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# Effects of Thyroxine Hormone on the Reproductive Efficiency and Gene Expression of Thyroid Hormone Receptors in the Eggs of Caspian Trout (*Salmo caspius*)

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**ABSTRACT**

Caspian trout (*Salmo caspius*, Kessler 1877) is an anadromous brown trout species of ecological and conservation significance. Thyroxine (T4) plays a crucial role in oocyte maturation, fertilization, and early development in teleosts; however, its specific effects on Caspian trout embryos remain insufficiently understood. This study investigated the influence of exogenous T4 immersion on fertilization, eyeing, and hatching rates (FR, ER, HR), endogenous T4 and T3 levels in eggs, and thyroid hormone receptor (THR) gene expression (*Thrα* and *Thrβ*). Fertilized eggs were exposed to four treatments for 60 minutes: D1 (control), D2 (0.25 mg $l^{-1}$), D3 (0.5 mg $l^{-1}$), and D4 (0.75 mg $l^{-1}$). Samples were collected from pre-fertilization through post-hatching to capture developmental changes. FR showed no significant differences among treatments, whereas ER and HR were significantly reduced only in the highest T4 concentration (D4, $p< 0.05$). Egg T4 levels were elevated in D2 during hatching, while T3 concentrations peaked in control eggs (D1), indicating dose-dependent modulation of yolk thyroid hormone dynamics. Two-way ANOVA revealed significant effects of time, T4 concentration, and their interaction on *Thrβ* expression ($p< 0.001$). In contrast, *Thrα* transcripts were undetectable throughout development, suggesting isoform-specific regulation or temporal suppression of this receptor during early ontogeny. Moderate T4 exposure improved hatching performance and stimulated *Thrβ* expression, whereas excessive supplementation impaired development, potentially due to metabolic overload, altered hormone conversion, or receptor downregulation. Collectively, these findings demonstrate that embryo-yolk thyroid hormone signaling in Caspian trout is sensitive to exogenous T4 levels and plays a critical role in supporting embryogenesis and morphogenesis. Understanding these hormone-mediated processes has important implications for hatchery practices and conservation strategies aimed at enhancing early life-stage survival in this threatened species.



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## Introduction

Teleost fish represent an excellent model for investigating the evolution and function of endocrine systems in vertebrates. Their thyroid hormone (TH) physiology is particularly informative due to the diversity and complexity of

TH signaling pathways. This complexity is rooted, in part, in a teleost-specific whole-genome duplication that provided a substrate for paralog evolution and functional diversification (Glasauer and Neuhauss, 2014; OECD, 2023). As a consequence, many teleost species retain multiple thyroid hormone receptor (THR) paralogs; for example, zebrafish possess thraa, thrab, and thrb. These paralogs give rise to several isoforms via alternative splicing, including truncated and domain-extended variants, consistent with subfunctionalization or neofunctionalization after duplication (Heijlen *et al.*, 2013). These receptors act as diverse sensors that are differentially expressed during various developmental stages, including early growth, metamorphosis, and maturation. The tightly regulated expression of THRs throughout the teleost life cycle underscores their essential role in coordinating developmental and physiological transitions (Lazcano and Orozco, 2018). Embryonic development in teleosts relies heavily on maternal inputs, such as nutrients, hormones, and reproductive mRNAs stored in oocytes during maturation (Campinho *et al.*, 2010). Similar to other vertebrates, fish synthesize thyroid hormones (THs) through the hypothalamus-pituitary-thyroid (HPT) axis. In teleosts, thyroid follicles, typically variable in shape and size, are located mainly in the pharyngeal region, extending along the afferent artery and into the head kidney (Deal and Volkoff, 2020). The primary THs, T4 and its biologically active form triiodothyronine (T3), are crucial for numerous physiological processes, including growth, morphological development, osmoregulation, pigmentation, and reproduction (Power *et al.*, 2001; Campinho, 2019). Because embryos lack thyroid follicles, the THs present in eggs are exclusively of maternal origin (Campinho *et al.*, 2010).

Exogenous application of hormones is an emerging method for improving larval development (Kang and Chang, 2004; Abdollahpour *et al.*, 2019; Alizadeh *et al.*, 2020). For instance, T4 immersion has been shown to reduce deformities, mortality, and the hatching period in Asian stinging catfish eggs (Nayak *et al.*, 2004). However, the benefits of THs on early growth are not consistent across all fish species (Sharma and Patiño, 2013).

In vertebrates, THRs are encoded by two main genes (α and β). Due to an additional round of gene duplication, teleosts possess three THR isoforms: *Thraa*, *Thrab*, and *Thrb*, each with unique functional roles (Glasauer and Neuhauss, 2014). These isoforms are often developmentally regulated, particularly during metamorphosis or significant life transitions (Lazcano and Orozco, 2018). Their presence in developing eggs before hatching suggests a functional role in early embryogenesis (Power *et al.*, 2001; Shibata *et al.*, 2020). Maternal THRs transcripts have been identified in unfertilized zebrafish eggs (Liu and Chan, 2000), and some of the nongenomic effects of T3 and T4 are mediated through surface integrin receptors (Habibi *et al.,* 2012). Since the first cloning of THRs in fish by Yamano *et al.* (1994), various studies have investigated THRs mRNA expression across developmental stages in multiple species, including *Salmo salar*, *Oncorhynchus kisutch*, *O. mykiss*, Japanese flounder *Paralichthys olivaceus*, turbot *Scophthalmus maximus*, Atlantic halibut *Hippoglossus hippoglossus*, zebrafish *Danio rerio*, orange spotted grouper *Epinephelus coioides*, Japanese eel, and Pacific bluefin tuna *Thunnus orientalis*. However, there is no consistent pattern in TRα and TRβ regulation across species, although these receptors are widely implicated in growth, reproduction, and thermal homeostasis (see more details in Lazcano and Orozco, 2018; Campinho, 2019; Quesada-García *et al.*, 2014; Deal and Volkoff, 2020).

The Caspian trout (*Salmo caspius*, Kessler 1877) is an anadromous brown trout species endemic to the western and southern Caspian Sea. It migrates upstream in early autumn to spawn and returns to the sea following smoltification (Björnsson *et al.*, 2011; Jalali and Mojazi Amiri, 2009; Kalbassi *et al.*, 2013). This species is classified as endangered by the IUCN and has experienced a dramatic decline in native populations due to threats such as overfishing, pollution, dam construction, and habitat destruction (Kiabi *et al.*, 1999; Niksirat and Abdoli, 2009; Vera *et al.*, 2011). Given the species' economic value of marine culture (Najafpour *et al.*, 2019), the nutritional quality of its fillets (Kalbassi *et al.*, 2013), and the importance of conserving its genetic diversity, understanding the hormonal regulation of its early development is vital. Thyroid hormones exert

diverse effects and are crucial for maintaining physiological homeostasis across vertebrates. Although there are fundamental similarities in thyroid function among vertebrate groups, the thyroidal system in fish exhibits several unique features. These differences arise from the remarkable diversity of fish anatomies, habitats, and life history strategies (Deal and Volkoff, 2020). Despite the known roles of T4 and THRs in fish eggs, no prior studies have examined their fundamental understanding of fish physiology in the Caspian trout. This study is the first to evaluate how T4 immersion at varying concentrations influences fertilization rate (FR), eying rate (ER), hatching rate (HR), egg hormone levels (T4, T3), and the expression of *Thra* and *Thrb* during embryogenesis in Caspian trout.

## Material and Methods

This study was carried out at the Shahid Bahonar Breeding and Cultivation Center in Kelardasht, Mazandaran Province, Iran. Broodfish were captured during their spawning migration in the Cheshme Kileh River (Tonekabon, Mazandaran) and transported to the hatchery for artificial breeding procedures. After a three-week acclimation period, wild mature female (3) and male (1) Caspian trout with a mean body mass of 4950.2 ± 30.5 g (mean ± standard deviation) and 1868 g, respectively, were maintained in 300-liter fiberglass tanks under standardized conditions (Bahre Kazemi *et al.*, 2010). The water in fiberglass tanks was a mixture of water from the nearby river and spring (9± 0.6°C). Fish were fed once daily with a commercial pellet (INICIO Plus, Biomar Co.; www.biomar.com). Animal use protocols were approved by the Iranian Fisheries Organization, which has jurisdiction over the Breeding and Cultivation Center.

During the experiment, a flow-through system supplied filtered river water at a constant rate of 14 l $min^{-1}$. The average values of physical water parameters, including temperature (measured using a mercury thermometer) and pH (measured using an Aqua pH meter, TPS, Australia), are summarized in Table 1. All experimental units were exposed to natural light conditions (11L:13D) throughout the study period.

**Table 1.** Water quality variables of Caspian trout

| Physical variables | means ± S.D |
|---|---|
| Temperature | 8-10°C± 1·4 |
| pH | 8.44± 0·4 |
| Dissolved $O_2$ (DO) | 9.55± 0·1 (mg $l^{-1}$) |
| Electrical conductance (EC) | 214.15 ± 6·7 (µS/cm) |
| Total dissolved solids (TDS) | 102.45 ± 2·1 (mg $l^{-1}$) |

## The T4 administration method

To prepare the T4 treatment solutions, 1 mL of 96% ethanol (Razi, Khuzestan, Iran) was used to dissolve T4 (Merck, Darmstadt, Germany), following established protocols (Abdollahpour *et al.*, 2019; Alinezhad *et al.*, 2020). Prior to sperm collection, all broodfish (3 wild femals+1 male) were anesthetized using clove powder at 100 ppm [duration (5-7 minutes) and recovery time (3-5 minutes)]. Following fertilization in hatchery water, eggs were immersed for 60 minutes (Akbari *et al.* 2014) in four separate 1-liter T4 solutions, as follows: Dos1 (D1) (the control: T4-free, hatchery water), D2 (0.25 mg $l^{-1}$), D3 (0.5 mg $l^{-1}$), and D4 (0.75 mg $l^{-1}$) of T4. The control group (D1) was exposed to hatchery water containing the same concentration of ethanol used in the T4 treatments. The concentrations and immersion duration were based on previous studies (Castillo *et al.*, 2013; Akbari *et al.*, 2014). After immersion, eggs were gently transferred to horizontal incubators under standard hatchery conditions. The samples were collected before fertilization (t1), at fertilization (t2), 24 hpf (t3), 48 hpf (t4), 25 dpf, at eyed stage (t5), at hatching (t6), and at yolk sac absorption (CYA) (t7) (See Fig. 1 for the sampling timeline). Each treatment was conducted in triplicate tanks. Collected eggs were preserved in RNAlater® (QIAGEN, RNeasy Protect Mini Kit, Cat. Nos. 74124, 74126) for subsequent RNA extraction and qPCR analysis. Samples were stored at 4°C for 24 hours, then transferred to –80°C for long-term preservation.

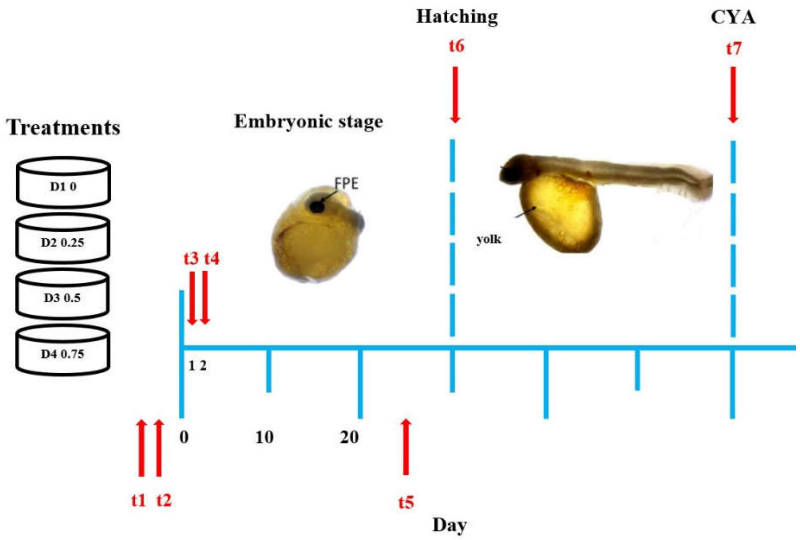


**Fig. 1.** Schematic drawing of the timing of sampling (red arrows) starting before fertilization (t1), at fertilization (t2), 24 hpf (t3), 48 hpf (t4), 25 dpf, at eyed stage (t5), at hatching (t6), and CYA (t7) in developmental stages of larval Caspian trout. D1 (the ctrl treatment: T4-free, hatchery water), D2 (0.25 mg $l^{-1}$), D3 (0.5 mg $l^{-1}$), and D4 (0.75 mg $l^{-1}$) of T. FPE: Fully pigmented eyes (Photos of Caspian trout embryonic development are from Najafpour *et al.*, 2019). The eggs were immersed (60 minutes) and D1 (the control: T4-free, hatchery water).

### Calculating the FR, ER, and HR

To calculate the FR, ER, and HR, dead eggs and embryos were regularly removed, and the following formula was used:

% FR= (number of fertilized eggs/total number of fertilized eggs)× 100

%ER= (Total number of eyeing eggs/total number of fertilized eggs)× 100

% HR= (Total number of alevins/total number of eyeing eggs)× 100

### Sample preparation and hormone assays

Approximately 90 g of fertilized eggs (11/1 number per g representing around 1000 eggs) were collected at five developmental stages: 24 hpf (t3), 48 hpf (t4), 25 dpf (at eyed stage, t5), and hatching time (t6). Samples were first homogenized using an Ultra Turrax T25 homogenizer (Janke and Kunkel Labortechnik, Staufen, Germany) in five volumes of ice-cold phosphate-buffered saline (PBS, 0.1 M, pH 7.2). A 300 µL portion of the homogenate was then transferred to new tubes and further homogenized in 6 mL of ice-cooled ethanol. The mixtures were placed on a horizontal shaker for 10 minutes at 4°C, followed by centrifugation at 1600× g for 15 minutes at 4°C. The supernatants were carefully transferred to clean tubes. A second round of homogenization was performed using 2 mL of cooled methanol instead of ethanol. After centrifugation, the supernatant was again transferred to new tubes. All collected supernatants were then evaporated at 56°C for 24 hours to remove the methanol. Next, to extract THs, 5 µL of methanol, 200 µL of chloroform, and 50 µL of barbital buffer (0.1 M, pH 8.6) were added to the dried extracts. The mixtures were placed on a horizontal shaker for 10 minutes, after which the upper phase containing the THs was collected, dehydrated at 56°C for 3 hours, and finally reconstituted in 1 mL of barbital buffer containing 0.1% gelatin. All final extracts were stored at –70°C until analysis (Abdollahpour *et al.*, 2019; Alizadeh *et al.,* 2020). Thyroxine and T3 concentrations were quantified using commercial ELISA kits (Diazist, Iran), following the manufacturer's instructions. Absorbance readings were obtained using an ELISA reader (Epoch 2, Microplate Spectrophotometer, Vermont, USA). Hormone concentrations were calculated from a standard curve and expressed as ng $g^{-1}$ of egg tissue (Abdollahpour *et al.*, 2019).

### RNA extraction

Frozen pieces of egg were thawed, homogenized in lysis buffer (β-Mercaptoethanol) at 6400 RPM 2× 10 s; Bertin (Precellys 24 bead homogenizer) and centrifuged at 5000 g for 1 minute at room

temperature (Heraeus Pico 17 Centrifuge, Thermo Scientific). The silica-based columns (Aurum Total RNA mini kit, Bio-Rad) and Nanodrop spectrophotometer (Thermo Scientific, Wilmington, DE, United States) were used for RNA isolation (according to the manufacturer's instructions) and to determine total RNA concentration and/or purity, respectively (Malakpour Kolbadinezhad *et al.*, 2018). The isolated total RNA samples were then stored at –80°C. The iScript cDNA kit (Bio-Rad) was used for converting to cDNA according to the manufacturer's instructions (each sample, 1 mg of total RNA), and then, the samples were stored at –20°C.

**Gene isolation**

Consensus primers were designed from a conserved region of *β*-actin (*Actb*, *Sparus aurata*, Santos *et al.*, 1997), *Thrα* and *Thrβ* (*S. salar* TRβ (AF146775), *O. mykiss TRβ* (AF302246), *S. salar TRα* (AF302250), *O. mykiss TRα* (AF302245), *S. salar TRα1* (large isoform; AF302251) (Marchand *et al.*, 2001; Quesada-García *et al.*, 2014) by multiple sequence alignment. The primer sequences are shown in Table 2. The specific primers for *S. caspius* were designed with Primer3 (Rozen and Skaletsky, 2000) and initially tested for specificity by RT-PCR. The sizes of the PCR fragments exactly matched the expected size of all primer sets.

**Real-time PCR**

The PCRs were performed using 0.4 µl of sample cDNA, 0.08 mM dNTPs, 0.4 mM $MgCl_2$, 0.4 µM of each primer and 0.2U GoTaq® DNA polymerase (Promega, Madison, WI, USA) and 1 µl of 5× Green GoTaq® reaction buffer in 10 µl reaction volumes for beta-actin PCRs and Phusion Flash (Thermo Fisher Scientific) master mix for the other interested genes. Primers were designed using Primer3 (Rozen and Skaletsky, 2000) and were initially tested for specificity by RT–PCR. The beta actin expression was stable across treatments and developmental stages.

GoTaq reactions consisted of an initial denaturation at 95°C for 2 min followed by 30 cycles of 95°C for 30 s, annealing at 60°C for 30 s, extension at 72°C for 5 s, and a final extension for 5 min at 72°C. The Phusion Flash reactions consisted of an initial denaturation at 98°C for 10 s followed by 35 cycles of 98°C for 1 s, annealing at 56, 58, or 60°C for 5 s, extension at 72°C for 5 or 10 s, and a final extension for 1 min at 72°C. The PCR products were separated on 2% agarose TBE (Tris-borate-EDTA) gels at 80 V to confirm the size of the amplicons. All gels were stained with GelRed, and images were acquired using a Fujifilm LAS-4000 Mini luminescent image analyzer (Fujifilm, Tokyo, Japan). A DNA ladder 1kB or 100 bp (Bioron GmbH, Ludwigshafen, Germany), depending on the amplicon size, was run on every gel to determine the size of the amplification products. Single bands of the correct predicted size for each PCR were cut and cleaned using an Illustra GFX PCR DNA and Gel Band Purification Kit (GFX column, GE Healthcare, Carnaxide, Portugal), and directly sequenced (StabVida, Oeiras, Portugal). The product was confirmed using tBLASTx. The alignment was performed via BioEdit (Version 7.0.9.0; Hall, 1999), and ClustalW was used for sequence assembly.

Relative levels of mRNAs for the *thrβ* gene were quantified by real-time RT–PCR analysis using SYBR green with an iQ5 Multicolor Real-Time PCR Detection System (Bio-Rad). Each cDNA sample was diluted 25 times, and then 5 µl was added to a reaction mix containing 10 µl of 2× iQ SYBR Green Supermix (Bio-Rad) and 250 nM of each primer in a total volume of 20 µl. The cycle profile was as follows for the given primer pairs: 95°C for 3 min, followed by 35 cycles of 95°C for 10 s, 56, 58, or 60°C for 30 s, and 72°C for 3-5 s. The generation of a melt curve for every PCR product and preparation of a dilution series was used to confirm the specificity of the assays and check the efficiency of the reactions, respectively. *βact* was used as the reference gene. The expression levels of the genes of interest were analyzed based on cycle threshold (CT) values by using the comparative CT method ($2^{-\Delta\Delta CT}$ method) (Livak and Schmittgen, 2001). In addition, a subset of samples was run on 2% agarose TBE (Tris-borate-EDTA) gels to confirm the presence of a single amplified product at the predicted correct size.

**Table 2.** The primer names and oligomer sequences were used in this research.

| Primer names | Oligomer sequence | Product size (bp) | Fish | Accession no | Primer efficiency (%) |
|---|---|---|---|---|---|
| *SSB1 (5′)* | F: 5'TCGCTGTCGGCATGGCAACA | 261 | *S. salar* | AF302251.1 | 102.2 |
| *SSB2 (3′)* | R: 3'TGACCAATGTCCTCAGGCAA | | | | |
| *SSA1 (5′)* | F: 5'GCACAACATTCCCCACTTCT | 117 | *S. salar* | AF146775.1 | 98 |
| *SSA2 (3′)* | R: 3'AGTTCGTTGGGACACTCCAC | | | | |
| *FB-actin* | F: 5'ACTGGGACGACATGGAGAAG | 281 | *Danio rerio* | EF406272 | 100 |
| *RB-actin* | R: 5'CAGAGCGTAACCCTCGTAGAT | | | | |

*F= Forward; R=Reverse

### Statistical analysis

The set of eggs was randomly distributed into different groups. Data are presented as the mean±standard deviation (SD). The results were analyzed after testing the normal distribution of the data using the Kolmogorov–Smirnov test. Statistical differences in FR, ER and HR were determined using one-way analysis of variance (ANOVA) followed by the post hoc Student–Newman–Keuls (SNK) test (SigmaPlot 11.0 Systat Software, Inc.) in eggs exposed to different hormone concentrations. A two-way ANOVA with subsequent Duncan's Multiple test was used for comparison of mean values resulting from the various treatments (effect of concentration, time, and interaction effect on gene expression) at a significance level of $p < 0.05$. SigmaPlot 11.0 Systat Software, Inc. software was used for statistical analysis. The fiducial limit was set at 0.05. The ggplot2 package in R, version 4.3.1, is dedicated to data visualization and the creation of charts.

## Results

### Concentrations effects of T4

Statistical analysis revealed no significant differences in the FR (98.5-94%) among the treatment groups ($p > 0.05$). However, a significant decrease in ER and HR was observed at the highest T4 concentration (D4, 0.75 mg $l^{-1}$) ($p < 0.05$; Fig. 2B, C). The highest ER (70%) and HR (82%) values were recorded in D1 (control) and D2 (0.25 mg $l^{-1}$), whereas the lowest rates were found in D4 (Fig. 2B, 2C). Overall, there appeared to be an inverse relationship between T4 concentration and both ER and HR.

### T4 and T3 levels in eggs

There were no significant differences in T4 levels across sampling points (fertilization, 24 hpf, 48 hpf, and eyed stage) for most treatment groups. However, at fertilization, T4 levels in the 0.75 mg $l^{-1}$ group were significantly lower than in the other treatments ($p < 0.05$), while the highest T4 levels were recorded at hatching in the 0.25 mg $l^{-1}$ group ($p < 0.05$; Fig. 3A).

In contrast, T3 levels were generally highest at fertilization and declined over time. Significantly lower T3 levels were detected in the at eyed stage of the 0.5 mg $l^{-1}$ group and in the fertilization stage of the 0.75 mg $l^{-1}$ group (D4) compared to the other groups ($p < 0.05$; Fig. 3B). In addition, T3 levels were significantly higher in 48 hpf of the 0.25 mg $l^{-1}$ group, Fertilization stage of the 0.5 mg $l^{-1}$ group, Fertilization, 48 hpf, and at eyed stages of the 0.75 mg $l^{-1}$ group than in the at eyed stage of the 0.5 mg $l^{-1}$ group and 24 hpf of the 0.75 mg $l^{-1}$ group ($p < 0.05$; Fig. 3B).

### Gene expression of TRs (*Thrα* and *Thrβ*)

Two-way ANOVA indicated that both T4 concentration and developmental stage, as well as their interaction, had a significant effect on *Thrβ* gene expression ($p$ values for time ($p < 0.001$), treatment ($p < 0.001$), and their interaction ($p < 0.001$); Fig. 4). Among these, concentration was the most influential factor. A significant increase in *Thrβ* expression was observed across all T4-treated groups at hatching (t6) ($p < 0.05$), followed by a marked decline at the completion of CYA in all groups except the control (D1). The *Thrβ* expression peaked at t6 and was lowest at t5 (at eyed stage) across all treatments, except in D4, where the expression was relatively stable. Interestingly, *Thrα* gene expression was not detected at any developmental stage. Duncan's post hoc test confirmed significant differences between all-time points and treatment groups ($p < 0.05$), supporting the conclusion that T4 concentration and developmental timing both significantly influence *Thrβ* expression during embryogenesis in Caspian trout.

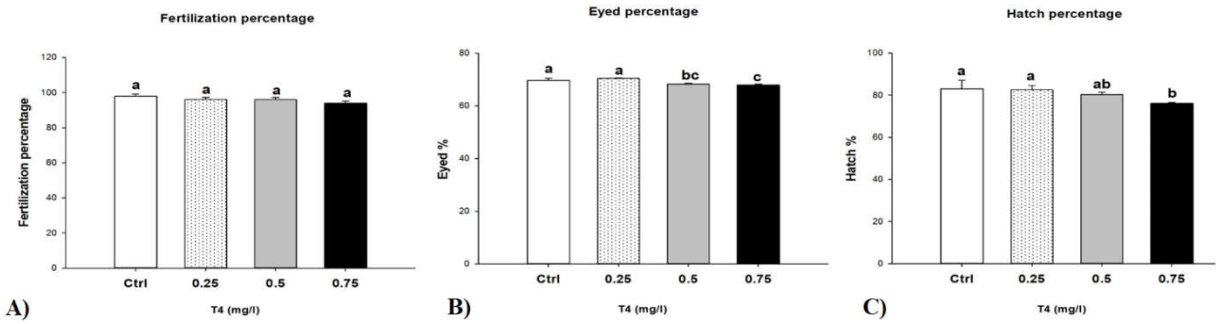


**Fig. 2.** The reproductive performance in the eggs of Caspian trout *S. caspius*: A) Revealing FR; B) Revealing ER; C) Revealing HR. These were conducted on the eggs of Caspian trout *S. caspius* following immersion in different T4 hormone concentrations, including D1 (the ctrl treatment: T4-free, hatchery water), D2 (0.25 mg $l^{-1}$), D3 (0.5 mg $l^{-1}$), and D4 (0.75 mg $l^{-1}$) of T4. Values are presented as the mean ± SD (n = 9). Different lowercase letters (a, b, c, d) above the bars indicate statistically significant differences among treatment groups (one-way ANOVA followed by SNK, $p < 0.05$).

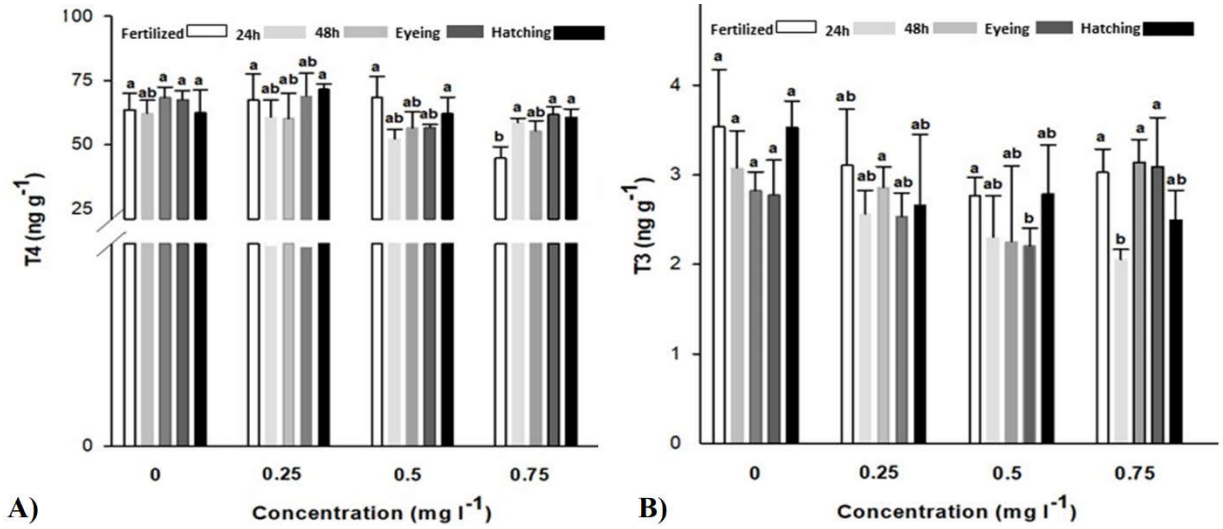


**Fig. 3.** Thyroid hormones concentrations in the eggs of Caspian trout *S. caspius*: A) T4 concentration; B) T3 concentration. These were conducted on the eggs of Caspian trout *S. caspius* following immersion in different T4 hormone concentrations, including D1 (the control: T4-free, hatchery water), D2 (0.25 mg $l^{-1}$), D3 (0.5 mg $l^{-1}$), and D4 (0.75 mg $l^{-1}$) of T4. Values are presented as the mean ± SD (n= 9). Different lowercase letters (a, b, c, d) above the bars indicate statistically significant differences among treatment groups (one-way ANOVA followed by SNK, $p < 0.05$).

## Discussion

This study investigated the effects of different concentrations of T4 immersion on reproductive performance, TH levels, and TR gene expression during early embryonic development in Caspian trout. The results provide the first evidence that exogenous T4 modulates early developmental outcomes, yolk TH dynamics, and *Thrβ* transcriptional activity in this species. Dose-dependent and stage-specific responses observed across FR, ER, HR, egg TH content, and receptor gene expression highlight the sensitivity of Caspian trout to thyroidal manipulation during embryogenesis. These findings underscore the importance of species-specific endocrine regulation. Overall, T4 exposure elicited concentration-dependent effects on gene expression and developmental parameters, with higher concentrations negatively affecting early growth and survival. To our knowledge, there are

no previously published data on the effects of T4 on development (FR, ER, HR), egg TH (T4 and T3) levels, or the gene expression of *Thrβ* and *Thrα* in the Caspian trout. While FR remained largely unaffected across T4 concentrations, elevated T4 (0.75 mg $l^{-1}$) significantly decreased embryonic ER and HR, suggesting a threshold beyond which T4 becomes deleterious. Moderate concentrations (0.5 mg $l^{-1}$) improved HR, consistent with previous reports in rainbow trout, grass carp, and common carp, where T4 promoted survival and early development (Jamili *et al.*, 2004; Kiapour *et al.*, 2024). Similarly, Sterlet sturgeon larvae treated with 1 g $l^{-1}$ T4 showed increased growth and survival (Alinezhad *et al.*, 2020).

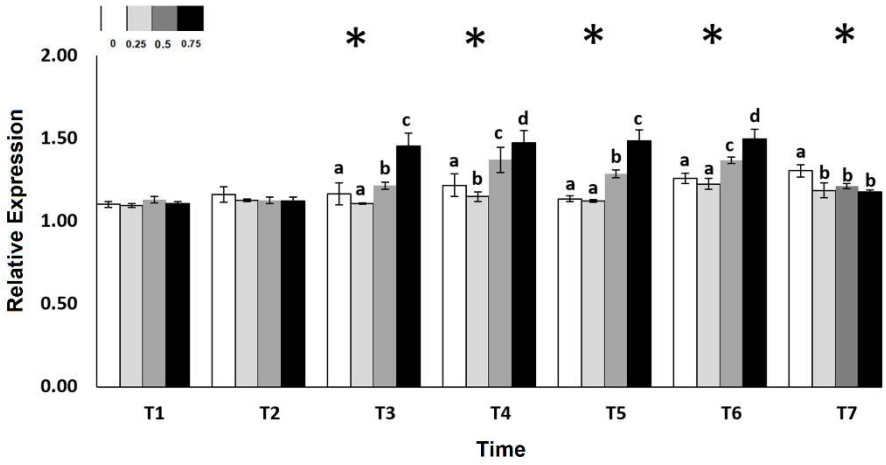


**Fig. 4.** Detection of *Thrβ* expression in the eggs of Caspian trout *S. caspius*: Relative mRNA expression of *Thrβ* (n= 9), THRs of Caspian trout *S. Caspius* eggs immersion in various hormone concentrations of T4, based on concentration and time represented by a two-way interaction plot. T1: before fertilization, T2: at fertilization, T3: 24 hpf, T4: 48 hpf, T5: 25 dpf, at eyed stage, T6: at hatching and T7: CYA. Different letters indicate a significant difference between concentrations within each time point. The stars indicated a significant difference between the interaction of the treatment and time, two-way analysis of variance (ANOVA). (*p* values for time ($p < 0.001$), treatment ($p < 0.001$), and their interaction ($p < 0.001$); see text for details).

Similarly, the beneficial effects of T4 at concentrations of 0.01, 0.1, 0.5, and 1 mg $l^{-1}$ on HR in grass carp and common carp have been reported (Jamili *et al.*, 2004). Furthermore, increased growth, HR, and survival were observed in Sterlet Sturgeon (*Acipenser ruthenus*) larvae treated with 1 g $l^{-1}$ of T4 (Alinezhad *et al.* 2020).

Early life stages of fish rely on the catabolism of yolk amino acids and proteins, the main sources of fuel to generate a constant surfeit of ammonia, for energy production during the period of yolk sac absorption (Zimmer *et al.*, 2017). The effect of THs, depending on doses and temperature, on fish ammonia production and/or excretion has already been documented. The adverse effects at high concentrations may be explained by accelerated yolk catabolism, which increases ammonia accumulation and depletes essential nutrients required for proper embryogenesis (Zimmer *et al.*, 2017). This aligns with findings in rainbow trout, where T3 exposure increased embryonic mortality without affecting growth (Raine *et al.*, 2003). These observations emphasize that the effects of THs on early development are both dose- and species-dependent, and that excessive T4 may disrupt metabolic homeostasis and energy allocation (Deal and Volkoff, 2020), environmental conditions, and complex endocrine interactions. These include cross-talk between THs, growth hormone, and cortisol through the HPT axis (Brown *et al.*, 2014), in addition to other currently unidentified mechanisms.

It is also important to note that the beneficial effects of THs on early development are not universal among teleosts. Adverse outcomes such as premature differentiation, morphological abnormalities, e.g., in brown trout, toxic or ineffective responses, e.g., in zebrafish, and

common carp have been documented (Brown et al., 2014). Negative effects of T4 immersion have also been reported in Atlantic salmon (Roche and Leblond, 1952) and alligator gar (Castillo *et al.*, 2013).

The presence of THs in the yolk of eggs and embryos of teleost fish has been well documented (Power *et al.*, 2001). The yolk serves as the primary source of THs during early embryogenesis, and our study confirms that T4 concentrations in eggs exceeded T3, consistent with most freshwater teleosts (Raine *et al.*, 2004). The highest T4 exposure (0.75 mg $l^{-1}$) resulted in lower yolk T4 levels at fertilization, likely due to rapid uptake by developing tissues and early activation of TH receptors (Power *et al.*, 2001; Kang and Chang, 2004). Similarly, the decline in T3 observed at 24 hpf suggests active utilization during organogenesis and morphogenesis, supported by enzymatic conversion and outer-ring deiodinase activity prior to endogenous TH synthesis (Raine *et al.*, 2004; Castillo *et al.*, 2013).

These findings highlight a dynamic interplay between exogenous hormone exposure and endogenous metabolism, where T4 availability directly influences early development and hormone homeostasis. The interspecific differences in yolk TH levels (e.g., rainbow trout, sturgeon, sheepshead minnows) likely reflect species-specific TH storage, metabolism, and receptor sensitivity (Brown *et al.*, 2014; Abdollahpour *et al.*, 2019; Deal and Volkoff, 2020). However, further investigation is necessary to clarify the specific mechanisms underlying these observations and to determine the developmental implications of exogenous T4 exposure on TH metabolism.

Detecting THR expression at different times before egg hatching probably represents their functions (Shibata *et al.*, 2020). Our study demonstrates that *Thrβ* expression is both time- and concentration-dependent, supporting the notion that TRs are functionally active prior to thyroid follicle maturation (Power *et al.*, 2001; Shibata *et al.*, 2020). Expression of TR isoforms, including *Thrα* and *Thrβ*, has been reported in several teleost species such as rainbow trout, Atlantic salmon, coho salmon, and zebrafish. Also, TR isoforms expression has been identified in flounder, Japanese flounder, halibut, Pacific bluefin tuna, Atlantic cod, Fathead minnow, orange-spotted grouper, sea bream, and eel (e.g., Vergauwen *et al.*, 2018; Lazcano and Orozco, 2018; Campinho, 2019; Quesada-García *et al.*, 2014; Deal and Volkoff, 2020). Expression patterns of *Thrβ* and *Thrα* in Caspian trout eggs mirror those observed in other teleosts, including rainbow trout, Atlantic salmon, zebrafish, and sea bream (Liu *et al.*, 2000; Nowell *et al.*, 2001). However, no universal regulatory pattern emerges across species, indicating that THR expression is finely tuned to species-specific developmental trajectories (Lazcano and Orozco, 2018). In sea bream, *Thrβ* expression peaks at hatching and then declines, whereas *Thrα* expression remains elevated (Nowell *et al.*, 2000). In zebrafish, *Thrα* expression typically surpasses that of *Thrβ* during embryogenesis (Liu *et al.*, 2000), and both genes show increased expression following T4 treatment after hatching (Liu and Chan, 2002). In the present study, a significant effect of both time and hormone concentration, as well as their interaction, on *Thrβ* expression suggests activation of the thyroid axis (Raine *et al.*, 2004; Power *et al.*, 2001). The observed maintenance or transient increase in *Thrβ* expression at moderate T4 concentrations (0.5 mg $l^{-1}$) coincided with higher HR, suggesting that optimal receptor activation supports embryonic development, whereas excessive T4 exposure suppresses *Thrβ* transcription, potentially as a protective feedback response. Stage-specific declines in *Thrβ* expression may reflect both changes in circulating TH levels and differential expression of deiodinase isoforms, which regulate local T3 availability (Raine *et al.*, 2004). These findings highlight the importance of temporal regulation of TH signaling and receptor-mediated effects during early development.

The lack of decline in *Thrβ* expression at D4- T5 could be due to species-specific differences or the embryo's capacity to maintain TH homeostasis under higher exogenous hormone exposure (Raine *et al.*, 2004; Liu and Chan, 2002). Furthermore, variation in developmental strategies across species (Reddy *et al.*, 1992) might influence these patterns. The decrease in *Thrβ* expression in the CYA stage, except for D1, could reflect changes in external TH concentrations, alterations in endogenous hormone release during development, undetected

*Thrα* expression, and the dynamic levels of T3 and T4 observed in Caspian trout embryos and larvae.

## Conclusion

This study demonstrates that exogenous T4 modulates early embryonic development, yolk TH dynamics, and *Thrβ* gene expression in Caspian trout in a concentration- and stage-dependent manner. Moderate T4 exposure (0.25-0.5 mg $l^{-1}$) enhanced hatching rates and supported receptor activation, whereas high concentrations (0.75 mg $l^{-1}$) negatively affected embryonic and hatching success, likely due to metabolic overload, nutrient depletion, or receptor downregulation. These results highlight the sensitivity of Caspian trout embryos to thyroidal manipulation and emphasize the importance of careful hormone dosing in both experimental and hatchery settings. The observed changes in *Thrβ* transcription, together with dynamic yolk T4 and T3 levels, suggest that TH signaling plays a critical role prior to the functional maturation of thyroid follicles, supporting organogenesis and morphogenesis during early development. The absence of detectable *Thrα* expression may indicate species-specific regulatory mechanisms or differential isoform activity during embryogenesis. While these findings provide important insights into Caspian trout endocrine regulation, further studies are needed to evaluate long-term effects of early T4 exposure on post-hatching growth and survival, to clarify the functional roles of THR isoforms via transcriptomics or gene-editing approaches, and to explore interactions between T4 and other endocrine factors such as cortisol during development and environmental adaptation. A comprehensive understanding of TH regulation in Caspian trout will inform conservation strategies and optimize hatchery practices, improving survival and growth during early life stages.

## Author contribution

SMK designed the experiments. SMK and ShJ performed the experiments, SMK and AF analyzed the data, and SMK wrote the draft of the manuscript. SN, AF, MG, and MTA were also involved in writing and editing the manuscript.

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