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Research Article

Temporal expression of thyroid hormone regulating genes (*tsh-b, tsh-r, dio2* and *dio3*) and their correlation with annual reproductive cycle of the Indian freshwater catfish, *Heteropneustes fossilis* (Bloch).

Dinesh Raj Pant
Fish Endocrinology Laboratory, Department of Zoology, University of Delhi, Delhi- 110007, India
Kumari Ila
Fish Endocrinology Laboratory, Department of Zoology, University of Delhi, Delhi- 110007, India
Uma Bharati Sahu
Fish Endocrinology Laboratory, Department of Zoology, University of Delhi, Delhi- 110007, India
Neeta Sehgal*
Fish Endocrinology Laboratory, Department of Zoology, University of Delhi, Delhi- 110007, India

*Corresponding author. E mail: nsehgal@zoology.du.ac.in

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Abstract
Photoperiod and temperature are well-established environmental cues for gonadal growth in seasonally reproducing organisms. The photoperiod is known to regulate seasonal reproduction via induction of thyroid hormone regulating genes in the saccus vasculosus (SV) of fishes. However, SV is absent in many seasonally breeding fishes, including *Heteropneustes fossilis*. *H. fossilis* is a long-day breeding catfish in which gonadal recrudescence begins six months earlier than spawning. The present study attempted to analyse the expression of thyroid hormone-regulating genes (*tsh-b, tsh-r, dio2* and *dio3*) in the brain, liver and ovary. In the brain, upregulated expression of *thyrotropin-beta subunit* (*tsh-b*), *deiodinase2* (*dio2*) and *deiodinase3* (*dio3*) genes is concomitant with the increasing photoperiod and temperature in nature, which may appear to regulate seasonal reproduction. Both deiodinases, *dio2* and *dio3*, were also upregulated in the liver and ovarian tissue during the gonadal growth phase. The upregulation of deiodinases may enhance the metabolism and activity of tissues, thereby facilitating their respective roles. The expression of these genes was also assessed in the brain, liver, ovary, kidney, skin, spleen and gill tissues during the spawning period. The ubiquitous expression of deiodinases may point to enhanced activity in their organ-specific role. The present study speculates that expression of *tsh-b, tsh-r, dio2* and *dio3* genes during the reproductive phase of *H. fossilis* might be involved in the regulation of seasonal reproduction.

Keywords: Deiodinase enzymes, Saccus vasculosus, Seasonal reproduction, Thyroid, Thyrotropin

INTRODUCTION

A burgeoning literature suggests that seasonal reproduction is an evolutionarily adaptive trait in most organisms inhabiting the sub-tropical and temperate regions (Migaud *et al.,* 2010). Reproduction in the most favourable period of the year is timed by the endogenous rhythm of an organism, thus ensuring the maximal survival of the young ones (Skoglund *et al.,* 2011). The endogenous rhythm synchronizes the physiology of an organism with the external environment and is entrained by external cues like the duration of daylight, precipitation and temperature (Husse *et al.,* 2015). The favourable physiological conditions for seasonal reproduction are guided through the mediation of external as well as internal factors that are translated into the neuroendocrine physiological changes, like elicitation of melatonin production by scotophase (Migaud *et al.,*

2010; Feng *et al*., 2019; Kupprat *et al*., 2020; Nisembaum *et al*., 2021).

There has been a long-standing interest in exploring the interplay of molecular mechanisms of photoperiod and temperature-responsive elements that regulate seasonality in reproduction. The reproductive phase of a seasonally breeding fish is initiated by GnRH release from the hypothalamus and is subsequently facilitated by GnRH-GtH-gonadal steroid-regulated endocrine mechanism (Zhang *et al*., 2009; Guh *et al*., 2019; Hafeez and Ahmed, 2021). At a specific latitude, the length of the photoperiod is fixed at a particular time of the year, and as a consequence, day length is considered a reliable environmental factor influencing seasonal reproduction (Nakane *et al*., 2013; Polat *et al*., 2021; Garcia *et al*., 2022). Likewise, the average seasonal water temperature also varies, making it another important cue for the seasonal reproduction of fishes (Sundararaj and Vasal, 1976; Chaube and Joy, 2002; Pankhurst and King, 2010; Raimondo, 2012; Polat *et al*., 2021; Garcia *et al*., 2022). Several endocrine mechanisms involving melatonin (Chaube and Joy, 2002; Falcon *et al*., 2010; Hafeez and Ahmed, 2021), kisspeptin (Shahjahan *et al*., 2013; Shao *et al*., 2019; Chaube *et al*., 2020) and thyroid hormone (Parkinson and Follett, 1994; Tovo-Neto *et al*., 2018; Ma *et al*., 2020) have been shown to play a part in the regulation of seasonal reproduction via GnRH release. The expression of deiodinase enzymes in the liver can be correlated with its metabolic activity and plasma thyroid levels (Van Der Geyten *et al*., 1998; Eales, 2019). Nakane *et al.* (2013) have suggested the role of long-day induced thyroid hormone regulating genes in the seasonality of reproduction. A perusal of literature shows that thyroid stimulating hormone (TSH) regulates basal metabolic rate via thyroid hormones, i.e., thyroxine (T4) and triiodothyronine (T3). On the other hand, thyroid hormone metabolism is regulated by the action of deiodinase enzymes. Although deiodinases are expressed ubiquitously, the liver is the major site for peripheral deiodination (MacLatchy and Eales, 1992; Eales, 2019).

The role of the thyroid hormone regulating genes is well known in birds and mammals, including Japanese quail (Yoshimura *et al.,* 2003; Morris *et al*., 2020), red-headed buntings (Trivedi *et al.,* 2019) and hamsters (Watanabe *et al.,* 2004; Sáenz de Miera *et al*., 2018). In these animals the long day signal induces the pars tuberalis (PT) to secrete thyrotropin. The PT-TSH acts locally on the ependymal lining of the 3rd ventricle in the medial basal hypothalamus via thyrotropin-receptor (*tsh -r*) (Yoshimura *et al.,* 2003; Trivedi *et al*., 2019), that enhances the *dio2* and suppresses the *dio3* gene expressions, resulting in a high titre of intraventricular T3. Intra-ventricular thyroid hormone (T3) acts on specific neurons and induces the release of GnRH. Their role in the seasonality of reproduction has been elucidated in few fishes, namely *Oncorhynchus masou* (Nakane *et al.,* 2013), *Gymnocypris przewalskii* (Tian *et al.,* 2019) and *Gadus morhua* (Doyle *et al*., 2021) *Scomber japonicus* (Ohga *et al*., 2023). Though the fish pituitary lacks pars tuberalis, the long-day induced thyrotropin and deiodinase enzymes have been localized in the saccus vasculosus (Nakane *et al*., 2013; Maeda *et al*., 2015) circumventricular organ located posterior to the pituitary.

The saccus vasculosus possesses three types of cell population; coronet cell, cerebro-spinal fluid (CSF) contacting cell and supporting cell. Several neuropeptides were localized in the SV under long photoperiod treatment that includes kisspeptins and kisspeptin receptors (Chi *et al.,* 2017) leptin and melatonin receptors (Chi *et al.,* 2019) in Atlantic salmon (*Salmo salar*), and thyrotropin beta, thyrotropin receptors, deiodinases and rhodopsin family genes in coronet cells of SV of masu salmon (*Onchorhynchus masou*). The coronet cells have apical crowns of globule tipped cilia and express both photoreceptive and secretory functions (Nakane *et al.,* 2013; Maeda *et al*., 2015). However, in many seasonally breeding fishes, including *H. fossilis*, SV is absent (Narsimhan, 1970).

The annual ovarian cycle of *H. fossilis* is divisible into four phases, namely, preparatory phase (February to April), pre-spawning phase (May-June), spawning phase (July- August) and post-spawning phase (September to January) (Sundararaj and Vasal, 1976; Chaube *et al*., 2020; Chaube *et al*., 2022). The absence of saccus vasculosus in *H. fossilis* makes it imperative to identify the molecular mechanisms and regulatory sites of photoperiod and temperature-induced seasonal reproduction in this fish. The objective of the present study was to assess the role of photoperiod and temperature in regulating the seasonality of reproduction in the Indian freshwater catfish *H. fossilis* by studying the expression pattern of genes, namely, *thyrotropin-beta subunit* (*tsh-b), thyrotropin-receptor (tsh-r), deiodinase 2 (dio2)* and *deiodinase 3* (*dio3).*

MATERIALS AND METHODS

Animal collection

Female specimens of the Indian freshwater catfish, *H. fossilis* (body weight: 20–30 g), were collected from the river Yamuna and its tributaries near Delhi, National Capital Region (NCR) at coordinates 28.704°N 77.102° E, in the second week of every month in the year 2019. The fish were kept in a glass aquarium (60cm × 30cm × 30cm) containing 40 L of water. The fish were acclimatized in the laboratory conditions with a photoperiod regime of 12L: 12D and a water temperature of 25±1ºC. The fish were fed with *ad libitum,* the lab-prepared fish feed.

Sample collection

Six female catfish were anesthetized using phenoxyethanol (0.001%), weighed and decapitated. Ovaries were excised and weighed. Tissue samples from ovaries, the whole brain (along with the pituitary) and the liver were processed for total RNA isolation. To assess the distribution of photoperiodic responsive genes in other peripheral tissues, total RNA was also collected from the spleen, skin, gills and kidney tissue in July.

Characterization and analysis of expression of *tsh-b, tsh-r, dio2* and *dio3 genes*

Tissue samples were homogenized in TRI-reagent (T9424, Sigma Aldrich), and total RNA was isolated using the manufacturer's protocol (Chomczynski and Sacchi, 2006) and quality was assessed using the nanodrop spectrophotometer (Thermo Fisher) at the wavelength of 260/280nm. cDNA was prepared from total RNA by using a cDNA synthesis kit (K1642 Thermo Fisher Scientific) using the manufacturer's protocols. The quality of cDNA was checked by the PCR amplification of the *β-actin* gene and separated on 1% agarose gel electrophoresis.

The nucleotide sequences of the *tsh-b, tsh-r, dio2* and *dio3* genes of various fish species were retrieved from NCBI. Degenerated primer sets (Table 1) were designed for *tsh-b, tsh-r, dio2* and *dio3* genes from the conserved regions of the aligned homologous sequences. Partial stretches of *tsh-b, tsh-r, dio2* and *dio3* genes were amplified by conventional PCR. Amplified products were resolved on the 1% agarose gel by electrophoresis and eluted using a gel elution kit (QIAquick® Ref. 28704). The products were sequenced via the Sanger dideoxy chain terminator method and specific primer sets for each of the genes were designed from the obtained sequences (Table 2). Primers were again designed to amplify the whole coding sequence of *tsh-b.*

Quantitative Real-time PCR

Quantitative Real-time PCR (qPCR) reactions were run in 384 well plates (Micro Amp 4309849 Applied Biosystems) on a Real-time PCR machine (7500 Applied Biosystems Fast Dx). Each reaction was run in triplicate and contained 5µL of 2X Power SYBR™ Green master mix (A25742 Applied Biosystems), 1µL of cDNA template, 1µL of forward and reverse primers each and 2µL of nuclease-free water. In no template control (NTC), template cDNA was replaced by nuclease-free water. Melt curve analysis was performed at the end of PCR, to confirm the amplification of a desirable single product only. *β-actin* gene was taken as an endogenous control.

The relative fold change was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). The cycle threshold (Ct) values of the triplicate reactions were averaged for the calculation. The C_t average of *β-actin* was subtracted from the target to obtain ΔCt, thereafter, the calibrator was subtracted from ΔCt to obtain $\Delta\Delta Ct$. The relative fold change was calculated using the formula $2^{-\Delta\Delta Ct}$ method.

Statistical analysis

The one-way ANOVA ($p<0.05$) was performed by employing the Tukey test using IBM-SPSS 25.0 software. The values were presented as mean ± SEM. The Heat map was generated using log2 of mean relative fold change. To assess the relationship between GSI and the expression of various genes, correlation graphs were plotted between the mean relative fold change in the expression of genes and GSI.

Ethical clearance

The fish were cared and treated according to the procedures established by the Institutional Animal Ethics Committee (IAEC) at the University of Delhi under the Committee for control and supervision of Experiments for Animals (CPCSEA) (File no. DU/ZOOL/IAEC-A/01/2019).

RESULTS

Characterization of *tsh-b, tsh-r, dio2* and *dio3 genes*

The amplified products of the degenerated primer pairs were sequenced and partial nucleotide sequences were obtained. Thereafter, primers for quantitative Real Time PCR were designed and the sequences were submitted to the National Center for Biotechnology Information (NCBI) database, *tsh-b* (Genbank: MW355447.1), *tsh-r* (GenBank accession No: MW355448.1), *dio2* (GenBank accession No: MW355445.1) and *dio3* (GenBank accession No: MW355446.1).

Annual expression profile of *tsh-b, tsh-r, dio2* and *dio3 genes*

Seasonal changes in the expression of above-mentioned genes and GSI of the *H. fossilis* was measured every month during a reproductive cycle. The GSI of *H. fossilis* started increasing in the early preparatory phase, but a significant increase was observed in April. The gonadal growth accelerated in May-June (pre-spawning phase) and reached the maximum in July-August (spawning phase). The increase in GSI during these three phases coincided with the increasing photoperiod and temperature in nature. The release of matured oocytes during the spawning phase led to a significant decline in the GSI (Fig. 1).

Expression pattern of thyrotropin-beta subunit (*tsh-b*)

Relative fold change of *tsh-b* mRNA were quantified in the brain, liver and ovary and depicted in the Figs. 2, 3,

4 and 5. The *tsh-b* relative mRNA abundance in the brain was evident during the pre-spawning phase. The gene starts upregulating in the preparatory phase and attains its peak from April to July (late preparatory to spawning phase). It again drops to a minimal level during the post-spawning phase and maintains it throughout (Fig. 2). In both liver and ovary tissue, *tsh-b* does not show any circannual variation and remains at a minimal level throughout the year (Figs. 3 and 4). The differential tissue analysis was also done in the spawning phase and found the expression of *tsh-b* exclusively expressed in the brain tissue (Fig. 7a).

Expression pattern of thyrotropin receptor (*tsh-r*)

The annual relative fold change of *tsh-r* mRNA in the brain, liver and ovary are depicted in Figs. 2, 3, 4 and 5. The *tsh-r* does not exhibit mRNA annual variation in the brain, liver and ovarian tissues. However, in differential tissue analysis it appeared evidently in the liver and brain tissue. On the contrary, other tissues viz. ovary, kidney, skin, spleen and gills, also exhibited a minimal level of expression (Fig. 7b).

Expression pattern of deiodinase 2 (*dio2*)

The annual relative fold change of *dio2* mRNA was quantified and depicted in Fig. 2 to 5. The *dio2* expression begins upregulating in the brain, liver and ovary in the preparatory phase of catfish and it attains its peak in May (Figs. 2, 3, 4 and 5). The expression remains high in the May, June and July months in the liver and ovary (pre-spawning to spawning), but in the brain it decreases gradually after May. The average day temperature is maximum in May, and that decreases in following month, in contrary the photoperiod is maximum in June, this may suggest the brain dio2 is responsive to temperature. The expression of *dio2* in all three tissues declined to a minimal level again in September. The *dio2* expression remained constant throughout the post-spawning phase. The differential tissue analysis was also done and found that the *dio2* mRNA was expressed ubiquitously during the spawning phase, with maximum fold expression in the gills followed by the brain, liver, ovary and spleen tissue. The kidney and skin tissue also expressed minimal *dio2* gene expression (Fig. 7c).

Expression pattern of deiodinase 3 (*dio3*)

The annual relative fold change of *dio3* mRNA was quantified and depicted in Fig. 2 to 5. The *dio3* expressions also began upregulating and attained peak in the preparatory phase (March in ovary tissue and April in the brain and liver). The *dio3* mRNA remained upregulated throughout summer, drops to a minimal level in September, and remained low throughout the post-spawning phase. The differential tissue analysis was also done and found that the *dio3* mRNA was expressed ubiquitously during the spawning phase with a maximum relative fold change in the liver, ovary and

Table 1. Degenerative primer pairs used for the amplification of desired genes

Gene	Primer sequence	Product size (bp)
tsh-b	Forward 5' CCA GAG ACA TGA TGT TTG CTC C 3' Reverse 5 GTC CAA TCT GAC TCT GAG TGG 3'	484
tsh-r	Forward 5' GAG TTG TCA GTT TAC ACA TTG ACC 3' Reverse 5' GCA CCA GCA GGA TCT TGG AGT T 3'	478
dio2	Forward 5'AAC TTT GGC TCG GCC ACC TGA C 3' Reverse 5' CAT TGT TAT CCA TGC AGT CGG CCA 3'	277
dio3	Forward 5' AGC TGC TCC TGA CCG CCG TTC ATG 3' Reverse 5' CGC TCG AAA TAA GCG CCG TAC GC 3'	281

Table 2. The primer pairs used for qPCR

Gene	Accession No.	Sequence of primer pair	Product size (bp)
β-actin	FJ409641.2	Forward 5' CGA AGA CGA CAG GAT TTG CT -3' Reverse 5' GTT TGA AGC GCT CGT CTC TC -3'	105bp
tsh-b	MW355447.1	Forward 5' GCT GTA CCT ATC AGG ACG TG -3' Reverse 5' TGT GGG CAC ACT CAT CAC TG -3'	141bp
tsh-r	MW355448.1	Forward 5' TGC TGT AAT GCT CGG GGG TT -3' Reverse 5' GGT AAC TGC TCA CCC CTA ACG -3'	74bp
dio2	MW355445.1	Forward 5' GTT CCC GTT CGA GGT GAA GAA - 3' Reverse 5' CAT TGT TGT CCA TGC AGT CGG CC -3'	125bp
dio3	MW355446.1	Forward 5' GTA CCA GAT CCC GCG CC -3' Reverse 5' ACG AGT TGT CCA TGG TGT CC -3'	110bp

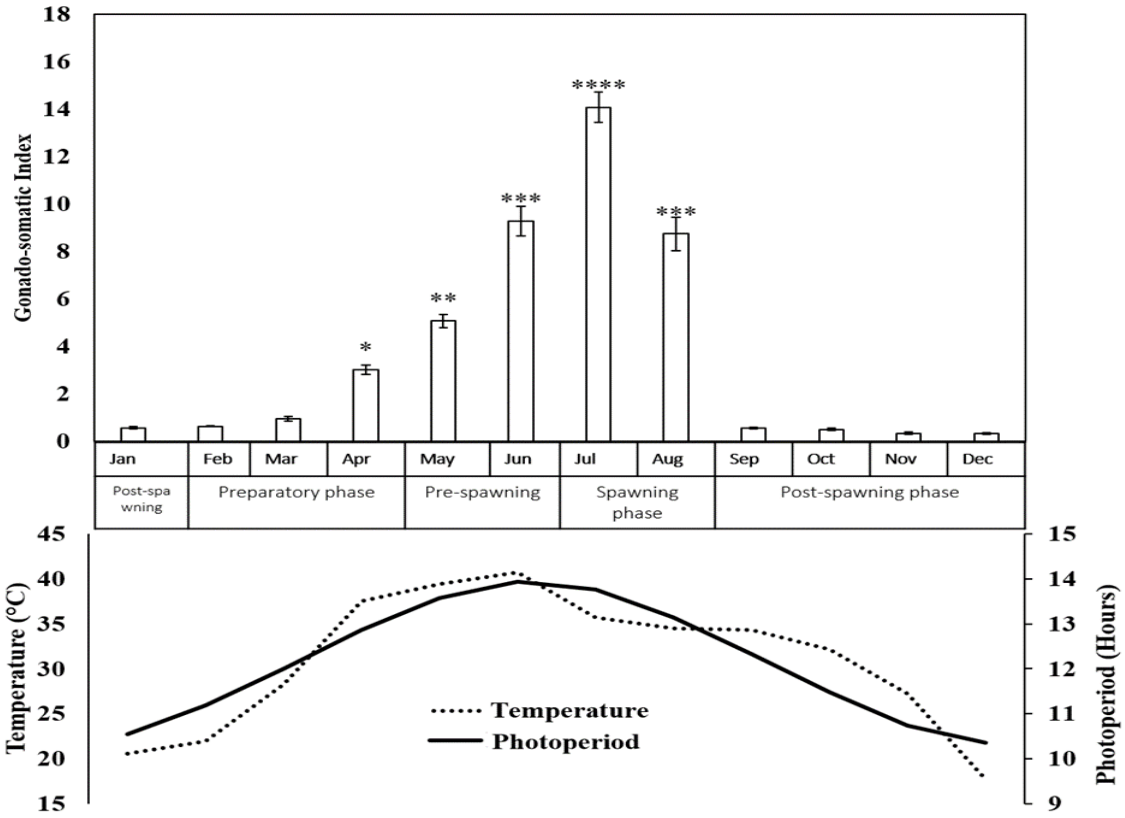

Fig.1. *Annual mean gonado-somatic index (GSI) of Heteropneustes fossilis, in correlation with the seasonal variation the photoperiod and temperature annual variation in photoperiod and air temperature in Delhi. Data values are expressed as mean±SEM. Bars with different superscripts are significantly different (n=6 and p<0.05)*

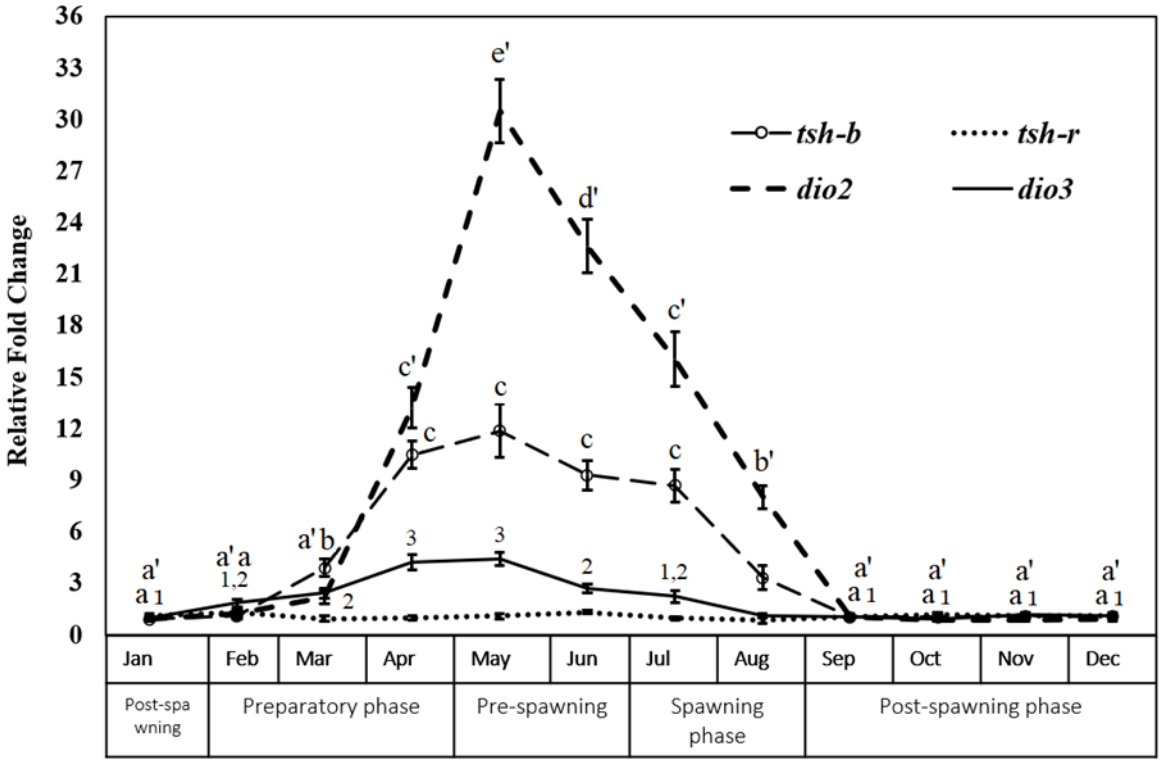

Fig. 2. *Relative gene expression ($2^{-\Delta\Delta Ct}$) of thyrotropin-beta (tsh-b), thyrotropin-receptor (tsh-r), deiodinase 2 (dio2) and deiodinase 3 (dio3) in the brain of Heteropneustes fossilis during the annual reproductive cycle in the year 2019. Data values are expressed as mean±SEM. Bars with different superscripts are significantly different (n=6 and p<0.05)*

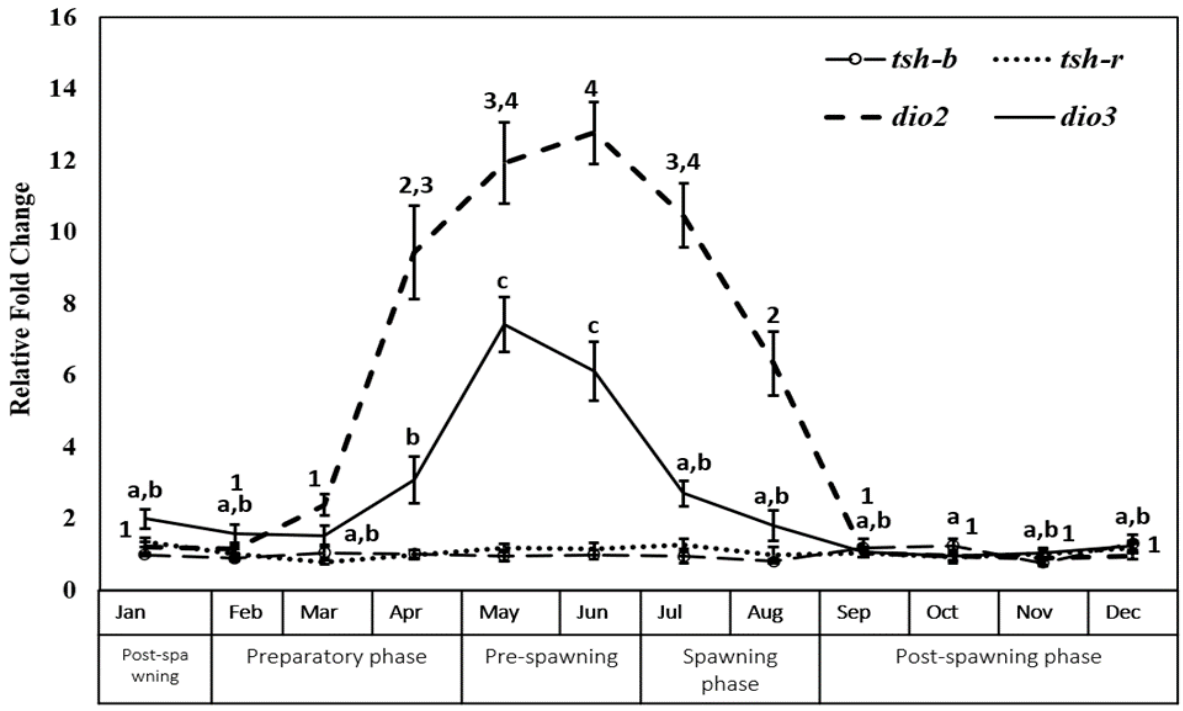

Fig. 3. *Relative gene expression ($2^{-\Delta\Delta Ct}$) of thyrotropin-beta (tsh-b), thyrotropin-receptor (tsh-r), deiodinase 2 (dio2) and deiodinase 3 (dio3) in the liver of Heteropneustes fossilis during the annual reproductive cycle in the year 2019. Data values are expressed as mean±SEM. Bars with different superscripts are significantly different (n=6 and p<0.05).*

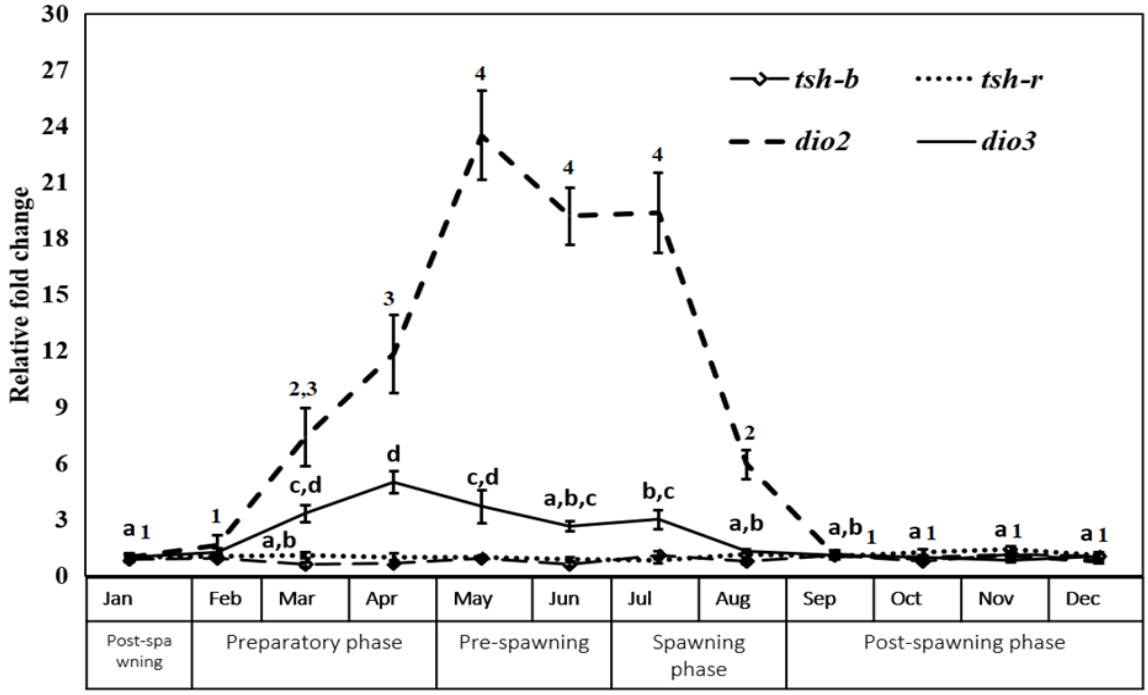

Fig. 4. *Relative gene expression ($2^{-\Delta\Delta Ct}$) of thyrotropin-beta (tsh-b), thyrotropin-receptor (tsh-r), deiodinase 2 (dio2) and deiodinase 3 (dio3) in the ovary of Heteropneustes fossilis during the annual reproductive cycle in the year 2019. Data values are expressed as mean±SEM. Bars with different superscripts are significantly different (n=6 and p<0.05).*

gills (Fig. 7d).

Correlation analysis

The scatter plot correlation was performed and the correspondence between and among the above-mentioned genes mRNA transcripts relative fold change and GSI was analyzed and depicted in Fig. 6. The brain *dio2* mRNA relative fold changed with *dio3* and *tsh-b* mRNA abundance in brain showed a positive correlation with coefficient of determinant (R^2) values of

0.42 and 0.52, respectively (Fig 6a (i) and (ii)). The brain *dio2* mRNA relative fold change also positively correlated with the GSI of catfish with an R^2 value of 0.76 (Fig. 6a (iii)). A positive correlation was also seen in the liver *dio2* relative fold change with liver *dio3* and GSI, with R^2 values of 0.57 and 0.61, respectively (Fig. 6b (i) and (ii)). A positive correlation was also seen in the ovary *dio2* relative fold change with ovary *dio3* and GSI, with R^2 values of 0.49 and 0.35, respectively (Fig. 6c (i) and (ii)).

DISCUSSION

Thyrotropin, or thyroid stimulating hormone (TSH), is a heterodimer glycoprotein hormone mainly synthesized in the pars distalis (PD). It consists of two protein chains, namely, common glycoprotein-alpha (CG-A) and thyrotropin-beta (TSH-B). The CG-A subunit is shared by all glycoprotein hormones, whereas the TSH -B subunit is unique to TSH. It is released into the blood and acts primarily on thyroid follicles via thyrotropin receptors for its synthesis and secretion. The pars tuberalis of mammals (Watanabe *et al.,* 2004) and birds (Yoshimura *et al*., 2003; Mishra *et al*., 2017) synthesize extra-PD TSH under photoperiod-regulated seasonal reproduction. However, in fishes photoperiod dependent seasonality regulating TSH is synthesized in the saccus vasculosus of *Onchorhynchus masou* (Nakane *et al.,* 2013), brain of *Gymnocypris przewalskii* (Tian *et al.,* 2019) and pituitary stalk of *Salmo salar* (Fleming *et al.,* 2019; Irachi *et al.,* 2021).

The HPT axis TSH and long-day stimulated TSH also differ in nature, which may prevent the physiological crosstalk. Like in mammals, TSH from PD and PT bears the same alpha and beta chain amino acid sequence, though they manage to prevent the crosstalk due to the differences in their glycosylation pattern (Ikegami *et al.,* 2014). However, in Atlantic salmon (*Salmo salar)* two paralogs of thyrotropin beta (*tsh-ba* and *tsh-bb*) were expressed in distinct pituitary cells. The *tsh-bb* was found to be expressed in the anterior pituitary near the stalk and upregulated during the spring season. On the other hand, *tsh-ba* is expressed in the PD and does not exhibit seasonal variation (Fleming *et al.,* 2019).

The thyrotropin receptor (TSH-R) belongs to the GPCR superfamily, having seven transmembrane domains (Hsu *et al*., 1998; Marcinkowski *et al*., 2019). The presence of TSH-R in the target tissue is equally important to facilitate the action of TSH. In the present study, thy-

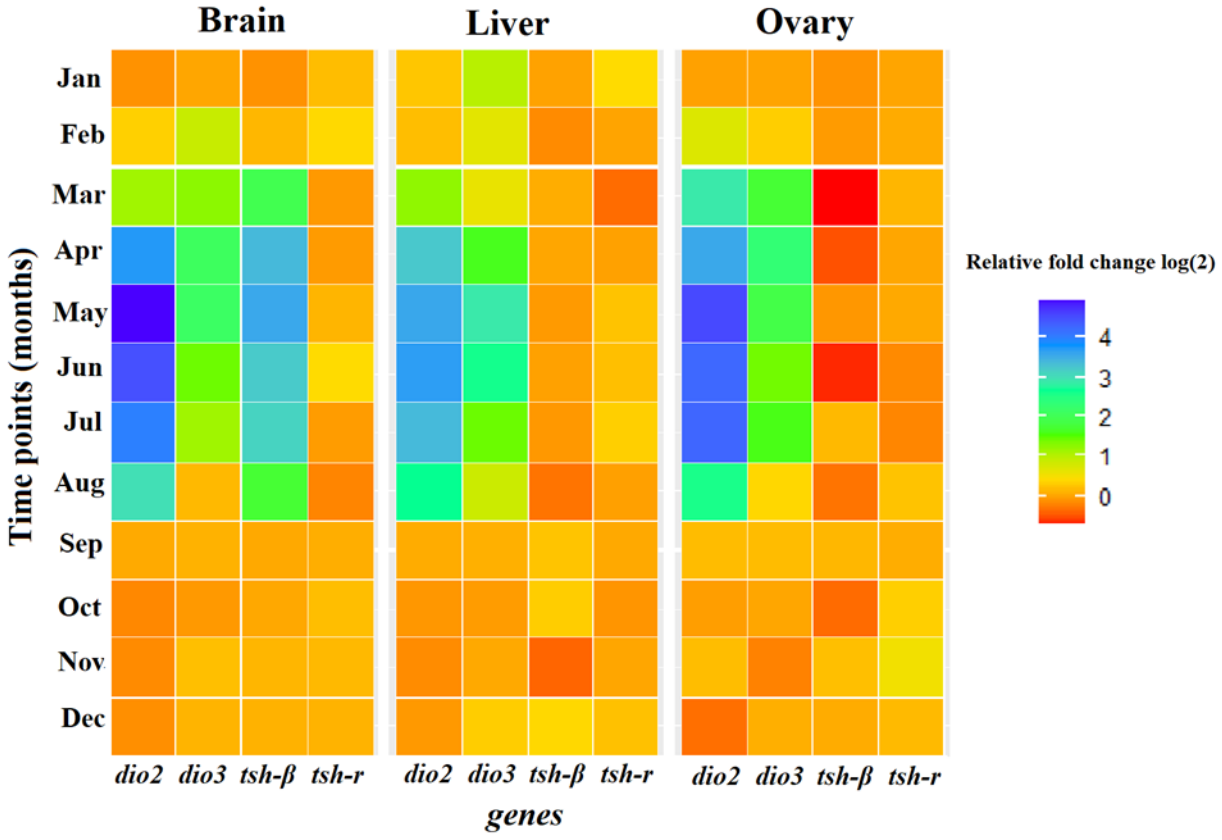

Fig. 5. *Heat map generated from log (2) mean relative fold change of thyrotropin-deiodinase axis genes. log (2) of thyrotropin-beta (tsh-b), thyrotropin-receptor (tsh-r), deiodinase2 (dio2) and deiodinase3 (dio3) mRNA relative fold change ($2^{-\Delta\Delta Ct}$) in brain, liver and ovarian tissue*

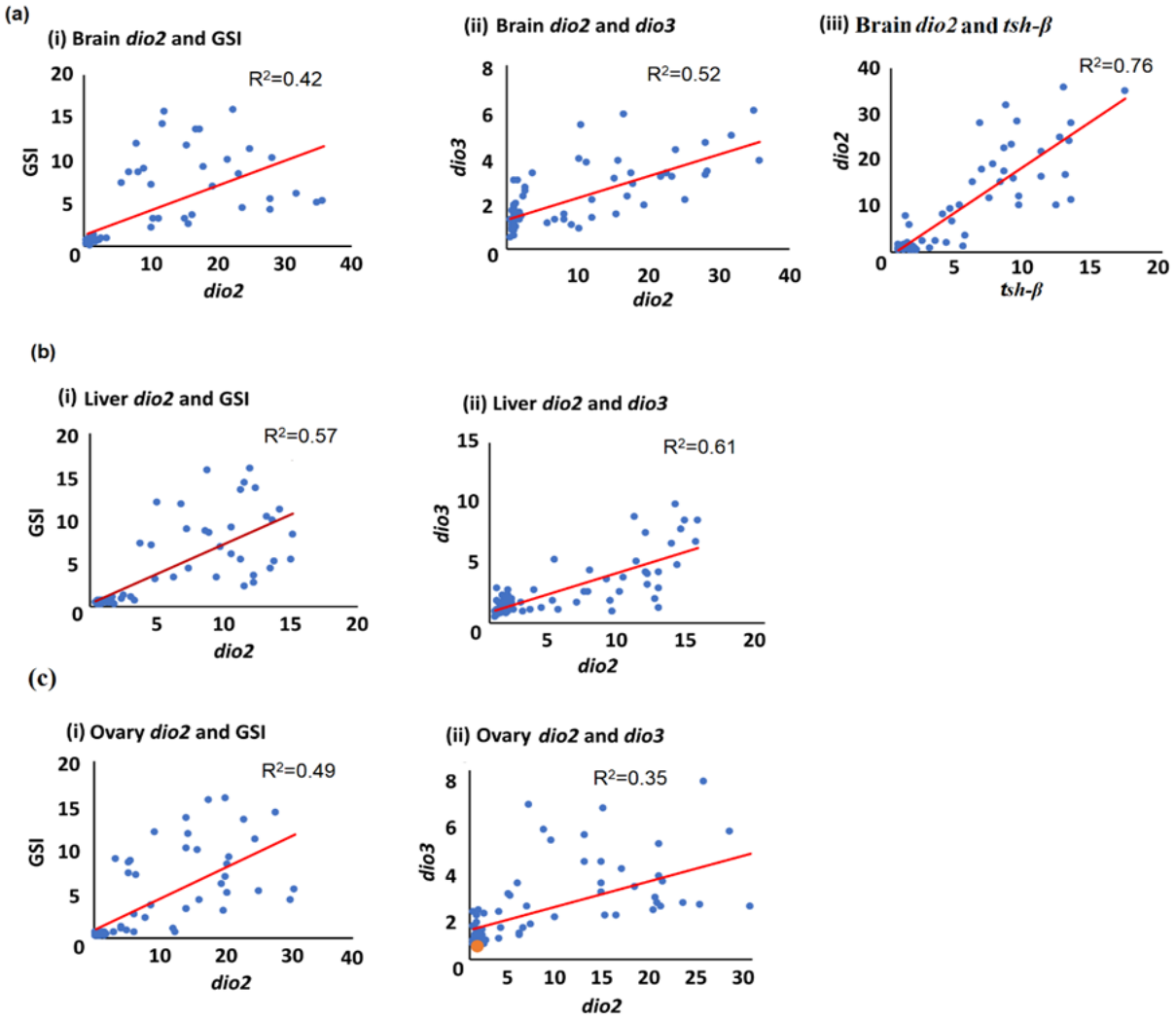

Fig. 6. *Scatter plot Correlation analysis;* ***(a)*** *Between mean relative fold change of deiodinase 2 (dio2) expressed in the brain with pituitary and (i) gonado-somatic index (GSI), (ii) deiodinase 3 (dio3) and ii) thyrotropin-beta (tsh-b) expressed in the brain with pituitary;* ***(b)*** *Between mean relative fold change of deiodinase 2 (dio2) expressed in the liver and (i) GSI and (ii) deiodinase 3 (dio3) expressed in the liver; © Between mean relative fold change of deiodinase 2 (dio2) expressed in the ovary and (i) GSI and (ii) deiodinase 3 (dio3) expressed in the ovary*

rotropin-receptor (*tsh-r*) does not exhibit circannual variation in the brain, liver, and ovary. However, during gonadal growth phase, *tsh-r* upregulation was reported in the SV of *Oncorhynchus masou* (Nakane *et al.,* 2013) and in the ovary of *Ictalurus punctatus* (Goto-Kazeto *et al.,* 2003). On the other hand, the increased thyrotropin-ligand, downregulates *tsh-r* expression via the inducible cAMP early repressor in rat thyroid gland (Lalli and Sassone-Corsi, 1995; Marcinkowski *et al*., 2019). The minimal expression of *tsh-r* may infer the constant sensitiveness of tissue. Deiodinases enzymes belong to the selenoprotein family, which carries out the deiodination reaction facilitated by the deiodinase 2 (Dio2) and deiodinase 3 (Dio3) enzymes encoded by the genes *dio2* and *dio3* respectively. The Dio3 enzyme catalyzes inner ring deiodination of both T4 and T3 hormones that results in inactivated thyroid hormones rT3 and T2 respectively. In contrast, Dio2 facilitates the outer ring deiodination of T4 that result into a more potent T3 hormone (Germain and Galton, 1997; Bianco *et al.,* 2002; Luongo *et al*., 2019; Russo *et al*., 2021).

Expression of *tsh-b, tsh-r, dio2* and *dio3* in the brain

In the present study, the concurrent upregulation of the brain *tsh-b, dio2* and *dio3* genes has been observed during the preparatory phase, which remains high throughout the pre-spawning and spawning phase. The *dio2* transcript abundance is positively correlated with the transcript abundance of *tsh-b* and *dio3* also with the GSI. The *tsh-b* and *dio2* genes upregulation with gonadal growth along with the long photoperiod appeared earlier in *O. masou* (Nakane *et al.,* 2013) and *Gymnocypris przewalskii* (Tian *et al.,* 2019) and temperature in *Emberiza bruniceps* (Trivedi *et al.,* 2019). These up-

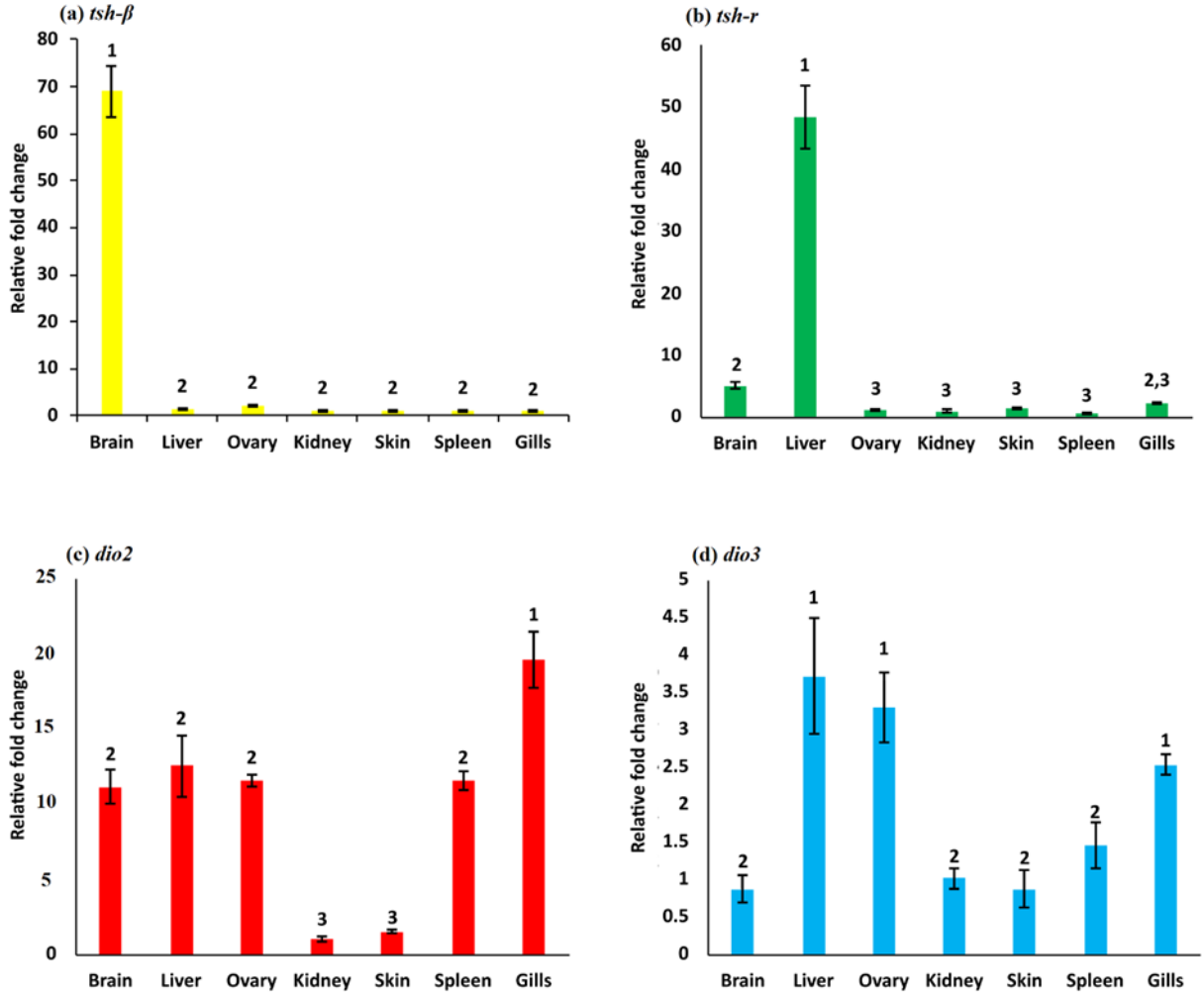

Fig. 7. *Relative gene expression ($2^{-\Delta\Delta Ct}$) of* ***(a)*** *thyrotropin-beta (tsh-β),* ***(b)*** *thyrotropin-receptor (tsh-r),* ***(c)*** *deiodinase 2 (dio2) and* ***(d)*** *deiodinase 3 (dio3) expressed in different tissues of Heteropneustes fossilis during the spawning phase. Data values are expressed as mean±SEM. Bars with different superscripts are significantly different (n=6 and p<0.05).*

regulated brain *dio2* in the present study may activate the hypothalamic GnRH via local activation of the T3 hormone, thereby may begin the reproductive phase as previously seen in *O. masou* (Nakane *et al.,* 2013), *G. przewalskii* (Tian *et al.,* 2019), *Gadus morhua* (Doyle *et al*., 2021) and *Scomber japonicus* (Ohga *et al*.,2023).

The present study showed *dio3* upregulation during increasing photoperiod and temperature that may suggest their involvement in reproduction. On the contrary, previous studies have reported the downregulation during long photoperiod conditions, thereby T3 catabolism is prevented e.g., in *Salmo salar* (Irachi *et al.,* 2021), *Onchorhynchus masou* (Nakane *et al.,* 2013), and *Gymnocypris przewalskii* (Tian *et al.,* 2019). The hypothalamic *dio3* is also sensitive to temperature (Wambiji *et al.,* 2011a), light (Nakane *et al.,* 2013), and nutritional status (Wambiji *et al.,* 2011b). In *O. masou*, the post-transcriptional event regulates *dio3*, and causes variation in protein expression under photo-stimulation (Nakane *et al.,* 2013). The DIO2 and DIO3 enzymes have contradictory roles in thyroid hormone metabolism, so three possibilities for their co-expression are: First, *dio3* expression can exist independently of *tsh-b* and *dio2*. Second, the increased T3 thyroid hormone may induce *dio3* in the brain tissue to self-inactivate*, like* in the gills of the rainbow trout (Van der Geyten *et al.,* 2005). Third, the net result of *dio2* and *dio3* co-expression appeared to be similar to the Deiodinase 1 (Dio1) enzyme, which catalyzes both the outer and inner ring deiodination of thyroid hormone (Kohrle, 1999). The *tsh-b* was localized in the pituitary stalk and SV, however photo-responsiveness is exhibited by the *tsh-b* in pituitary stalk only. On the contrary, both *tsh-b* and *dio2* were expressed in the saccus vasculosus (SV) of the *O. masou* (Nakane *et al.,* 2013). In *H. fossilis* the SV is absent. Despite this, in the present study, circannual variation was observed in the brain in the *tsh-b*, *dio2* and *dio3*. Therefore, it is speculated on their involvement in reproduction, and the *tsh-b* and *dio2* may be expressed in the photoreceptive nuclei of the brain,

which have SV homology, or in the hypothalamus, like in the Atlantic salmon and higher vertebrates (Irachi *et al.,* 2021; Yoshimura *et al.,* 2003). The SV has been attributed to several functions that include secretory (Stahl and Seite, 1960), osmoregulation, deep-sea pressure perception (Sueiro *et al.,* 2007), glycogen metabolism (Narsimhan and Sundararaj, 1971), and reproduction (Nakane *et al*., 2013; Chi *et al*., 2017; Chi *et al*., 2019).

Expression of *tsh-b, tsh-r, dio2* and *dio3* in the liver

In the present study, the upregulated expression of *dio2* and *dio3* genes appeared from the preparatory to spawning phase in the liver and ovary, concurrently with the GSI. The liver *dio2* and *dio3* are known to regulate the plasma thyroid hormone metabolism in the Nile tilapia (Van Der Geyten *et al.,* 1998), *Sciaenops ocellatus* (Leiner *et al.,* 2000), *Salmo salar* (Eales and Brown, 1993) and *Carassius auratus* (Deal and Volkoff, 2021). At low temperatures, the hepatic *dio2* enzymatic activity decreases, as seen in *Gadus morhua* (Cyr *et al.,* 1998) and *Salmo garidneri* (Cyr and Eales, 1988), *Ctenopharyngodon idella* (Li *et al*., 2021) and *Oncorhynchus mykiss* (Pavlov *et al*., 2022). In contrast, the higher temperature regulates the rate of reaction in molecular, biochemical, and physiological processes (Pankhurst and King, 2010). The expression of deiodinases in the liver is sensitive to nutritional status (Walpita *et al.,* 2007; Wambiji *et al.,* 2011b; Mahardini *et al*.,2020; Deal and Votkoff, 2021), estradiol (Kwonm *et al.,* 1999), growth hormone (MacLatchy *et al.,* 1992; Ma *et al*., 2021) and stress (Walpita *et al.,* 2007; Shi *et al*., 2018). The vertebrate liver plays a crucial role in female gametogenesis as it synthesizes the female-specific egg yolk protein vitellogenin. Plasma T3 accelerates or induces vitellogenesis via upregulating estrogen receptor-alpha (e*r-a*) in the hepatocytes of goldfish (Nelson and Habibi, 2016). T3 has been shown to downregulate estrogen receptors and aromatase enzymes (Nelson *et al.,* 2010).

Annual expression profile of *tsh-b, tsh-r, dio2* and *dio3* genes in ovarian tissue

Ovarian *dio2* and *dio3* upregulation during the reproductive phase may specify the increased activity of ovarian tissue, including steroidogenesis and gametogenesis. The *dio2* upregulation may result in increased local T3, which is known to facilitate the oocyte maturation and estradiol production in *Salmo garidneri* (Cyr and Eales, 1988) and incorporation of yolk proteins and associated nutrients in the oocytes of *Crysiptera cyanea* (Hur *et al.,* 2020). The ovarian *dio2* is also upregulated in the ovary of *Gymnocypris przewalskii*, and is speculated to facilitate gonadal steroidogenesis and gametogenesis (Tian *et al.,* 2019).

In present study, the collateral upregulation and downregulation of *dio2* and *dio3* in the liver and ovary may regulate an instant activation and deactivation of locally activated thyroid hormone that may prevent crosstalk with neighbouring tissues as suggested by Walpita *et al.,* (2007), or T3 induced *dio3* activation *like* in the gills of the rainbow trout (Van der Geyten *et al.,* 2005) and rat cardiac muscle (Sabatino *et al*., 2020).

Expression profile of*, tsh-b, tsh-r, dio2* and *dio3* genes in early spawning phase

The expression of *tsh-b* appeared only in the brain, which possesses the pituitary gland, the primary site for thyrotropin production. The *tsh-r expressed ubiquitously during the* spawning phase may suggest the sensitivity of the tissues to the thyrotropin hormone. Deiodination by the peripheral tissue has been observed in all vertebrate groups, i.e., mammals (Kohrle, 1999; Köhrle and Frädrich, 2022), birds (Hughes and McNabb, 1986; Lepine and Verreault, 2022), amphibians (Galton, 1992; Laslo *et al*., 2019), reptiles (Sciarrillo *et al.,* 2009; Chang *et al*., 2018) and fish (Eales and Brown, 1993; Seale *et al*., 2021). Also, in the present study, theubiquitous expression of *dio2* and *dio3 has been observed.* The expressions in gills might be due to the presence of thyroid follicles that serve as a primary site of thyroid hormone metabolism (Fournie *et al.,* 2005). Additionally, the gills deiodinases are involved in osmoregulation (Flemings *et al*., 2019; Irachi *et al*., 2021). Functional divergence of *dio*2 appeared in catadromous migrating fish *Salmo salar*, in which the different paralogs of *dio2* genes were expressed in the gills and brain tissue. The *dio2a* was significantly upregulated under saline treatment. They also suggested that *dio2*a expression in gills may facilitate the organ phenotypic plasticity essential for migration (Lorgen *et al.,* 2015; Irachi *et al*., 2021). Since fish do not possess intact thyroid glands like higher vertebrates, the liver is a major tissue for peripheral deiodination, as seen in *Sander vitreus* (Picard-Aitken *et al.,* 2007; Eales, 2019). The liver also regulates the plasma T3: T4 thyroid level in vertebrates (MacLatchy and Eales, 1992; Kohrle, 2000; Eales, 2019). However, the collateral expression of *dio2* and *dio3* in other tissues may infer their local thyroid hormone metabolism, which may avoid crosstalk with neighbouring tissues (Walpita *et al.,* 2007; Kohrle and Fradrich, 2022). Increased plasma T3 has also been shown to induce *dio*3-regulated inactivation, as evidenced by increased *dio*3 activity in the liver and gills of rainbow trout (Van der Geyten *et al.,* 2005) and Nile tilapia (Van Der Geyten *et al.,* 1998), and in mammals (Kohrle and Fradrich, 2022). The increased activity of *dio2* was also seen in the spleen. The local activation of T3 facilitates the enhanced immune response in *Onchorhynchus mykiss* (Quesada-

Garca *et al.,* 2014) and *Mus musculus* (Provinciali *et al.,* 1991), *Mesocricetus auratus* (Verma and Haldar, 2019).

Conclusion

Thyroid hormone is an important hormone for the basal metabolic rate of an organism, and its metabolism is regulated by the deiodinases. In addition, TSH is known to induce thyroid production in the thyroid follicles and is involved in seasonal reproduction of vertebrates. The present study speculated that brain thyrotropin and deiodinases presumably guides the seasonal reproduction in *Heteropneustes fossilis*, and deiodinase upregulation in the liver and ovary may increase the local thyroid hormone level subsequently credible for the vitellogenesis and the gametogenesis respectively. In future works in-situ localization of these genes in the brain tissue may give better insight to understand the molecular mechanism underlying seasonality.

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Conflict of interest

The authors declare that they have no conflict of interest.

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