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The reality of the use of growth hormones in fish (Rui (*Labeo rohita*), Catla (*Catla catla*), and Monosex Tilapia (*Oreochromis niloticus*) production

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ABSTRACT

The unobservable use of hormones in fish production is becoming an alarming issue worldwide. To reveal the fact in Bangladesh, 144 fish samples (rui (*Labeo rohita*), catla (*Catla catla*), and monosex tilapia (*Oreochromis niloticus*)) were collected from different fish farms and markets of Mymensingh district. The market samples had two sources (Mymensingh and Rajshahi district). The steroid hormonal (testosterone, estrogen, and progesterone) residue was analyzed by HPLC-UV detection. A standard questionnaire survey was conducted where most farmers (80%) denied using the hormone in fish production. Among the analyzed samples of all three fishes, hormonal residues were detected in approximately 98% of samples, and around 92% contained residues above the ADI. Among the contaminated samples, 70% of samples had a single residue and 30% had multiple residues. The testosterone and progesterone hormonal residue was detected in all three fishes in both farm and market samples and ranged (above ADI) from 2.1 to 16.96 μg/kg and 31.47–731.57 μg/kg ($p < 0.05$) respectively. The estrogen hormone residue was only detected in market samples (Rajshahi district) of rui and catla and no residue was detected in tilapia fish and the hormone level (above ADI) ranged from 8.23 to 40.13 μg/kg. This study revealed that the use of hormones varies on the attitude of farmers based on the local culture pattern as estrogen hormone residue was only detected in market samples. The consumption of contaminated fish at such concentrations may cause many health hazards in humans, especially in children. Thus, this study reveals a new alarming fact to focus on, and an effective monitoring system should be implemented as soon as possible for public health concerns.

1. Introduction

The steroid hormonal use in fish production is becoming a concern worldwide. Steroid hormones (estrogen, progesterone, testosterone) are used in fish farming to increase growth when one sex of a species can grow bigger and faster than the other sex (Li et al., 2018). It is used to increase the growth, weight, and size of the fish for more economic gain (Hoga et al., 2018). In fish species, steroid hormones work by binding with the activated hormone-receptor complex in DNA that activates specific genes and thus increases protein production (Khalil et al., 2011). However, the use of hormones in fish farming can have harmful effects on humans, animals and the environment due to indiscriminate use and hormone-dependent parameters.

Studies have shown that synthetic steroid hormones were widely used to increase growth in many fish species, and among them carp fish and tilapia were the most common (Das et al., 2022; Islam et al., 2015; Zhai et al., 2022). Regarding the hormone residue in fishes, Liu et al. (2017) found that the steroidal hormone accumulated in crucian carp (*Carassius auratus*), carp (*Cyprinus carpio*) and silvery minnow (*Anabarilius alburnops*) in aquaculture environments in Dianchi Lake, China. It was also reported by Wang et al. (2012) and Cheng et al. (2012) that in yellow croaker (*Larimichthys polyactis*) collected from local supermarkets in Changchun, China and in tilapia (*Oreochromis mossambicus* and *Oreochromis niloticus*) purchased from supermarkets in Taipei City, Taiwan had steroidal hormone residue in their tissue samples.

In Bangladesh, the demand for fish has increased in recent years due

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to population growth and the constant search for a healthy diet (Tidwell and Allan, 2001). However, due to natural and man-made hazards, natural fish populations have declined during the last several decades. It has increased the effort in techniques development for more fish production in hatcheries and farms (Rottmann et al., 1991). In Bangladesh, many fish species culture is now in progress on farms and rivers; such as rui (*Labeo rohita*), catla (*Catla catla*), monosex tilapia (*Oreochromis niloticus*) and so on (Ahmed et al., 2013; Hosen et al., 2019). But these fish species do not gain weight or undergo reproduction spontaneously in the culture system (Rottmann et al.,1991). Thus, various techniques have been developed to improve growth and spontaneous reproduction, such as hormone-induced breeding, growth and transgenesis (Alimuddin et al., 2010).

Steroid hormone has many harmful effects on consumers. In children, it causes early puberty, advances in bone age, modification of sexual characteristics and cancer development (Bergman et al., 2013). It also causes feminization effects in males, infertility, reduced fertility, inhibition of the development of sexual organs and sex reversal in females (Hoga et al., 2018). To prevent consumers from suffering from possible health risks, the use of steroid hormones in fish culture has been banned or limited in many countries and organizations (Li et al., 2018). The Joint FAO/WHO Expert Committee on Food Additives (JECFA) established an acceptable daily intake of testosterone, progesterone, and estrogen at the rate of 2 μg/kg, 30 μg/kg, and 0.05 μg/kg body weight, respectively (JECFA, 2000).

Nevertheless, it is necessary to know the hormonal contamination level in fish flesh as these hormones pose human health hazards if they exceed acceptable dietary intake (ADI). Many methods are used for the analysis of steroid hormones in fish, but in this study, the HPLC-UV detection method was used for analysis. The main objective of this study was to investigate whether or not fish have steroid hormones in their flesh at a harmful concentration at the time of marketing and also know the farmers' knowledge, attitudes and practices regarding hormone use.

2. Material and methods

2.1. Selection of study area

For the study area, the Mymensingh district of Bangladesh was selected as this area has numerous fish farms and was popular for fish production. The fish samples were categorized into farm and market fish samples. For farm samples, three Upazilas of Mymensingh district, namely Mymensingh Sadar, Muktagacha, and Trishal were selected. In Mymensingh district, the market fishes came from two areas- Mymensingh and Rajshahi district fishes. For market samples, two renowned markets (Mechua Bazaar and Mymensingh city corporation market) and one local market (Bangladesh Agricultural University K R market) were selected for sample collection as this market is the central and busiest market of the Mymensingh district.

2.2. The survey

A cross-sectional survey was conducted to assess the knowledge of farmers on hormone use in fish farming. The survey was carried out using a well-structured questionnaire for the farmers. The survey covered three Upazilas (Mymensingh Sadar, Trishal, and Muktagacha) of the Mymensingh districts. A total of 70 farms were surveyed. The questionnaire used in the study was written both in English and Bengali. The educational status, farm size, feed type (commercial or homemade), use of the hormones in fish production (yes or no), knowledge regarding hormonal residue, culture system (monoculture or polyculture), veterinarian suggestions etc., were taken into consideration during the questionnaire survey (questionnaire added to the supplementary material).

During the survey, the educational status of the farmers was categorized from illiterate to graduate and classified as illiterate (no alphabetical knowledge), primary (standard one to five), junior secondary (standard six to eight), secondary (standard nine to ten), higher secondary (intermediate), and graduate (both graduate and post-graduate). The farms were categorized into small, medium and large according to the no. of ponds following Ntsama et al. (2018).

2.3. Selection of farms for fish samples

The farms were categorized according to the use of hormones during fish production based on a survey. Fish farms were randomly selected from both categories. From three Upazila (Mymensingh Sadar, Muktagacha, and Trishal) two farms were selected; thus, a total of six farms were selected for the study.

2.4. Selection of fish

Fish that were easy to culture, fast-growing, cheap, and very demandable in the local market were selected for this study. Tilapia (*Oreochromis niloticus*) and carp fish – rui (*Labeo rohita*), catla (*Catla catla*) were selected for this experiment as they fulfilled the criteria.

2.5. Selection of hormone

Hormones were selected based on the literature review and then supported by a questionnaire survey. The literature review revealed that steroid hormones act as a growth hormone for fish. Some of them are also used for the production of monosex populations in fish. Thus the steroid hormones (testosterone, estrogen, and progesterone) were selected for this experiment.

2.6. Sample collection

Fish (Rui, Catla, and Tilapia) samples were collected in September 2021. For each fish, four samples were collected from each farm, and eight samples were collected from each market. A total of 48 samples were collected, of which 24 were farm samples, and 24 were market samples. Thus, for three fish species, a total of 144 samples (48 Rui, 48 Catla, and 48 Tilapia) were collected, of which 72 were farm samples and 72 were market samples. Whereas, the fish samples from the Government fish farm were used as negative controlled fish.

The mature fish were collected in a clean, transparent airtight zipper bag, and each bag was properly labelled with sample numbers, sources, collection date and species name. Then, the samples were transferred to the Department of Pharmacology lab, BAU, via icebox (4 °C). Then the samples were cut, washed, and chopped into pieces, weighted and collected into another zipper bag properly labelled and stored at −20 °C for further processing.

2.7. Chemical and reagents

Hormone reference standards were purchased from Sigma-Aldrich. The certified purities of these standards range from 98% to 99%. HPLC grade acetonitrile and methanol were purchased from Merck (Germany). Florisil (magnesium silicate) was purchased from Sigma, USA. The solvents acetone (Merck, Germany), Trichloroacetic acid (Merck, Germany), sodium chloride (Merck, Germany), magnesium sulfate (Merck, Germany), aluminium oxide (Merck, Germany) used in this study were laboratory reagent grade. The syringe filters (0.45 μm) were obtained from Merck, Germany.

Before usage, all glassware was cleaned with detergent, sonicated, rinsed with distilled water followed by acetone and then heated for 2 h at 180 °C.

2.8. Sample extraction

The extraction of fish samples for hormone analyses was performed

according to (López-garcía et al., 2018) with slight modifications. The refrigerated sample was thawed and chopped into small pieces. The chopped sample was then blended with mortar and pestle. The blended 2 g sample was taken in a falcon tube and added 2.5 ml of deionized water. The sample was then vortex for 1 min and allowed to stand for 5 min at room temperature. Subsequently, the 7.5 ml of acetonitrile-containing formic acid (1% v/v) solution was added to the sample. Then the solution was blended using a rotary blender for 1 h. The mixture was then allowed to stand for 10 min at room temperature. The sample was centrifuged for 10 min at 4500 rpm and filtered into another falcon tube.

Following that, a cleanup stage was completed by adding 1.25 ml of extract to a solution containing 50 mg of aluminium oxide and 50 mg of florisil. After 15 s of vortexing, the mixture was centrifuged at 1400 rpm for 8 min. Finally, it was filtered through a 0.45 μm syringe filter and injected into the chromatographic system.

2.9. High-performance liquid chromatography-ultra violet (HPLC-UV)

High-performance liquid chromatography (Dionex Ultimate 3000 UHPLC) equipped with a pump, an auto-sampler, a UV–Vis detector, C18 reverse-phase column (Acclaim® 120, 5 μm, 120 Å, 4.6 × 250 mm), and a monitor with Chromeleon software 6.80 version were used for the analysis. Washing began by priming the syringe 3–5 times using an 80:20% (IPA: water) wash buffer solution. The external needle was primed with a 300 μl and a 100 μl IPA solution, respectively. Purge pump with a system-programmed draw speed of 3 ml/min for solvent lines A and B. Set the UV detector at a wavelength of 242 nm and the temperature of the column compartment to the desired temperature (30 °C). Before every analysis, run the data acquisition baseline for 30–45 min to ensure the system is stable.

The water and acetonitrile in a multi-step gradient system were used as the mobile phase (Table 1). Separation was performed at 30 °C temperature at a flow rate of 1.0 ml min-1 or 1.5 ml min-1; the injection volume was 10 μl for both standard solutions and sample extracts by the auto sampler (G1329A). The detection was done using a UV–Vis detector set to 242 nm.

2.10. Standard preparation and calibration curves

The individual stock solution of all standard hormones (17-α-methyltestosterone, β-estradiol, and progesterone) was prepared by dissolving 2 mg of each hormone into 10 ml of HPLC-grade methanol. Further, it was diluted by mobile solvent (HPLC grade acetonitrile) in the following order; 200 μg/ml, 100 μg/ml, 50 μg/ml, 25 μg/ml, 12.5 μg/ml respectively. The stock solution was stored at 4 °C for further use.

The spiking of samples and the creation of matrix-matched standards were both done using the working standard solutions. The plot was made by the area under the peak versus the concentration, and these data points were fitted using a simple linear regression to obtain the equation for the standard curves. Based on the slope of the standard curve, the amount of each hormone in each sample was calculated (Fig. 1a) and (Fig. 1b).

Table 1
The multi-step mobile phase gradient program.

Time (min)	Water (%)	Acetonitrile (%)	Flow (ml min-1)
0	100	0	1
3.5	80–70	20–30	1
5	50	50	1.5
7.5	40	60	1
9.5	20	80	1
10	80	20	1
15	70	30	1

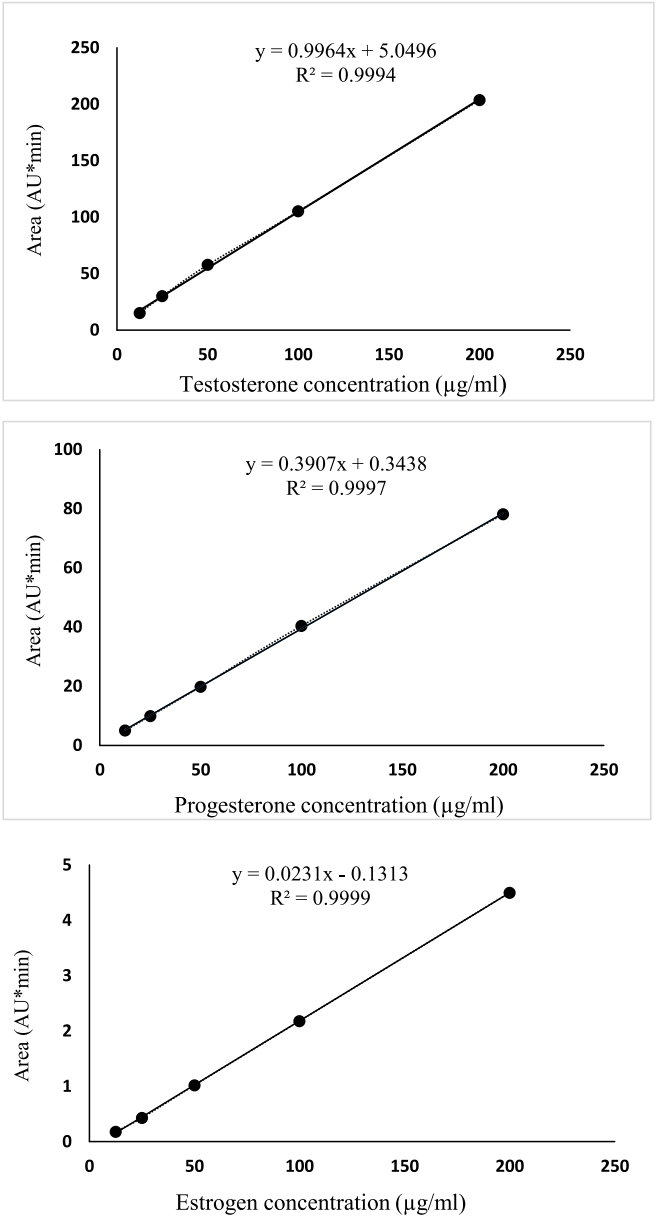

Fig. 1a. Calibration curve of standard hormones (a) Testosterone, (b) Progesterone, and (c) Estrogen.

2.11. LOQ and LOQ

The limit of quantification (LOQ) was defined as the lowest concentration of the analyte that could be quantified with acceptable precision and accuracy. The LOD was defined as the lowest concentration of the analyte in a sample that could be detected but not necessarily quantified. The LOQ and LOD were determined using the standard deviation of the y-intercept of regression analysis (σ) or the residual standard deviation of a regression line and the slope (S) as follows LOD = 3.3 σ/S and LOQ = 10 σ/S (Soranganba and Singh, 2018).

In the present study, LOD ranged from 0.97 to 3.66 μg/kg, and LOQ ranged from 2.93 to 11.09 μg/kg (Table 2).

2.12. Calculation of residual concentration

The standard curve of three (3) hormones was formulated according to the straight-line equation $y = mx + c$.

Where y represented the area of the sample (AU*min), *m* was a slope,

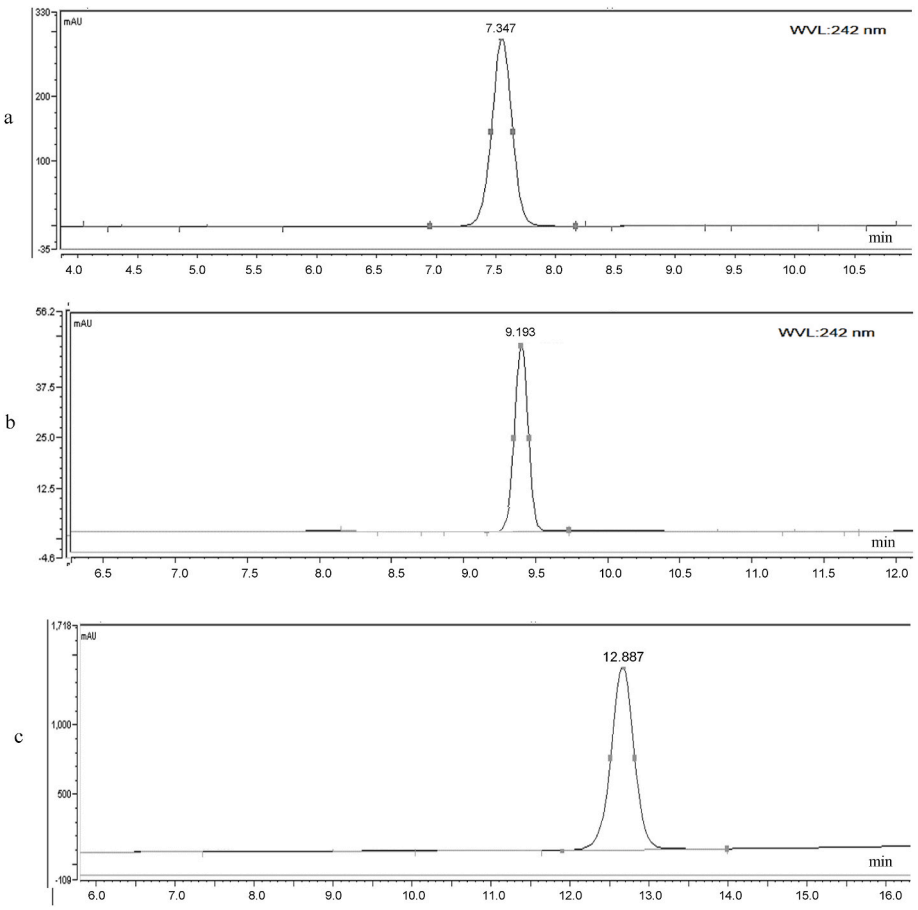

Fig. 1b. Chromatographic picture of standard hormones (a) Testosterone, (b) Estrogen, and (c) Progesterone.

Table 2
Retention time, correlation coefficient, the limit of detection (LOD), the limit of quantification (LOQ) and recoveries of selected hormone.

Compound	Retention time ±SD (min)	Correlation Coefficient/R	LOD (μg/kg)	LOQ (μg/kg)	Recoveries	
					Added (μg/ml)	Recovery (%)
Testosterone	7.347 ± 0.151	0.9994	0.97	2.93	10	92%
Progesterone	12.887 ± 0.197	0.9999	3.66	11.09	10	87%
Estrogen	9.193 ± 0.38	0.9997	2.58	7.84	10	74%

c was the intercept, and x was the concentration of the unknown sample (μg/kg). The m and c were obtained from a specific hormone's standard curve, and y was the area obtained from the detected hormone in the sample. Therefore, the equation was

$$x = (y\text{-}c)/m$$

Where y represented the area covered by hormone in the sample (AU*min), *m* was the slope of the standard curve, c was the intercept of the standard curve, and x was the residual concentration of hormone in the sample (μg/kg).

3. Results

3.1. Questionnaire survey

The survey results found that only a few farmers (20%) accepted using the hormone in fish production (Fig. 2). Farmers admitted to using only testosterone in tilapia for monosex population production. Again the farmers (20%) had the knowledge of hormonal residue and withdrawal period (Fig. 2). The survey also revealed that the farmers used

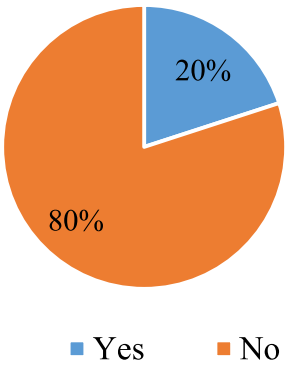

Fig. 2. Knowledge of farmers regarding the use and withdrawal period of the hormone in fish production.

hormones on their farms without consulting the veterinarian. They mainly used hormones at the suggestion of other farmers, medicine sellers, or feed company representatives.

Among the surveyed farms, most are medium category (60%), followed by small (20%) and large (20%) (Fig. 3). From the perspective of educational status, the majority of the farmers accomplished secondary education (40%), followed by primary (20%), graduate (20%), and illiterate (20%) (Fig. 4).

It was also known that all the farmers used commercial feed on their farms and practised monoculture fish production (one species in single ponds) for tilapia and polyculture fish production for rui and catla.

3.2. Sample analysis

Hormonal residues were detected in approximately 98% of all three types of fish samples. Among the hormone-contaminated samples, around 92% of samples contained hormonal residues above the acceptable dietary intake (ADI) established by the Joint FAO/WHO Expert Committee on Food Additives (Fig. 5). Of the analyzed samples, around 70% samples contaminated with a single residue, and 30% samples contaminated with multiple residues (Fig. 6).

3.2.1. Testosterone hormone

Among the analyzed samples of rui, all the farm samples (24) had residues of testosterone hormone ranging from 1.69 to 5.57 μg/kg. Among them, 92% (22) samples exceeded the value of ADI (2 μg/kg), the lowest value was 1 times higher, and the highest value was 3 times higher than the ADI. About 75% (18) of the market samples of rui had testosterone hormone residue ranging from 2.31 to 7.14 μg/kg, and all of them exceeded the ADI value. The lowest value above ADI was 1 times higher, and the highest value was 4 times higher (Table 3).

About 92% (22) of farm samples of catla tested positive for testosterone hormone, which ranged from 1.43 to 5.96 μg/kg. Among them, 67% (16) samples exceeded the value of ADI and ranged from 2.1 to 5.96 μg/kg, which was 1–3 times higher than the ADI. The 67% (16) market samples of catla were positive for testosterone hormone and ranged from 1.20 to 7.19 μg/kg. Among them, 50% (12) samples exceeded the value of ADI and ranged from 2.16 to 7.19 μg/kg, which was 1–4 times higher than the ADI. From the above analysis of catla fish, farm samples had more contaminated and above ADI samples than market samples (Table 4).

All the farm (24) samples of tilapia were positive for testosterone hormone, ranging from 1.15 to 6.87 μg/kg and among them, 96% (23) samples exceeded the ADI value, ranging from 2.83 to 6.87 μg/kg, which was 1–3 times higher than the ADI. All market samples of tilapia (24) were positive for testosterone hormone, ranging from 2.54 to 16.96 μg/kg and all of them exceeded the ADI value and were 1–8 times higher than the ADI.

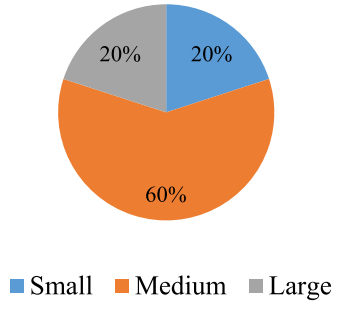

Fig. 3. Size of the surveyed farms.

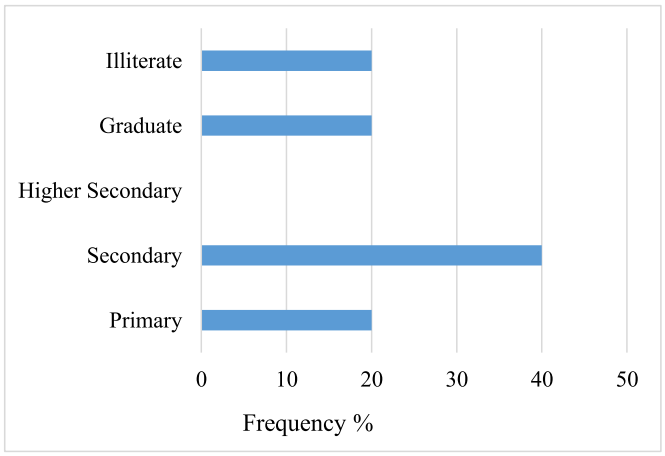

Fig. 4. Farmer's educational status of the surveyed farms.

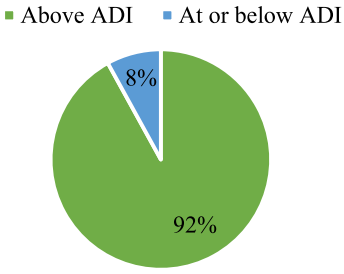

Fig. 5. The percentage of hormone contamination in fish samples related to ADI.

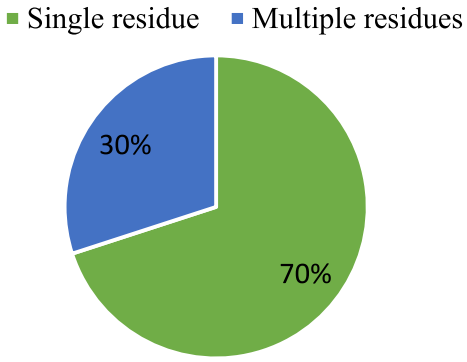

Fig. 6. The percentage of hormonal residues detected in the fish samples.

3.2.2. Progesterone hormone

In rui fish, the progesterone hormone tested positive in 16.67% of farm samples but all were below the ADI (30 μg/kg) level and ranged from 1.08 to 1.89 μg/kg. Whereas in market samples, the progesterone hormone tested positive in 58% (14) of samples and ranged from 31.47 to 427.26 μg/kg. Among the contaminated samples, all the samples exceeded the ADI value and 1 to 14 times higher than the ADI.

In catla fish, the progesterone hormone tested positive in 33% (8) of samples (all samples of Muktagacha farm), ranging from 10.07 to 48.12 μg/kg. Whereas about 25% (6) contaminated samples exceeded the value of ADI, which was 1–2 times higher than the ADI. The

Table 3
Percentage of hormone contaminated fish samples above ADI.

Fish types	Hormone types	Frequency of contamination	
		Farm samples	Market samples
Rui (*Labeo rohita*)	testosterone	92%	75%
	progesterone	–	58%
	estrogen	–	42%
Catla *(Catla catla)*	testosterone	67%	50%
	progesterone	25%	33%
	estrogen	–	21%
Tilapia (*Oreochromis niloticus*)	testosterone	96%	100%
	progesterone	29%	17%
	estrogen	–	–

progesterone hormone had residue in 33% (8) market samples (only K. R market) and ranged from 34.05 to 46.98 μg/kg. Among the contaminated market samples, all the samples exceeded the ADI value and 1 to 2 times higher than the ADI (Table 4).

In tilapia fish, the progesterone hormone was detected in 29% (7) of farm samples (only Muktagacha farm) ranging from 293.6 to 718.9 μg/kg and all of them exceeded the ADI value, which was 10–24 times higher than the ADI. Whereas for market samples, 25% (6) of samples had progesterone residue and ranged from 4.57 to 731.57 μg/kg. Among them, 17% (4) samples exceeded the ADI value; the lowest value was 24.3 times higher, and the highest value was 24.4 times higher than the ADI. In tilapia fish, the farm samples had more contaminated and above ADI samples than market samples (Table 3).

3.2.3. Estrogen hormone

In rui fish, there were no samples tested positive for estrogen hormone in farm samples. In the market samples, the estrogen hormone tested positive in 42% (12) of the samples and ranged from 8.23 to 34.35 μg/kg. Among the contaminated samples, all the samples exceeded the ADI (0.05 μg/kg) value; the lowest value was 165 times, and the highest value was 687 times higher than the ADI (Table 4).

In catla fish, there was no positive farm samples for estrogen hormone. The estrogen hormone tested positive in 21% (5) of the market samples (only K. R market) and ranged from 32.63 to 40.13 mg/kg. Among the contaminated market samples, all the samples exceeded the ADI value; the lowest value was 653 times and the highest value was 803 times higher than the ADI.

In tilapia fish, there were no positive samples for farm and market for estrogen hormone (Table 3).

3.2.4. Multi-hormonal residue

In rui fish, testosterone and progesterone hormone combination was detected in only one farm sample. And all the 4 samples possessed testosterone above the ADI level but progesterone was below the ADI level. Whereas about 13 market samples had multiple hormonal residues and among them, 5 samples had (testosterone and progesterone) residues, 3 samples had (testosterone and estrogen) residues and 5 samples had all three hormonal residues. All the market samples had multi-hormonal residue above the ADI level (Table 5).

In Catla fish farm samples, there were 8 samples that had multiple hormonal residues (testosterone and progesterone) and among them, 6 samples contained testosterone and progesterone above the ADI level. Whereas 5 market samples of catla had combined estrogen and progesterone hormone residue and all of them exceeded the ADI value (Table 5).

In tilapia, 7 farm samples had multiple hormonal residues (testosterone and progesterone). Among them, 6 samples had testosterone exceeding the ADI value and all 7 samples exceeded the ADI value of progesterone. In the case of market samples, 6 fishes had combined testosterone and progesterone hormone residue in their flesh. Among the multi-residue contaminated market samples, all samples having testosterone exceeded the ADI value whereas, 4 samples of progesterone exceeded the ADI value in tilapia (Table 5).

4. Discussion

In this survey, most of the farmers (80%) stated that they didn't use any hormones in fish production, but after the survey, it was observed that hormonal residue was evident both on the farm and market fish samples. As no other report was available on the attitude of farmers using hormones it could be assumed from this experiment that farmers were using hormones either intentionally or ignorantly during fish production in Bangladesh. Whether it is intentionally or ignorantly could not be differentiated as there was no evidence of the support of the farmers' answers. But from the socio-economic perspective, it could be assumed that hormones (other than testosterone) were not available in the market to use in fish production. Moreover, the cost of the hormones was very high and difficult to make the production cost-effective by using them regularly.

In the study, farm size or educational background had no impact on hormonal residue detection in Bangladesh. It was reported by Shamsuzzaman and Biswas (2012) that there was no impact on farm size or educational background of farmers to use antibiotics in shrimp farms in Bangladesh. It was also observed that none of the farmers referred to a

Table 4
Summary of single hormone residue contamination levels in fish samples from Bangladesh.

Fish samples	Type of samples	Name of hormone detected	No. of contaminated samples	No. of samples exceeding the ADI	Ranges of hormone detected (μg/kg)	Ranges of hormone above ADI (μg/kg)	ADI (μg/kg)
Rui (*Labeo rohita*)	Farm samples (24)	testosterone	24	22	1.69 to 5.57	2.18 to 5.57	2
		progesterone	4	0	1.08 to 1.89	1.08 to 1.89	30
		estrogen	–	–	–	–	0.05
	Market samples (24)	testosterone	18	18	2.31 to 7.14	2.31 to 7.14	2
		progesterone	14	14	31.47 to 427.26	31.47 to 427.26	30
		estrogen	12	12	8.23 to 34.35	8.23 to 34.35	0.05
Catla *(Catla catla)*	Farm samples (24)	testosterone	22	16	1.43 to 5.96	2.1 to 5.96	2
		progesterone	8	6	10.07 to 48.12	31.5 to 48.12	30
		estrogen	–	–	–	–	0.05
	Market samples (24)	testosterone	16	12	1.20 to 7.19	2.16 to 7.19	2
		progesterone	8	8	34.05 to 46.98	34.05 to 46.98	30
		estrogen	5	5	32.63 to 40.13	32.63 to 40.13	0.05
Tilapia (*Oreochromis niloticus*)	Farm samples (24)	testosterone	24	23	1.15 to 6.87	2.83 to 6.87	2
		progesterone	7	7	293.56 to 718.94	293.56 to 718.94	30
		estrogen	–	–	–	–	0.05
	Market samples (24)	testosterone	24	24	2.54 to 16.96	2.54 to 16.96	2
		progesterone	6	4	4.57 to 731.57	729.25 to 731.57	30
		estrogen	–	–	–	–	0.05

Table 5
Summary of multiple hormone residue contamination levels in fish samples from Bangladesh.

Fish samples	Type of samples	Combination of multi-hormonal residue	No. of contaminated samples	No. of samples exceeding the ADI for each hormone	Ranges of hormone detected (μg/kg)	Ranges of hormone above ADI (μg/kg)	ADI (μg/kg)
Rui (*Labeo rohita*)	Farm samples (24)	a. testosterone & progesterone	4	4	2.87 to 3.74	2.87 to 3.74	2
				–	1.08 to 1.89	–	30
	Market samples (24)	a. testosterone & progesterone	5	5	2.31 to 7.14	2.31 to 7.14	2
				5	209.15 to 420.17	209.15 to 420.17	30
		b. testosterone & estrogen	3	3	3.5 to 4.62	3.5 to 4.62	2
				3	8.23 to 34.21	8.23 to 34.21	0.05
		c. testosterone, progesterone & estrogen	5	5	3.63 to 4.77	3.63 to 4.77	2
				5	31.47 to 427.26	31.47 to 427.26	30
				5	8.25 to 14.69	8.25 to 14.69	0.05
Catla *(Catla catla)*	Farm samples (24)	a. testosterone & progesterone	8	6	1.43 to 5.35	2.1 to 5.35	2
				6	10.07 to 48.12	30.17 to 48.12	30
	Market samples (24)	a. progesterone & estrogen	5	5	34.05 to 46.98	34.05 to 46.98	2
				5	32.63 to 40.13	32.63 to 40.13	30
Tilapia (*Oreochromis niloticus*)	Farm samples (24)	a. testosterone & progesterone	7	6	1.15 to 5.45	2.83 to 5.45	2
				7	293.56 to 718.94	293.56 to 718.94	30
	Market samples (24)	a. testosterone & progesterone	6	6	3.49 to 16.96	3.49 to 16.96	2
				4	4.57 to 731.57	730.67 to 731.57	30

veterinarian for a prescription, and most (80%) didn't know about the hormonal residue and withdrawal period. A similar practice regarding veterinarian suggestions was found in Cameroon, where most farmers (75.6%) used agrochemicals (antibiotics, pesticides and fertilizers) in fisheries without consulting a veterinarian (Ntsama et al., 2018). It was also reported that academic and economic background had little or no impact on the knowledge of hormonal use in fish production. This study may be the first data regarding farmers' knowledge of hormonal use, withdrawal period and residue in fish farms (especially rui, catla, and tilapia) in Bangladesh. Moreover, this study was one of a few in the world which was also related to farmers' knowledge and attitude towards hormonal use in fish production.

The use of testosterone in tilapia for monosex population production is a common practice worldwide (Utete and Muposhi, 2012; Jensi et al., 2016). Testosterone is used in monosex population production at the time of gonadal sexual differentiation for up to 30 days (Almeida, 2013; Utete and Muposhi, 2012). But the safety dose (below ADI) needs to be checked before being marketed (Duarte et al., 2002). In this study, the farm and market samples were collected during marketing (5–6 months). So it is expected that there will be no hormonal residue in fish samples at the time of marketing. But in this study, testosterone residues above ADI in farm and marketed fish samples indicated following an improper withdrawal period. It was reported that in Bangladesh 59% of the farmers applied steroids as growth promoters in beef cattle fattening all year round, and 36% of farmers used hormones for the fattening for 3 months; before being marketed (Kamal et al., 2019). If either of the practices was performed for testosterone hormone in tilapia fish production, then it could be a great concern.

In this experiment, high concentrations of testosterone hormone residues were detected in rui and catla fish in farm and market samples. Though the use of testosterone in rui and catla for monosex population production is not well known (Hoga et al., 2018). In this study, it was observed that all the farmers practised a polyculture system in rui and catla production. The source of testosterone in farm samples (monoculture or polyculture) is still unknown and surprising. Marketed fish samples (rui and catla) also contained testosterone above ADI. The possible source of testosterone contamination was either feed containing hormones, as all the farmers used commercial feed or came from other sources (water bodies, environmental pollution, etc.) or farmers were using hormones in fish production intentionally or ignorantly.

The progesterone hormone was mainly used in fish for spawning and therapeutic purposes and in fathead minnow (*Pimephales promelas*) spawning it was used for 21 days (DeQuattro et al., 2012). If progesterone hormones were used for spawning purposes, there would be no residue at the marketing time. In tilapia, using progesterone hormone for any purpose has not been reported so far. But in this study, tilapia and catla tested positive for progesterone residue above ADI for their farn and market samples. Interestingly all the farm samples of rui tested negative for progesterone which raised the question of maintaining a proper withdrawal period in different farms. However, the presence of progesterone in tilapia and catla (both farm and marketed) above ADI is an eye-opener to detect the source of this hormone.

The estrogen hormone in fish is used for producing all female stock (feminization), especially in carp fish. However, the withdrawal period needs to be checked while producing the female stock (Piferrer, 2001). In this study, no farm samples of fish (rui and catla) had estrogen hormone residue, but the market samples had estrogen hormone residue above ADI. The estrogen hormone residue in only market samples implies that farmers from other regions (Rajshahi district) used estrogen hormone for fish production and local culture patterns played an important role in that case.

The multi-hormonal residue was common in all three fish species. In farm samples, the combination of testosterone and progesterone hormone residue was found. This combination of progesterone and testosterone was detected in all three species but not all samples. In market samples, 3 types of combination were present (i. testosterone and progesterone; ii. testosterone and estrogen; and iii. testosterone, progesterone and estrogen) in some of the samples. Whereas, in all types of contaminated samples the hormonal residues were above the ADI level. It has been found out that the market fish had two areas of sources (Mymensingh and Rajshahi). Contaminated market samples of Mymensingh contained a combination of testosterone and progesterone only. It could be assumed that the farmers from Mymensingh districts used testosterone and progesterone combinations for fish production. Whereas, samples of Rajshahi district origin had different combinations of hormonal residue (estrogen and progesterone; and testosterone, progesterone and estrogen). Whereas, estrogen hormone was common in Rajshahi district samples (rui and catla). It could be assumed that estrogen was used in the production of carp fishes in that area. Though the result suggested the probable use of estrogen in carp fish production, there is no documentation available among the scientific community or policymakers. Moreover, the farmers are not affirming this fact. From this analysis, it revealed that testosterone and progesterone hormones were used widely in fish production. Whereas, the use of estrogen hormone depended on the local culture pattern.

The people of Bangladesh regularly consume these types of fish. The continuous exposure of these hormones to contaminated fish causes many health disorders in children and adults (Hoga et al., 2018). Children are particularly at high risk because they are in a growing phase when puberty has not yet developed and have disorders like precocious

pseudo puberty, negative repercussions on growth, modification of sexual characteristics and cancer development (Alves et al., 2007; Bergman et al., 2013). In adults, consuming these hormone-contaminated fish causes feminization effects in males, infertility, reduced fertility, inhibition of the development of sexual organs and sex reversal in females (Hoga et al., 2018).

There are various rules regarding the usage of hormones in different countries. The European Union forbids the use of the hormone in aquatic organisms (EC, 2003, 2008), while other countries like the United States, Australia, Canada, New Zealand, and Argentina permit the use of natural steroid hormones like testosterone, progesterone, and 17-estradiol but they evaluated maximum residue levels (MRLs) (Duarte et al., 2002). But in Bangladesh still, there are no monitoring systems developed. No regulatory law has developed regarding the use of hormones in fish production. Furthermore, a mass and extensive study is needed for fish feed analysis for possible sources of hormone contamination. This work is evidence of the indiscriminate use of steroid hormones in fish. Thus it is expected that the respective Government or the consumers should be aware of these things, and a monitoring system should be developed as soon as possible.

5. Conclusions

Hormonal residue and its associated health hazards are very alarming issues nowadays all over the world. The level of hormones detected in this study can cause health hazards in humans, especially in children. Due to the indiscriminate and intentional misuse of the hormone in fish production, hormonal residues enter the human food chain. This issue is needed full attention from our respective authorities and Government. As no monitoring system has been established yet, a new law and strict monitoring system should be implemented for hormonal use in fish production. This study also emphasized that mass awareness regarding hormonal residue-contaminated fish consumption should be raised. An extensive study regarding hormonal residue in different fish and fish feeds should be needed immediately.

6. Limitation

Conducting this study, we encountered a few limitations. In this experiment, following the analysis of the survey, samples were collected. During the survey only the tilapia farmers accepted the use of testosterone. This survey didn't reveal any data about estrogen and progesterone use in fish farming in Bangladesh. As a result, emphasis was not given to those two hormones to investigate widely. Based on this analysis feed and water samples were not also tested as the hormone sources as testosterone was only used through mixing with water for monosex. After analysis, the presence of estrogen, progesterone and testosterone in all kinds of samples raised a question about the source and method of use of these hormones in fish culture. Another small-scale investigation was performed to identify the source of hormones in water (water, water purifiers, fish and veterinary products used in farming available in Bangladesh) and no clue was linked to the source of estrogen and progesterone. Yet analysis of water, feed, water purifiers or fish and veterinary products used in farming is to be performed.

CRediT authorship contribution statement

Popy Khatun: did the research and MS thesis in this project. **Pritam Saha:** helped PK in sample collection and Formal analysis. **Md Zahorul Islam:** helped in thesis co-Supervision. **Arup Islam:** helped in manuscript drafting. **Md Anwarul Islam:** helped in visualising, conceptualising, Project administration and Funding acquisition of the study. **Purba Islam:** designed, visualized, conceptualized, project administered, Funding acquisition, method Validation, manuscript review to editing and overall Supervision of the whole research.

Declaration of competing interest

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

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.crfs.2024.100709.

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