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Effect of climatic factors on sexual maturity *Labeo rohita* fish

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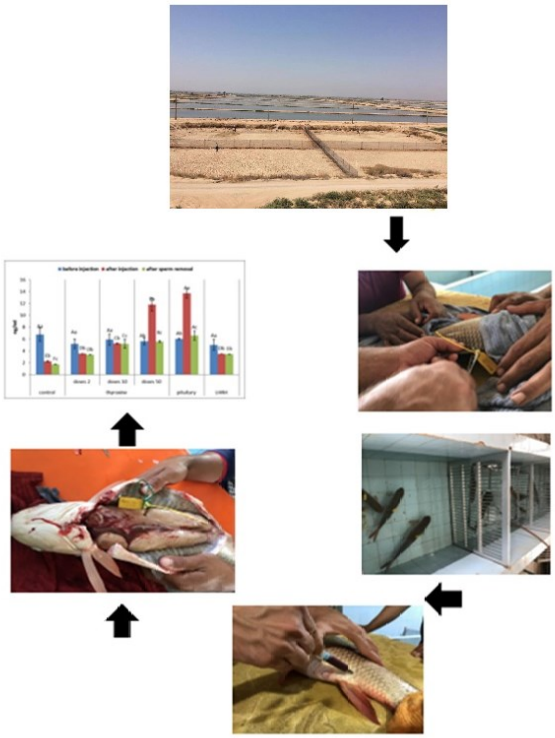
Graphical abstract



Abstract

This study examined the varying levels of thyroxine hormone impact (at doses of 2, 10, and 50 µg g^{-1} BW) as an environmentally responsive hormone on the fatty acid and sexual steroid profiles in male *Labeo rohita* fish. Each treatment group comprised six male fish with an average weight of 5.4 ± 0.58 kg. Blood samples were collected from male Rohu fish pre-and post-injection, as well as following sperm retrieval from the caudal peduncle. Biotechnical parameters were evaluated post-fertilization. Among male breeders, the pituitary treatment and the 50 µg g^{-1} BW thyroxine treatment exhibited the highest levels of estradiol, testosterone, 17-alpha hydroxyprogesterone, cortisol, LH, as well as testicular protein and fat content. Whereas the lowest levels were observed in the LHRH recipient and control groups ($p < 0.05$). Moreover, the saturated fatty acids C16:0 and C18:0, monounsaturated fatty acids C18:1n9c and C24:1n9, along with polyunsaturated fatty acids C20:3n6 and C22:6n3 in male breeders, were found to be the predominant fatty acids in the testes under hormonal treatment. The pituitary treatment and the 50 µg g^{-1} BW thyroxine treatment demonstrated a greater total percentage of testicular fatty acids compared to other interventions ($p < 0.05$). These findings emphasize the heightened efficacy of pituitary hormone and subsequent thyroxine hormone administration at a dose of 50 µg g^{-1} BW, relative to lower thyroxine doses and LHRH. The aforementioned treatment can increase the levels of sex hormones that are important for sexual responsiveness, emphasizing their pivotal role in facilitating successful artificial reproduction in male Rohu breeders.

Keywords: Climate Change, Sexual maturity, Hormone, Thyroid, Artificial reproduction

1. Introduction

Climate change poses new challenges to the sustainability of fisheries and aquaculture systems on which 520 million people depend, with almost 3 billion people relying on fish as a major source of animal protein. Climate change can directly impact fish reproduction, global fish stocks, and supply for consumption, or indirectly affect fish prices, or the costs of goods and services required by fishermen and fish farmers (Mitra *et al.*, 2023). These impacts on aquaculture may include global warming, saltwater intrusion, sea level rise, reduction in oceanic productions, changes in circulation patterns, alterations in monsoon patterns, occurrences of extreme weather events, water stress, changes in inland water hydrological regimes, increased diseases, social impacts on farmers and breeders, effects on biodiversity, and impacts on the provision of feed for farmed fish (Servili *et al.*, 2020).

At present, climate change, including the sharp rise in temperature and the decline in natural breeders due to overfishing, as well as reduced egg viability, reduced sperm motility, reduced ovulation of eggs, low fertilization rates, and high losses during incubation periods due to ecological changes are considered to be significant challenges in the breeding of various fish species (Degani, 2020). The endocrine system establishes a relative gradual coherence between the external environment and the internal state of the body. Consequently, reproductive behaviors align

well with sexual maturity and favorable environmental conditions simultaneously. Messages associated with internal and external factors such as photoperiod and water temperature are received by sensory organs and accumulate in the brain (Froehlich *et al.*, 2022). Subsequently, the central nervous system synthesizes informational cues. If the recorded messages are in a positive direction, for example, an increase in temperature or photoperiod, the hypothalamus stimulates the production and release of gonadotropin-releasing hormones and regulates the synthesis and release of pituitary gonadotropins (Muhala *et al.*, 2021).

Thyroid and growth hormones play a significant role in the growth and early development stages of teleost fish. Additionally, these hormones are effective in the early stages of metamorphosis, growth, metabolism, and osmoregulation (Tovo-Neto *et al.*, 2018). Thyroid hormones are essential for sexual differentiation, maintaining sex ratio balance, and influencing gonadal maturation. Moreover, considering that thyroid hormone receptors are present in the ovary and testis, this hormone also affects the evolution of sexual gonads (Deal and Volkoff, 2020), making them suitable candidates for studying climate change effects on sexual maturity.

Indian carp are considered among warm-water fish species, ranking second in global production after Chinese carp. Rohu fish (*Labeo rohita*) is an excellent species among Indian carp and is particularly favored as one of the most delicious farmed carp on the Indian subcontinent. Numerous studies highlighted the positive impact of combining species of Indian and Chinese carp in earthen ponds compared to monoculture of Chinese carp alone, which can significantly increase fish production (Brown *et al.*, 2014). In recent years, the occurrence of mass mortalities among warm-water fish, especially phytophagous and carp species, in Iranian aquaculture farms has posed a crisis to the aquaculture industry (Lin *et al.*, 2021). The use of new species that are both productive and more resistant to environmental conditions can contribute to the development of this industry and the mitigation of existing threats. However, since they are not native to Iran and at the same time there are strong climate changes and a significant increase in air temperature, their hormonal behavior needs to be further investigated (Lindmark *et al.*, 2022). Breckels and Neff (2013) The effects of elevated temperature on the sexual traits, immunology and survivorship of a tropical ectotherm, Pilakouta *et al.* (2022) effects of temperature on mating behavior and mating success and Ribeiro Reis *et al.* (2023) effect of temperature on the early sexual development of tambaqui *Colossoma macropomum*. Considering the above conditions, this study aimed to investigate the effects of different doses of influential hormones on the sexual maturity of male Rothu breeders (*L. rohita*) that may result from increases or decreases in temperature.

2. Materials and Methods

2.1. Fish breeders

Male Rohu fish (5.4 ± 0.58 kg) were utilized from aquaculture breeders in Dezful County (Khuzestan Province, Iran). Male fish were identified with an elongated body

The male fish were identified by their slender bodies and placement of the genital path behind the genital papilla, then transferred to cement tanks and anesthetized with 30 cc EG/100 L H_2O. Six breeders were allocated for each treatment and control group.

2.2. Preparation of Thyroxine hormone

Many studies have demonstrated the effect of temperature on fish physiology, considering it as one of the most crucial environmental factors affecting the growth and survival of these organisms (Servili *et al.*, 2020). Therefore, the effect of temperature changes on sexual maturity was investigated in this study by examining different doses of thyroxine hormone. Thyroxine, commercially known as Thyroxine Sodium or T4 (Iran Hormone, Iran), was purchased. This hormone was used at three doses of 2, 10, and 50 µg g^{-1} BW of breeder fish were selected (Khalil *et al.* 2011).

2.3. Hormonal treatments

The Luteinizing Hormone-Releasing Hormone (LHRH) hormone dose was determined according to Naeem *et al.* (2013), focusing on inducing sexual maturity in male breeders using LRHR analog. After injection, fish were placed in tanks with water circulation. The mentioned hormone was dissolved in dimethyl sulfoxide solution (1 cc). Doses of 2, 10, and 50 µg g^{-1} BW were prepared and injected into the ventral caudal fin. The breeders in the control treatment were not injected with hormones but with distilled water.

2.4. Hormonal changes analysis in male and female Rohu breeders

In male fish, blood sampling was conducted pre-and post-injection, as well as after sperm retrieval. Blood sampling from the breeders was performed using a 5-mL plastic syringe, extracted from the caudal peduncle. Then, blood samples were transferred to ice-filled isolated containers and transported to the laboratory. Serum samples from each fish were centrifuged at 2000 RPM for 10 minutes and stored at -20°C until hormonal assays were performed (Degani, 2020). Testosterone and 17-beta estradiol were measured using the Spectria hormonal kit (Finland). Cortisol levels were measured using the RIA method, and 17-alpha hydroxyprogesterone was measured using the Immunotech hormonal kit (France). Furthermore, LH hormone was assessed using Immunotech kits (France) and I125 tracer by radioimmunoassay (RIA) method.

2.5. Testicular histochemistry

Testicular tissue samples were kept at 23°C to conduct histochemistric studies. Moisture, protein, fat, and ash content in the testes were measured using the AOAC method (1995). Moisture content measurement involved drying the samples at 105°C until a constant weight was achieved. Ash determination was carried out by burning the samples at 550°C for 12 hours in an electric furnace. Whereas protein content in the samples (total nitrogen × 6.25) was determined using the Kjeldahl method after sample digestion in 98% sulfuric acid. Fat extraction from

the samples was performed using Soxhlet extraction, and the fat was dissolved using ether.

2.6. Data Analysis

Statistical calculations were performed using SPSS version 23 software. One-way analysis of variance (ANOVA) was employed to examine the difference between the means of hormonal variables, tissue composition, and ovarian fatty acids. Tukey's post hoc test was utilized for multiple comparisons. A significance level of 0.05 was considered, and the data were reported as mean ± standard deviation.

3. Results

3.1. Changes in sexual steroid levels in male Rohu breeders

3.1.1. Estradiol

In male breeders (Figure 1-A), pre-and post-injection and after sperm release, the highest level of estradiol hormone was observed in the groups receiving pituitary and 50 µg g^{-1} BW of thyroxine. While the lowest level was measured in the control and LHRH groups ($p<0.05$). In male breeders, the estradiol hormone level was lowest pre-injection and highest after injection, with a significant decrease in its level in all treatments after sperm collection ($p<0.05$).

3.1.2. Testosterone

Figure B-1 illustrates the trend of testosterone changes in male breeders. The highest level was measured in the groups receiving thyroxine, pituitary, LHRH, and control before injection in male fish ($p<0.05$). After sperm extraction, a decreasing trend in testosterone levels was observed in all treatments ($p<0.05$). In the male breeders, the lowest testosterone levels were observed in the groups that received thyroxine after sperm collection, in the pituitary group after sperm collection, in the LHRH group after injection and sperm collection, and in the control group after injection ($p<0.05$).

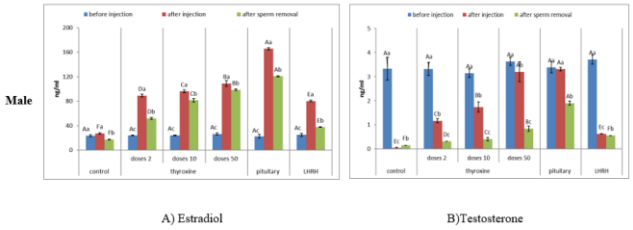


Figure 1 The hormone changes trend in A) Estradiol and B) Testosterone in Rohu (*L. rohita*) treated with hormones effective in sexual maturity effects of temperature. Lower and upper case letters indicate significant differences in each treatment and between treatments (P<0.05).

3.1.3. Cortisol

The cortisol hormone level in male breeders had the lowest amount after sperm collection, except in the pituitary treatment (29.20 ng mL^{-1} after the second injection and 57.15 ng mL^{-1} after sperm collection). Notably, no significant difference was found between other treatments before and after injection ($p<0.05$) (Figure 2).

3.1.4. 17-Alpha-Hydroxyprogesterone

In male breeders, the lowest level of the 17-alpha-hydroxyprogesterone hormone was observed in the control and LHRH treatments, while the highest value was measured in the groups receiving 50 µg g^{-1} BW of thyroxine and pituitary ($p<0.05$). Additionally, according to the changes trend observed in this hormone (Figure 2), male breeders had the highest level of 17-alpha-hydroxyprogesterone hormone post-injection and the lowest level pre-injection ($p<0.05$).

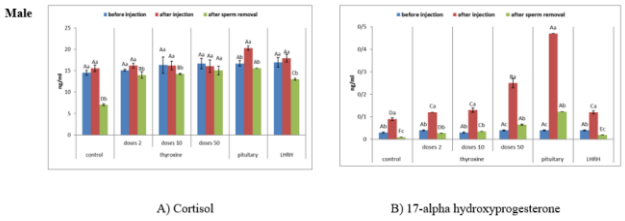


Figure 2 The hormonal changes trend in A) Cortisol, B) 17-alpha hydroxyprogesterone in Rohu (*L. rohita*) treated with hormones effective in sexual maturation effects of temperature. Lower and upper case letters indicate significant differences in each treatment and between treatments (P<0.05).

In the female breeder, the LH hormone exhibited the highest level pre-injection, significantly higher compared to other times ($p<0.05$). After injection, the highest level of this hormone was measured in all treatments after the second injection (Figure 3). In the male breeders, except for the LHRH treatment, where there was no significant difference in LH hormone levels after injection (3.51 ng mL^{-1}) and after sperm collection (3.45 ng mL^{-1}) ($p> 0.05$), the lowest level was observed after sperm collection in the other treatments ($p < 0.05$).

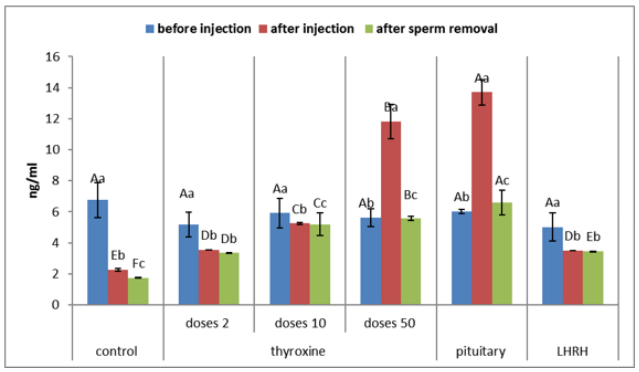


Figure 3 Changes in LH hormone of male Rohu fish (*L. rohita*) treated with hormones effective in sexual maturity effects of temperature. Lower and upper case letters indicate significant differences in each treatment and between treatments (P<0.05).

3.1.5. Fatty acids

The changes in fatty acids in the male breeder overall studied treatments are shown in Table 1. In the male breeder, the saturated fatty acid C12:0 was only measured in the treatments receiving thyroxine, whereas the unsaturated fatty acid C18:1n9t was only detected in the LHRH and pituitary treatments. Furthermore, the unsaturated fatty acid C18:1n7t was measured in the pituitary, LHRH, and control treatments. C16:0 and C18:0 are saturated fatty acids, C18:1n9c and C24:1n9 are unsaturated fatty acids with a double bond, and C20:3n6 and C22:6n3 are fatty acids with multiple double bonds, which were the most abundant fatty acids in the testes of male breeder fish under hormonal treatments. The composition of identified fatty acids in the male breeder fish is shown in Table 4.

Table 1. Fatty acid composition of the testis of male Rohu fish (*L. rohita*) treated with effective hormones in sexual maturity effects of temperature

Treatment		Control	Thyroxine (µg BW)			Pituitary	LHRH
Parameter			Dose 2	Dose 10	Dose 50		
Saturated fatty acids	C12:0	n.d	0.008±0.004^{c}	0.12±0.01^{a}	0.10±0.06^{b}	n.d	n.d
	C14:0	0.21±0.04^{c}	0.014±0.03^{d}	0.42±0.002^{b}	1.90±0.03^{a}	0.082±0.008^{e}	0.81±0.005^{e}
	C16:0	17.32±0.33^{e}	0.18±32.55^{d}	19.19±0.32^{c}	20.39±0.34^{b}	23.57±0.16^{a}	17.75±0.75^{e}
	C17:0	0.87±0.00^{c}	0.83±0.005^{d}	0.98±0.05^{b}	1.05±0.09^{a}	0.89±0.03^{c}	0.89±0.01^{c}
	C18:0	33.9±0.12^{d}	75.12±0.18^{c}	12.79±0.22^{c}	13.84±0.08^{b}	15.31±0.19^{a}	10.31±0.036^{d}
	C20:0	0.09±0.01^{e}	n.d	0.17±0.008^{c}	0.29±0.01^{b}	0.37±0.009^{a}	0.10±0.026^{d}
	C22:0	0.0±61.01^{e}	79.0±0.01^{d}	0.90±0.02^{c}	1.06±0.015^{b}	1.28±0.01^{a}	0.62±0.03^{e}
	C21:0	0.0±02.001^{f}	09.0±0.003^{e}	17.0±0.0004^{b}	0.67±0.0007^{a}	0.10±0.007^{d}	0.11±0.00^{c}
	C24:0	33.1±0.08^{d}	4.0±12.20^{c}	5.01±0.31^{b}	5.27±0.01^{b}	6.66±0.27^{d}	5.22±0.18^{b}
Unsaturated fatty acids with a double bond	C16:1	0.0±40.02^{f}	67.0±0.005^{d}	1.07±0.003^{c}	1.43±0.16^{b}	5.68±0.08^{a}	0.55±0.01^{e}
	C17:1	07.0±0.001^{f}	14.0±0.07^{d}	0.25±0.018^{c}	84.0±0.01^{b}	0.88±0.007^{a}	0.11±0.003^{e}
	C 20:1n	10.0±0.09^{e}	0.0±21.0008^{d}	0.55±0.015^{b}	0.99±0.001^{a}	0.99±0.03 ^{a}	0.42±0.017^{c}
	C18:1n9t	^{a} 0.0±0.64	n.d	n.d	n.d	n.d	^{b} 0.043±007
	C18:1n9c	^{f} 0.02±43.1	^{d} 62.09± 3.0	^{c} 0.05±7.70	0.11 ^{b}± 8.87	^{a} 21.68±1.56	^{e} 3.03±0.13
	C18:1n7c	0.04^{f}± 06. 1	^{d} 43.2±1.0	^{b} 0.05± 1.84	^{a} 0.20± 2.24	^{c} 0.01± 1.51	^{e} 0.03±1.28
	C18:1n7t	0.0 ^{c}± 02.0	n.d	n.d	n.d	^{b} 0.02± 0.12	^{c} 0.004± 0.03
	C22:1n9	^{c} 23.0± 0.0	0.03 ^{a}± 49.0	^{a} 0.005±0.53	^{a} 0.02±0.54	0.005 ^{b}± 0.36	^{b} 0.03±0.34
	C24:1n9	0.11^{f}± 57.1	^{d} 81.15±2.0	0.14^{c}±2.95	^{b} 0.17± 3.09	^{a} 0.06±3.46	^{e} 0.07±2.09
Omega 6 fatty acids	C18:2n6t	^{a} 25.0±0.0	^{b} 0.01±0.18	^{b} 0.004±0.19	^{b} 0.01±0.18	^{b} 0.02±0.20	^{c} 0.03±0.12
	C18:2n6c	0.10^{d}±5.2	^{d} 0.009±27.2	0.003 ^{c}± 3.42	^{b} 0.01±4.70	^{a} 0.12±8.42	^{d} 0.11±2.20
	C20:2n6	n.d	^{b} 0.02±0.36	^{b} 0.01±0.37	^{c} 0.01±0.21	^{a} 0.01±0.42	n.d
	C20:3n6	^{d} 0.13±86.3	^{b} 0.22±51.11	0.008 ^{a}±12.37	^{a} 0.61±12.87	^{a} 0.60±13.40	^{c} 0.10±10.76
	C20:4n6	^{e} 0.01±97.1	^{c} 47.18±4.0	^{b} 0.15±5.24	0.32^{a}±6.54	0.22 ^{a}±6.55	^{d} 0.46±3.22
	C22:2n6	0.004^{e}±0.06.00	n.d	^{b} 0.06±0.44	^{c} 0.01±0.21	^{a} 0.02±1.07	^{d} 0.004±0.08
Omega 3 fatty acids	C18:3n3	0.08 ^{d}±42.0	0.03 ^{c}±43.0	0.03 ^{c}±0.45	^{d} 0.01±0.39	^{a} 0.10±3.69	0.01 ^{b}±0.78
	C20:5n3	^{e} 0.42±0.76	0.009^{d}±80.10	^{c} 0.11± 2.11	^{b} 0.02±2.25	^{a} 0.07±5.20	^{d} 0.09±1.80
	C22:6n3	^{e} 0.44±96.4	^{cd} 2.77±0.16	0.37 ^{b}± 18.73	^{b} 1.06±19.96	1.33 ^{a}±20.30	^{d} 1.20±14.81

Non-identical letters mean a significant difference at the level of 0.05% between treatments (P<0.05). n.d: Not recognized by the device

Table 2. Investigating the ratio between types of fatty acids in the testicles of Rohu fish (*L. rohita*) treated with effective hormones in sexual maturation effects of temperature

Parameter	Control	Thyroxine (µg BW)			Pituitary	LHRH
		Dose 2	Dose 10	Dose 50		
ΣSUF*	29.78±2.31 ^{d}	37.12± 3.01 ^{b}	39.75± 2.92 ^{b}	44.57±3.25 ^{a}	48.26±2.82^{a}	34.98±2.47 ^{c}
ΣMUF**	4.94±0.62 ^{f}	9.37±1.36 ^{Ad}	14.89±2.62 ^{c}	18.63±2.78 ^{b}	34.68±1.75^{a}	^{e} 7.89±1.05
ΣPUF***	^{f} 14.33 ±1.80	37.04±2.93 ^{d}	43.32±2.51 ^{c}	47.31±3.94 ^{c}	59.25±3.68^{a}	33.77±2.49 ^{e}
ΣPUF/ ΣSUF	0.34	0.25	0.34	0.39	0.58	0.23
Σω6	^{f} 8.19±1.55	18.3±79.66 ^{d}	22.2±0.72 ^{c}	24.3±71.7 ^{b}	30.2±6.36 ^{a}	16.4±38.39 ^{e}
Σω3	6.14±0.52 ^{c}	18.25±3.63 ^{b}	21.29±4.10 ^{b}	22.6±4.80 ^{ab}	29.19±3.18^{a}	17.39±3.82 ^{b}
Σω3/ Σω6	0.74	0.97	0.96	0.91	0.97	1.06

*SFA: saturated fatty acid; **MUFA: monounsaturated fatty acid; ***PUFA: polyunsaturated fatty acid.**

Small non-similar letters indicate significant differences in each row (P<0.05).

Table 3. Histochemical composition of the testis of Rohu fish (*L. rohita*) treated with effective hormones in sexual maturity (dry weight percentage) effects of temperature

Parameter	Control	Thyroxine (µg body weight)			Pituitary	LHRH
		Dose 2	Dose 10	Dose 50		
Protein	10.58±0.21 ^{d}	13.38±0.22 ^{c}	13.01±0.27 ^{c}	14.61±0.17 ^{b}	15.54±0.24 ^{a}	11.61±0.25 ^{d}
fat	1.30 ±0.06 ^{e}	1.44 ±0.03 ^{d}	1.24 ±0.03 ^{d}	1.02 ±0.14 ^{b}	0.98± 0.08 ^{a}	1.49 ±0.05 ^{d}
humidity	82.01 ±0.12 ^{a}	82 ±0.09 ^{a}	82.05 ±0.05 ^{a}	81.97±0.07 ^{a}	82.11±0.16 ^{a}	82.03±0.07 ^{a}
ash	2.13 ±0.03^{a}	2.01 ±0.42 ^{a}	2.17 ±0.08 ^{a}	2.14±0.04 ^{a}	2.15 ±0.03 ^{a}	2.16 ±0.04 ^{a}

Small non-similar letters indicate significant differences in each row (p<0.05)

Table 2 shows that breeders treated with pituitary hormone and thyroxine were richer in total saturated and unsaturated fatty acids and total omega-3 and omega-6 fatty acids. Total unsaturated fatty acids with multiple

double bonds were significantly higher in the thyroxine, pituitary, LHRH, and control treatments in male breeders than in female growers ($P < 0.05$).

3.1.6. Testes histochemistry

The histochemical composition of the testis is presented in Table 3. In male breeders, no significant difference was recorded in moisture and ash content among the examined treatments ($P > 0.05$). The pituitary recipients exhibited the highest protein content at 15.54%, and the thyroxine recipients at 50 µg g^{-1} BW showed the highest (11.61%) ($P < 0.05$). While the LHRH and control treatments exhibited the lowest protein content ($P < 0.05$). In both male breeders, the gonad fat content was higher in thyroxine recipient treatments (doses of 2, 10, and 50 µg g^{-1} BW) compared to other treatments ($P<0.05$).

4. Discussion

Fish are ectothermic organisms, which means that all their life processes are influenced by the water temperature, as most fish species lack physiological mechanisms to regulate their body temperature. Therefore, fluctuations in water temperature can have differential effects on populations and the survival of aquatic species. Water temperature plays a crucial role in controlling all reproductive processes, from gamete and maturation growth, spawning, sperm release, and egg hatching to larval growth and survival. The effect of temperature on the natural reproduction of aquatic organisms can vary depending on the timing of the annual thermal cycles and can be exhibited differently. Water temperature is one of the most important factors that trigger biological responses and effectively coordinate the final stages of maturation through its influence on the hypothalamic-pituitary-gonadal axis (Imsland *et al.*, 2019).

Thyroid hormones in fish blood change according to reproductive cycles. Previous studies documented that high levels of these hormones increase the gametes growth in breeders, as well as the development and differentiation in ovarian follicles (Landines *et al.*, 2010). Considering the importance of these hormones in the sexual maturation of fish, this study examined their effects, compared to commercial hormones, on the reproductive hormones and body indices of Rohu fish in the male gender. Rawung *et al.* (2022) demonstrated that testosterone acts as a precursor in the production of estradiol in both male and female genders. It increases gonadotropin levels through positive feedback before the onset of oocyte and sperm maturation. This induction leads to final oocyte maturation in female fish and spermatogenesis in male fish (Landines *et al.*, 2010; Rawung *et al.*, 2022). A similar trend regarding changes in testosterone levels was observed in the present study. Accordingly, all treatments (thyroxine, pituitary, LHRH, and control) exhibited the highest testosterone levels before injection and before the initiation of gonadal maturation stages. Subsequently, there was a decreasing trend after injection until the spermiation stage, suggesting the potential utility of this hormone for stimulating spermatogenesis (Naeem *et al.*, 2013). After the decrease in testosterone, estradiol content increased to initiate maturation stages immediately after injection. Kulkarni and Pruthviraji (2016) reported the highest testosterone levels in river-collected fish, including *L. rohita*, *Catla catla*, *Cirrhana mrigala*, and *L. fimbriatus*, just before maturation.

After injection into male fish, the production of the hormone estradiol increased in the testes. After discharge of the gonads, it reached its lowest level, indicating a similar trend in the male sex. The higher levels of estradiol in the pituitary and thyroxine recipient treatments, compared to LHRH, confirm the higher efficiency of these two hormones in the sexual maturation phenomenon. However, at lower levels of thyroxine, the decrease in testosterone and estradiol production, as hormones involved in sexual maturation, led to a decrease in plasma androgen levels and a decrease in male fish maturation. Similar results were noted in various studies such as Safian *et al.* (2016) on zebrafish (*Danio rerio*) and Swapana *et al.* (2006) on catfish (*Clarias gariepinus*). Morais *et al.* (2013) investigated the effect of thyroid hormone on spermatogenesis in zebrafish (*D rerio*), and found that the use of thyroxine increases both mitotic index in undifferentiated spermatogonia and in Sertoli cells of the testes. Similarly, Safian *et al.* (2016) demonstrated that thyroxine not only stimulated the formation of type a spermatocyte but also increased the storage of type B spermatocytes in the testes.

Our results showed that increasing the thyroxine level from 2 µg g^{-1} BW to 50 µg g^{-1} BW significantly enhanced the level of this hormone in males. Compared to the pituitary gland, all three treatments led to significantly lower levels of the 17-alpha-hydroxyprogesterone hormone ($P < 0.05$). Moreover, Abdollahpour and Falahatkar (2017b) assessed the effect of thyroxine hormone injection on growth performance and reproductive function of female Sterlet (*Acipenser ruthenus*). They reported that higher doses exhibited an anabolic effect, while lower doses reduced efficiency and showed catabolic effects, leading to abnormalities. This result was particularly evident in the composition of gonadal tissues and the higher percentage of protein in the thyroxine recipient treatments with a dose of 50 µg g^{-1} BW compared to doses of 2 and 10. In male fish, research conducted by Franca *et al.* (2015) and Schulz *et al.* (2010) indicated the impact of elevated or diminished levels of thyroid hormones, notably thyroxine, on diminishing testicular size and sperm production rate. They attributed this mechanism to thyroxine's influence on the maturation of Sertoli cells.

In the LHRH treatment, the levels of 17 alpha-hydroxyprogesterone hormone were higher compared to the control group, but significantly lower than those observed in the thyroxine and pituitary hormone recipient treatments ($P < 0.05$). The superiority of pituitary and thyroxine treatments over LHRH can be attributed, in part, to the site of action of the injected hormones within the HPG axis (Heraedi *et al.*, 2018). The pituitary hormone directly affects the gonads, while thyroxine hormone, regulated by thyroid-stimulating hormones (TSH released from the pituitary), is released from thyroid follicles and plays a role in maturity activities (Abdollahpour and Falahatkar, 2017a). On the other hand, LHRH hormone

stimulates the pituitary gland of the breeders, leading to increased timing and hormonal chain reactions. This results in the pituitary hormone having a shorter pathway and a stronger effect in implementing sexual maturation instructions compared to the other two hormones (Al Zaidy *et al.*, 2017).

Previous studies have demonstrated that appropriate doses of thyroxine exert their effects through increased protein synthesis, glycogenesis, and synergistic interaction with other growth hormones, while lower doses result in growth reduction and protein catabolism (Eales *et al.*, 2004). In males, the treatment that received 2 µg g^{-1} BW had the lowest level of sexual organ proteins compared to the other treatments and the control group ($P < 0.05$). In other words, thyroid hormones play a role in the gene expression stage by increasing the expression of growth hormones and IGF-I, thereby increasing the production of these hormones and promoting protein synthesis (Schmid *et al.*, 2003). These results were consistent with the findings of the present study in treatments with doses of 10 and 50 µg g^{-1} BW. Research on catfish has indicated that thyroxine stimulates the secretion of hormone Igf3 (insulin-like growth factor 3), igfbp3, and igfbp1, both of which are insulin-like proteins that bind to proteins, thus promoting gonad growth and development. Moreover, thyroxine enhances hepatic synthesis of vitellogenin in male fish by increasing estradiol levels, thereby facilitating increased fat and protein reserves in the gonads for use in sperm production (Safian *et al.*, 2016).

The comparison of hormone efficacy in relation to protein reserves in testicular tissue confirms the superior efficacy of pituitary hormone compared to thyroxine and the inferior efficacy of LHRH in increasing protein levels in the gonads. This serves as an important indicator for meeting protein requirements for egg and sperm production in male Rohu breeders. In a study on the induction of ovulation in silver carp breeders (*Hypophthalmichthys molitrix*) using LHRH-A hormone, the success of utilizing LHRH hormone has been recognized depending on the dose administered and the use of a dopamine antagonist, which may explain the reduced performance of this hormone in Rohu fish (Mabudi *et al.* 2013).

The cortisol dynamics exhibited no significant disparity in its levels between pre-injection stages and sperm discharge among recipients of pituitary treatments, as well as across various doses of thyroxine treatments ($P > 0.05$). Conversely, recipients of LHRH treatments and the control group exhibited the lowest cortisol levels post-gonadal discharge compared to other treatments ($P > 0.05$). Furthermore, the cortisol levels in these two groups were notably lower than in the pituitary and thyroxine recipient treatments ($P < 0.05$). Considering the impact of stress on cortisol levels (Mabudi *et al.*, 2013), it appears that the administration of pituitary and thyroxine hormones induces more stressful conditions for Rohu breeders compared to LHRH. Additionally, although there were no significant differences observed across various time points ($P > 0.05$), male fish exhibited the highest cortisol levels post-injection across all six treatments. This phenomenon may be attributable to breeder manipulation during hormone injection.

Dopamine, functioning as a catecholamine transmitter, assumes a pivotal role in mitigating sexual maturity in breeders exposed to stressful circumstances such as manipulation (Drose *et al.*, 2014). Based on the findings, breeders subjected to both pituitary and thyroxine recipient treatments, administered at a dosage of 50 µg g^{-1} BW, exhibited superior capacities to suppress stress and regulate dopamine levels compared to other treatments. This highlighted the elevated levels of LH hormone observed in these two treatments ($P < 0.05$).

Fatty acids play a crucial role in the reproductive cycle, experiencing various metabolic pathways, with certain forms stored in reproductive organs (Jerez *et al.*, 2006). Docosahexaenoic acid (DHA) is an essential fatty acid vital for gamete structure, with the highest levels observed in pituitary recipient treatments, followed by thyroxine recipient treatments injected at a dosage of 50 µg g^{-1} of thyroxine. Ascorbic acid (C20:4n-6) is among the key fatty acids in the reproductive pathway (Tocher, 2010), essential for prostaglandin production, which stimulates gamete maturation (Tocher, 2003). In male breeders, the pituitary and thyroxine treatments exhibited the highest levels, which corresponded to the higher percentages of estradiol and testosterone hormones. Carlos *et al.* (2009) noted the presence of elevated levels of eicosapentaenoic acid (EPA, C20:5n3) and DHA (C22:6n3) fatty acids in the gonads as indicative of heightened maturity. In male fish, the pituitary and 50 µg g^{-1} of thyroxine treatment groups had higher levels of these two hormones compared to the control, LHRH recipient treatments, and treatments receiving 2 and 10 µg g^{-1} of thyroxine ($P < 0.05$). Hence, it can be concluded that these two hormones have a greater effect on the sexual responsiveness of male Rohu fish.

Saturated fatty acids (ΣSUF) play a primary role in sperm formation activities in male breeders (Henderson *et al.*, 1987). Considering the results and the tendency of the secretion of the hormone estradiol, which is involved in the stages of spermatogenesis, the high content of saturated fatty acids in the pituitary and thyroxine recipient treatments compared to the control is justified.

5. Conclusion

According to our results, the reduction in thyroxine hormone levels, influenced by climatic changes, can significantly impact the decrease in sexual hormones and the reproductive response of males. It can be concluded that treatment with thyroxine (50 µg) was more effective in inducing maturity in male Rohu compared to lower doses of thyroxine and also LHRH hormone when considering reproductive hormone indices. Additionally, these two hormones increased the protein and lipid content in the testicles and thus increased the reproductive success of the Rohu fish.

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