENSGACG00000009897), Atlantic cod *Gadus morhua* (*Tsh $\beta$ a*; ENSGMOT00000018765, *Tsh $\beta$ b*; ENSGMOT00000012090, *Fsh $\beta$* ; ENSGMOT00000005957), Atlantic salmon *Salmo salar* (*Tsh $\beta$* ; NM\_001123528, *Fsh $\beta$* ; AF146152), rainbow trout *Onchorynchus mykiss* NM\_001124543, cave fish *Astyanax mexicanus* ENSAMXT00000011999, zebrafish *Danio rerio* ENSDART00000131080, common carp *Cyprinus carpio* AB003585, fathead minnow *Pimephalus promelas* EF590263, European eel *Anguilla anguilla* X73493, Japanese eel *Anguilla japonica* AB175833, quail *Cortunix cortunix* AF541922, red jungle fowl *Gallus gallus* ENSGALT00000004024, Guinea pig *Cavia porcellus* ENSCPOT00000008449, sheep *Ovis aries* ENSOART00000021912, and goat *Capra hircus* XM\_005677829.

**Supplemental figure 4** Alignment of the deduced aa sequence for vertebrate TSH $\beta$  compared with Atlantic cod and three-spined stickleback paralogs (TSH $\beta$ 2) and the gonadotropin FSH $\beta$  protein in Atlantic salmon *Salmo salar* and cod. Shaded regions indicate conservation of the amino acid residues with the conserved cysteine residues within the cysteine-knot region as described by (Hearn and Gomme 2000) are indicated by \* while the conserved glycosylated asparagine residue is indicated by #.

**Supplemental figure 5** Phylogenetic tree analysis of the vertebrate *Dio2* gene. The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model (Tamura and Nei 1993). The tree with the highest log likelihood (-2262.8587) is shown. Initial tree(s) for the heuristic search were obtained by applying the Neighbor-Joining method to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 19 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 204 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (Tamura et al. 2013). Accession numbers: red drum *Sciaenops ocellatus* KC999978, halibut *Hippoglossus hippoglossus* DQ856304, three-spined stickleback *Gasterosteus aculeatus* ENSGACT00000026661, fugu *Takifugu rubripes* ENSTRUT00000012441, Japanese medaka *Oryzias latipes* NM\_001136521, Atlantic cod *Gadus morhua* ENSGMOT00000022279, zebrafish *Danio rerio* (*Dio1*; ENSDART00000132596, *Dio2*; ENSDART00000146276), common carp *Cyprinus carpio* HE580224, spotted gar *Lepisosteus oculatus* ENSLOCT00000010823, cave fish *Astyanax mexicanus* ENSAMXT00000019169, Atlantic salmon *Salmo salar* (*Dio2a*; KP851704, *Dio2b*; KP851705), *Onchorynchus spp. (keta*; AB772421, *mykiss*; NM\_001124268, *nerka*; AB772420), red jungle fowl *Gallus gallus* ENSGALT00000044669, and sheep *Ovis aries* ENSOART00000003032.

**Supplemental figure 6** Alignment of the deduced aa sequence for vertebrate DIO2 compared with DIO1 in zebrafish. Shaded regions indicate conservation of the amino acid residues. The predicted iodothyronine deiodinase domain is highlighted in blue.



**Figure 1**

*Gadus morhua* EYA3  
*Danio rerio* EYA3  
*Gasterosteus aculeatus* EYA3  
*Ovis aries* EYA3  
*Gallus gallus* EYA3  
*Danio rerio* EYA1

```

-----
MDESQEVPELPTKKAHRHDPEVSQEGDSRSVVDSSDSPNRDES-----STQSNVNSYPPSSVTHLHSLPGA
-----
MEEEQDLPEQPVKKAKMQ---ESGEQTLSSQVSNPEVSDQKPE-----SSLASNLTMSEIIMTCTDYIP--
MEEEQDLPEQPVKKAKMQ---ESREQSLSHVSNTEVSDQKAES-----SSLGSNLPMSTEIMTCTDYIP--
-MEMQDLAS-PHSRVSGSSESPNGPNIDNSHINNNSMTPNGTEGDNITMLTTADWLLSSSSQSAAVKTEPMSSEIATSV
  
```

*Gadus morhua* EYA3  
*Danio rerio* EYA3  
*Gasterosteus aculeatus* EYA3  
*Ovis aries* EYA3  
*Gallus gallus* EYA3  
*Danio rerio* EYA1

```

-----
PD-----QSNQETISRSQGCVTENAYTHNAVTC-KDLATTTSTEYTSQMYQGSNTAVTAYASQVAFPSLGQSSMYSAFPQ
-----
-----SEYTOQVYQG-SDFCVALDVAS-----PAVTSYTGQVAYPPLAOSTVYSAFPQ
-----
-----RSSNDYTSQMYSA-KPYAHILSVFVS-----ETA--YPGQTQYQTLQOSQPYAVYPO
-----
-----RSSNDYTSQMYSA-KPYAHILSVFVS-----ETMSPYPGQTQYQALQOSQPYTIYPO
ADGSLDSFSGSAIGTSGFSPRQTHQFSPQIYPSNRPHYHILPTPSA-----QNMAAYGQTQYTTGMQCAAAYGTYPQ
  
```

*Gadus morhua* EYA3  
*Danio rerio* EYA3  
*Gasterosteus aculeatus* EYA3  
*Ovis aries* EYA3  
*Gallus gallus* EYA3  
*Danio rerio* EYA1

```

AGQTYGLPPFGAMWPGIKTETGLPEAPSVGQPGFLSFSSAYTSTQSGQLHYSYPSQGSSTTASVYSNIP-ATTASTVPA
SGQTYGLPPFGAMWPGIKTE--LPEAPSVGQPGFLSFSSAYTSTQPNQIHYSYPSQGSCTTSSVYTNIP-PSTAVTT-A
TGQTYGLPPFGAMWPGIKTETGQPEAPSGQPGFLSFSAAYTSTQPAQLHYSY--QGSSTTSSVYSSIPSAAAATTTST
ATQTYGLPPF-----ASSTNASLIPTSSAIANIPTAAVS
TTQTYGLPPFGALWPGMKPESGLIQTPSTSQHSVLTCTTGLTTSQPSPAHYSYSIEASTNASPVSTSSSTVNISTSAVA
PGQPYGISAYG----IKTEGGLTQAQSPGQSGFLSYSSSFSTPQTGQAPYSYQMQGGSFTTTSGLYAGS-NSLTNSTGF
  
```

*Gadus morhua* EYA3  
*Danio rerio* EYA3  
*Gasterosteus aculeatus* EYA3  
*Ovis aries* EYA3  
*Gallus gallus* EYA3  
*Danio rerio* EYA1

```

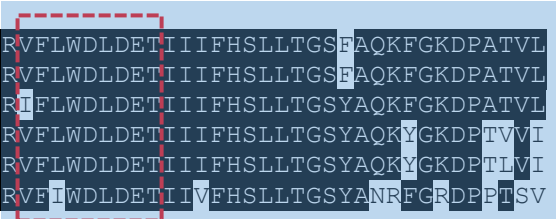
PAPNQEFSSYSSSLGPOAQFSQYYAPLPGYAPPGLPSSDSHGTDAAAGVAGYPAVKSESAVSAGL-----IA
AGTHQEFSSYNSVVG-QNQFSQYYVPPPSYMSAGLPSTDRDGAGVV-APGYPAIKTEGSASANLPNTTDASPGVTLPTGVA
TAAHQEFSSGYNLSG-QNQFSQYYTLPPSYVPAALPSSDDHGAGVG-AAGYSAAVKSEEAASAGLP-----PRGAA
SISNQDYPTYTILG-QSQYQACYP-SSSFGVTGQTNDAENTTLA-AATYQTEKPSVMVPAATPRLS-----SGDPS
SISQOEYPTYTILG-QSQYQTCYP-SSGFGVITPADSNAESTALA-TATYPSEKPNAMVPTRTVQRHSS-----AGDAS
NSTQDYPSYPTFG-QSQYAQYYN-SSPYTSPYMTSNNTSPITPSTTATYTLQEPSPSGITSQALTEQP-----TGE
  
```

*Gadus morhua* EYA3  
*Danio rerio* EYA3  
*Gasterosteus aculeatus* EYA3  
*Ovis aries* EYA3  
*Gallus gallus* EYA3  
*Danio rerio* EYA1

```

LPAGLALPTGARELE-DAGRRNSVGKAKGKAKKPDG-CPSTDSDLERVFLWDLDETI I I FHSLLTGSFAQKFGKDPATVL
LPAGMALPTGARDQD-EQNRKTPAGKAKGKAKKSDG-SQSTDNDLERVFLWDLDETI I I FHSLLTGSFAQKFGKDPATVL
LPTSVGVPAAGARDQD-EVGRRNSVGKAKGKGRSDN-SSPADSDLERIFLWDLDETI I I FHSLLTGSYAQKFGKDPATVL
PSPSLTQTTPSKDAD-DQSRKNMTGKNRGK-RKADA-SSSQDSELERVFLWDLDETI I I FHSLLTGSYAQKYGKDPPTVVI
TSPSLSRATASKESD-EQARKNIPGKNRGK-RKADT-SSSQDSELERVFLWDLDETI I I FHSLLTGSYAQKYGKDPPTLVI
YSTIHSPTPIKSDSDRLRRASDVKARGRRNNNPPPPSDLERVFLWDLDETI I I FHSLLTGSYANRFGRDPPTSV
  
```

**HAD motif I**



*Gadus morhua* EYA3  
*Danio rerio* EYA3  
*Gasterosteus aculeatus* EYA3  
*Ovis aries* EYA3  
*Gallus gallus* EYA3  
*Danio rerio* EYA1

```

NLGLQMEELIFELADTHLFFNDLEECDQVHVEDVASDDNGQDLSNYNFLADGFGSSGGGAPG--AGVPPGGVEWMRKLAFL
NLGLQMEELIFELADTHLFFNDLEECDQVHVDDVASDDNGQDLSNYNFSSDGFSGPSAGSGPGSATAVQGGVEWMRKLAFL
NLGLQMEELIFELADTHLFFNDLEECDQVHVEDVASDDNGQDLSITYNFLADGFNGSSGGGASGTTTGVQGGVEWMRKLAFL
GSGLTMEEMI FEVADTHLFFNDLEECDQVHVEDVASDDNGQDLSNYISFSTDGFGSGSGSGSHGSSVGVQGGVDWMRKLAFL
GSGLSMEEMI FEVADTHLFFNDLEECDQVHI EDVASDDNGQDLSNYNFSTDGFGSGSGSNANHSSSVGVQGGVDWMRKLAFL
SLGLRMEEMIFNLADTHFFNDLEECDQVHI DDVSSDDNGQDLSITYNFSTDGFHAAATSANLCLATGVRGGVDWMRKLAFL
  
```

**HAD motif II**

*Gadus morhua* EYA3  
*Danio rerio* EYA3  
*Gasterosteus aculeatus* EYA3  
*Ovis aries* EYA3  
*Gallus gallus* EYA3  
*Danio rerio* EYA1

```

RYRRLKEIYNAYKTNVGGLLSPMKRDLLLRLQSEIENVTDAWLS TALKSLLLIQSRGKCLNVLVTTTQLVPALAKVLLYG
RYRRLKEIYNGYKGNVGGLLSPMKRDLLLRLRSEIETVTDAWLS TALKSLLLIQSRGRCMVLVTTTQLVPALAKVLLYG
RYRRLKEIYSNFKGNVGGLLSPMKRELLRLQAEIESVTDTWLCTALKSLLLIQSRGKCMVLVTTTQLVPALAKVLLYG
RYRKVREIYDKHKS NVGGLLSPQRKEALQRLRAEIEVLTDSWLG TALKSLLLIQSRKNCVNIITTTQLVPALAKVLLYG
RYRRVREIYDKYKTNVGGLLSPQKREALQRLRTDIEVLTDSWLETALKSLLLIQSRKNCVNIITTTQLVPALAKVLLYG
RYRRVKEIYT TYKNNVGGLLGPAKREAWLQRLRAEIEALTDSWLTALKALTLIHSRNCVNIITTTQLIPALAKVLLYG
  
```

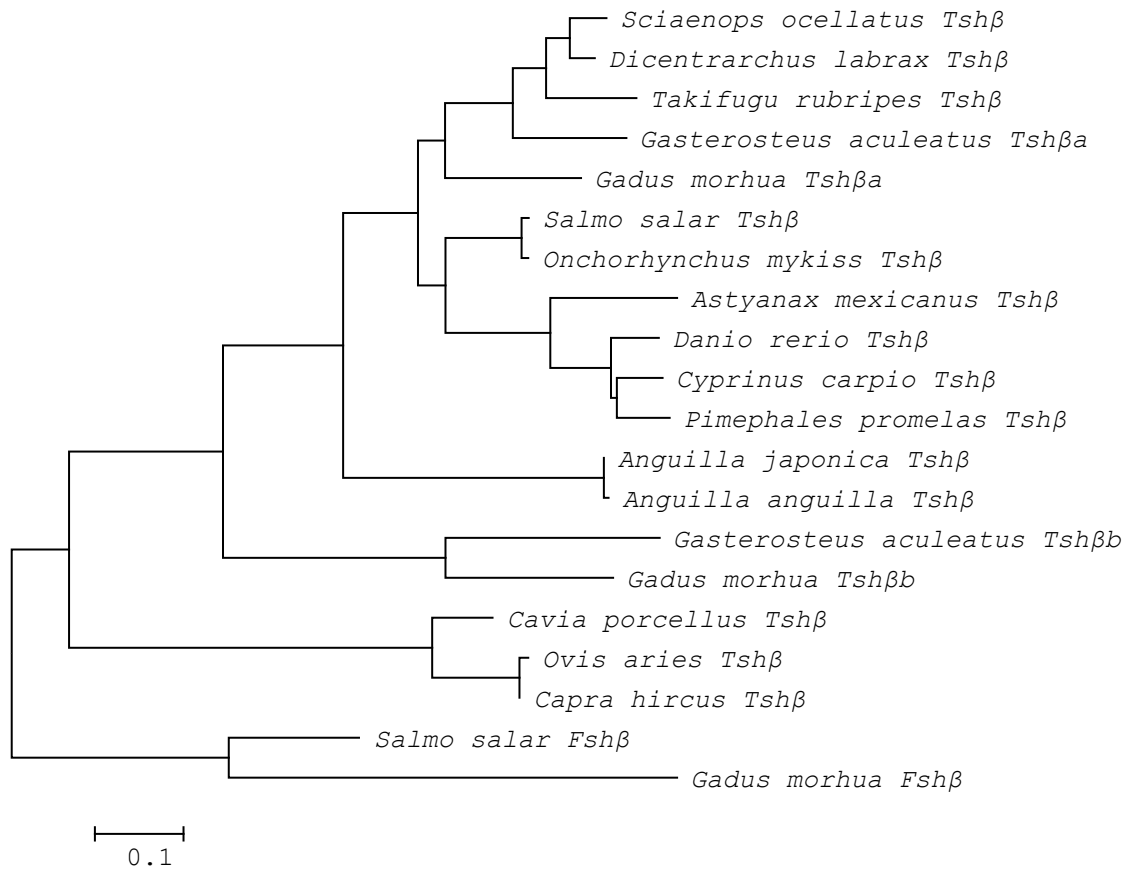
**HAD motif III**

*Gadus morhua* EYA3  
*Danio rerio* EYA3  
*Gasterosteus aculeatus* EYA3  
*Ovis aries* EYA3  
*Gallus gallus* EYA3  
*Danio rerio* EYA1

```

LGEVFSIDTIYSATKIGKESC FERIVSRFGKKVTVVIGDGRDEEFAAKQHNPFWRISTHGDLVSLHQALELDFL----
LGDVFPNIENIYSATKIGKESC FERIVSRFGKKVTVVIGDGRDEEFAAKQHNPFWRIS SHGDLISLHQALELDFL----
LGDVFPNIENIYSATKIGKESC FERIVSRFGKKVTVVIGDGRDEEFAAKQHNPFWRISTHGDLVSLHQALELDFL----
LGEIFPNIENIYSATKIGKESC FERIVSRFGKKVTVVIGDGRDEEFAAKQHNPFWRITNHGDLVSLHQALELDFL----
LGEVFPNIENIYSATKIGKESC FERIVSRFGKKVTVVIGDGRDEEFAAKQHNPFWRITNHADLVSLHQALELDFL----
LGVVFPNIENIYSATKIGKESC FERIVIQRFGRKVYVVVIGDGVVEEQGSKKHNPFWRIS SHSDLMALHHDLEYL----
  
```

**Figure 2**



**Figure 3**



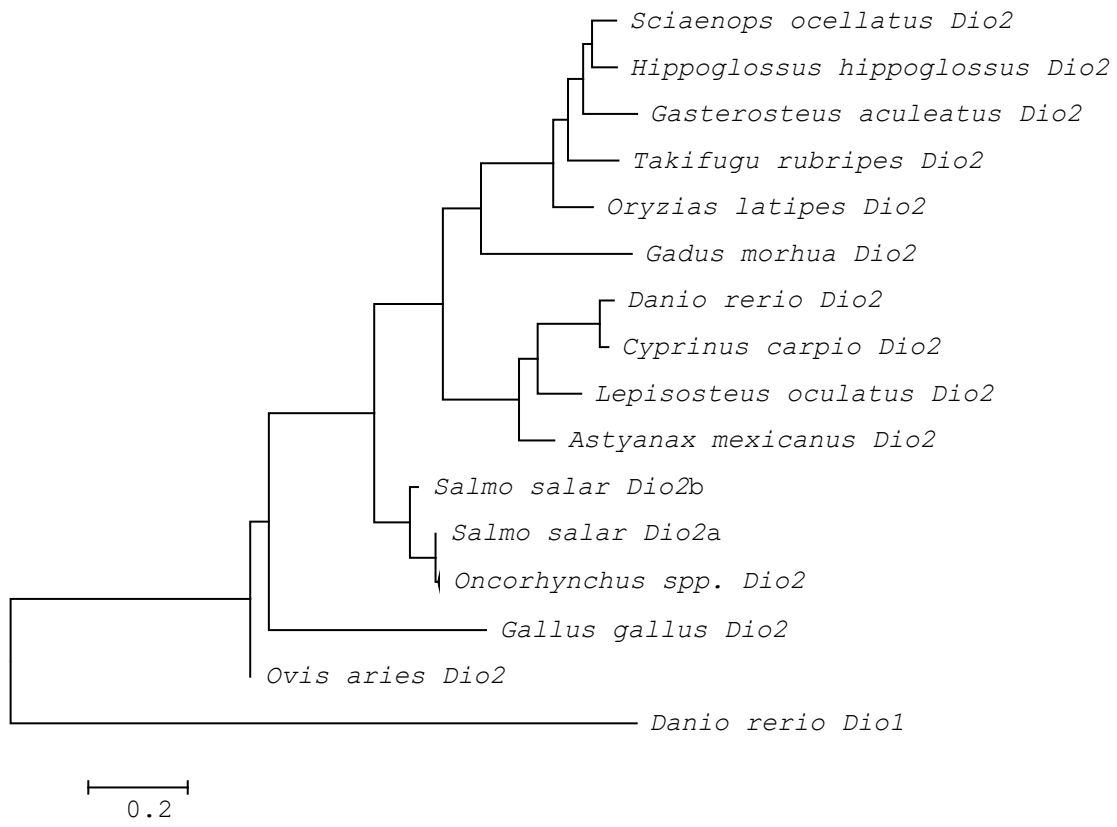
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Gadus morhua TSHβa  -----FSPAAPMCVPTDYTLTYVEKPECNFCVAINTTTCMGFCYSRDSNIGDLVGLRFLLRGCTYNQVE
Danio rerio TSHβ    --MS-LLYVIGMLGLLMKVAVPMCAPTDYTIYIERQECNYCVAVNTTTCMGFCFSRDSNIKELVGPFRFIVQRGCTYQEV
Gasterosteus aculeatus TSHβ --LS-PMYACRLLFLLLSPAVPTCFPTDFITMYVERPECDYCVAINNTTTCMGFCYSRDSNVRAIVGPRFLIQTGCTYDKVE
Ovis aries TSHβ    --MTAIFLMSMIFGLACGQAMSFICPTIYMMHVERKECAYCLTINTTTCAGYCMTRDVNGKLFPLKYALSQDVCTYRDFM
Gallus_gallus TSHβ --MSPFFMMSLLFGLTFGQTASVCAPESEYTIHVEKRECAVCLAINTTTCAGFCMTRDSNGKLLLLKSALSONVCTYKEMF
Gadus morhua FSHβ  -MQLVVMAAVLAMTWADQPCSFTRPTPTTIAVKS--CVRTESINTTMCEGQCYQEDP-----MDPGERPQQYTCSGDWA

Gadus morhua TSHβ  YRTAILPGCPSEGSLSFSYPVALSCHCGACNTAVDECAHRAS-SNRPTCTKPVRHIY----QSNFLLPF-----
Danio rerio TSHβ  YRTAVLPGCPSHADPHFTYPVALSCHCSTCKTHSDECALKTR-SAGMRC SKPVHHLYPE--ENNYAQAYWDQYE
Gasterosteus aculeatus TSHβ YRAALLPGCPIDSDPVFSYPVALSCRCGTCRTDSDECVHRAPGVGGARCTKPVRRIYPYPGOSTYMTPE-----
Ovis aries TSHβ   YKTAEIPGCPRHVTPYFSYPVAISCKCGKCNTDYSDCIHEAI--KTNYCTKP-----QKSYVVGFSI---
Gallus_gallus TSHβ YQTALIPGCPHHTIPYYSYPVAISCKCGKCNTDYSDCVHEKV--RTNYCTKP-----QKLCNM-----
Gadus morhua FSHβ  YEVKHFEGCLEG----VLYPVARSKCSLCSQSSNTDCERVLWRQPVSSCLT-----

```

**Figure 4**



**Figure 5**

<i>Gadus morhua</i> DIO2	MG-TGPE	DLLV	ALQILPGFFSNCLFLALYDSV	LLKRAVS	LLRASRSARGG	GEWQRALTSEGMR	SIWKGFLD	ANKQ	
<i>Danio rerio</i> DIO2	MG-LLSVDLLV	TLQILPGFFSNCLF	FVLYDSIV	LVKRVVS	LLSCSG-STG	EWQRMLTTAGVR	SIWNSFLD	AYKQ	
<i>Gasterosteus aculeatus</i> DIO2	MG-MASGGLRV	TLQILPGFFSNCLFLALYDSV	VLLKAVVS	LLSCSR-AAG	RGARRRMLTSAGLR	SVWRSFLD	AYKQ		
<i>Salmo salar</i> DIO2a	MG-AGSVDLLV	TLQILPGFFSNCLFLALYDSV	VLVKRLVS	LLSCSG-SGG	GEWQRMLTSAGLR	SIWNSFLD	AYKQ		
<i>Salmo salar</i> DIO2b 2b	MG-AASVDLLV	TLQILPGFFSNCLFLALYDSV	LVKRVVS	LLSCPG-GGG	GEWQRMLTSAGLR	SIWNSFLD	AYKQ		
<i>Gallus gallus</i> DIO2	MG-LLSVDLLI	TLQILP	VFFSNCLFLALYDSVI	LLKHMVL	FLSRKSARG	QTWRR--SSE	LQ-CNPHS	-----QG	
<i>Ovis aries</i> DIO2	MG-LLSVDLLI	TLQILP	VFFSNCLFLALYDSVI	LLKHVVL	LLSRKSTRG	Q-WRRMLTSEGMR	CIWKSFLD	AYKQ	
<i>Danio rerio</i> DIO1	MGS	AVGFAL	RKLFVYISAVLMV	CAAILRMSMLK	LLSFISPGRMRKIHMKG	--ERTT	MTQNP	KFRYEDWGP	AFSLAFIK

**Figure 6**

### **Supplemental references**

Hearn, M. T. W., and P. T. Gomme. 2000. "Molecular architecture and biorecognition processes of the cystine knot protein superfamily: Part I. The glycoprotein hormones." *Journal of Molecular Recognition* 13 (5):223-278. doi: Doi 10.1002/1099-1352(200009/10)13:5<223::Aid-Jmr501>3.3.Co;2-C.

Rayapureddi, J. P., C. Kattamuri, B. D. Steinmetz, B. J. Frankfort, E. J. Ostrin, G. Mardon, and R. S. Hegde. 2003. "Eyes absent represents a class of protein tyrosine phosphatases." *Nature* 426 (6964):295-8. doi: 10.1038/nature02093.