Residual 17β-Estradiol Levels in Climbing Perch (*Anabas testudineus* (Bloch, 1792)) Fry from Hormonal Sex Reversal Practices in a Small-Scale Thai Farm

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Abstract

Several small farms in Thailand mix 17β-estradiol (E2) with fish feed and rear *Anabas testudineus* (Bloch, 1792) fry for 21–28 d to obtain the female mono-sex. It is important to study the residues of hormones in *A. testudineus* to monitor the hormone residues derived from real farms. This study aims to investigate the residues of E2 in *A. testudineus* fry grown in cement tanks on a small farm in Thailand at 3 distinct times (April, June, and November) and the different of survival, length and weight were investigated. Three tanks were set as the control groups (AC) and 3 tanks were set as hormonal treatment groups (AH). Fish in the ponds were administered the hormone by mixing it into the feed at a rate of 60 mg/kg of feed. The fish samples were collected on the last day of hormone treatments in each month which were on day 21 except in November, the fish samples were collected on day 28. After 24 h of hormone withdrawal, the fish samples were collected to investigate the survival rate, weight, length, and hormone residues. The results showed significant differences in the length and weight of fish between the hormonal treatment group and the control group ($p<0.05$). There were no significant differences ($p>0.05$) between the survival rate of the hormonal treatment group and the control group. The residual hormone levels in fish samples collected on the last days in the AH groups showed high variation among rearing tanks and the hormone levels ranged from undetectable (ND) – 1575 ng/g while the hormone could not be detected in the AC groups. The highest hormone residue in a fish sample was observed in November. After hormone withdrawal for 24 h, the residual hormone could not be detected in all treatments. This research demonstrates that discontinuing the use of sex-inducing hormones at the appropriate time effectively reduces hormone residues in the fish. Appropriate reduction of hormone use in sex reversal not only lowers hormone consumption in agriculture but also minimizes residual accumulation in the environment.

Keywords: 17β-estradiol, *Anabas testudineus*, Small-scale farm, Thailand

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Introduction

Climbing perch (*Anabas testudineus* (Bloch, 1792)) is one of the most important fish in Asia. It can be found in Bangladesh, China, India, Malaysia, Myanmar, Pakistan, Philippines, Sri Lanka, and Thailand (Khatun et al., 2019). In Thailand, the major production of *A. testudineus* comes from aquaculture (Fishery Statistics Group, 2023). The production of *A. testudineus* has increased since 2020 with production in 2022 at 878 tons, which is equivalent to a production value of around 1.8 million US dollars (Fishery Statistics Group, 2023). Thai farmers usually rear *A. testudineus* in ponds and always use female mono sex for *A. testudineus* due to their higher production. The farmers apply fish feed containing 17β-estradiol to induce the female sex in fish fry for 21–28 d. This method is widely used worldwide due to the significant result of fish specification (Jul-a-dung & Komanpririn, 2007). After hormonal withdrawal, the farmers rear the female fish with a regular diet until they harvest the fish product (Jul-a-dung & Komanpririn, 2007). However, regarding the increase in *A. testudineus* production, the requirements of female mono-sex fish fry could require higher amounts of synthetic hormones needed to induce sex. Although mixing hormones in fish feed is practical for inducing fish sex, hormone residues

in fish should be monitored, as the accumulation of hormones in fish could be harmful to fish and human health (Hoga et al., 2018). Several studies have shown that the residues of 17α- methyltestosterone in fry of *Oreochromis niloticus* disappeared after ceasing fish feed containing hormones for 24–48 h (Yenpoeng et al., 2013; Vinarukwong et al., 2018). Moreover, Ruangsri (2020) indicated that the residues of 17β-estradiol in *A. testudineus* reared in circulated water systems in fiberglass tanks can be found after ceasing hormonal treatment for 28 d. However, the hormone residues cannot be detected several months before harvesting. However, those studies focused on laboratory procedures that were conducted by physicians and applied at low fish density. Until now, there has been little data on hormone residues in fish fingerlings produced on farms using different tools and procedures. Our previous study showed that Thai farmers typically use commercial hormones that differ from those used in the laboratory (Pianjing, 2021). The sex-reversal commercial hormones that farmers use contain 17β-estradiol as the major constituent at 97% and the rest are different kinds of sex hormone derivatives (Pianjing, 2021). The farmers rear the fish in ponds or huge cement tanks to produce many fries. They apply them to denser fry which need high amounts of water and fish feed. The utilization of feed-containing hormones could be higher than those studies that applied the procedures in the laboratory. Therefore, the objectives of this study were to investigate 17β-estradiol residues in climbing perch raised under commercial farm conditions that administered the hormone via feed to sexually undifferentiated fry. Subsequently, the effects of the hormone on survival rates and growth performance were evaluated. The data from this study could reflect the result of using hormones in farms and it may be useful for the monitoring policy of the Thai government.

Material and methods

1. Ethical approval

This study was carried out following the guidelines of Suan Sunandha Rajabhat University's Institutional Animal Care and Use Committee and the approval number is IACUC 64-001/2021.

2. Study area and the farm characteristic

The small extensive fishery farm in this study is located in Pathum Thani Province, near Bangkok. This farm produces Climbing perch fingerlings that are sold to farmers for further culturing as juvenile fish. The studied farm uses commercial 17β-estradiol to induce female sex in *A. testudineus* fingerlings. The farm applies the hormonal treatment by mixing the hormone with the commercial fry diet. This farm uses cement tanks to rear fry during the hormonal treatment process.

3. Material, fish fry and chemicals preparation

17β-estradiol (E2) for laboratory analysis was purchased from Sigma Aldrich (Sigma-Aldrich Corp., USA). Hexane was purchased from Honeywell B&J (Honeywell International Inc., USA). Acetonitrile and methanol (MeOH) were purchased from Merck (Merck & Co., Inc., USA). Formic acid was purchased from Themo Fisher Chemical (Thermo Fisher Scientific Inc., USA). Diestylstilbestos (DES) in dioxane or D8 was purchased from Cambridge Isotope Laboratories (Cambridge Isotope Laboratories, Inc., USA). The Commercial E2 for induction of fish's sex was purchased from Aldamex (Aldamex Co., Ltd, Thailand). The commercial 17 β-estradiol (E2) stock solutions were prepared by weighing 0.5 g of hormone and dissolving in 1 L of 95% ethanol. The fish feed contained hormones that were prepared following the recommendation of the standard protocol (Popma & Green, 1990) at a concentration of 60 mg/ 1 kg of fish feed. The prepared hormones were mixed with the fry's fish feed and the feed was kept indoors to evaporate the ethanol. The control groups were fed fish feed containing only 95% ethanol. The fries of *A. testudineus* were purchased from Pathum Thani Aquatic Animal Genetic Research and Development Center, Pathum Thani Province, Department of Fisheries Thailand. The farmer used the fries that were in the free-swimming stage or the absorbed yolk sac stage, which were suitable for hormonal treatment to specify the sex of the fish. The health status of the fish fry was assessed using 2 approaches as follows;

Behavioral observation (daily Routine) by observation of swimming pattern, and feeding response. At the end of each week, the 10–20 fish fries will be caught and put them in a beaker, and observe them closely for physical defects or external parasites.

Water quality monitoring by checking the water quality parameters that will affect fish fries' health including, ammonia, pH, temperature, total dissolve solid and dissolve oxygen.

4. Experimental design

In this study, the hormonal treatment was applied 3 times, in April, June, and November 2021. The selected periods followed the seasonal cycle of Thailand. April is summer and the weather is rather hot. During June, Thailand is in the rainy season, and November is cool. The weather influences the growth of fish fry, particularly the ambient air temperature. In November, which is the cold period, the fish intake less food and the Thai farmers take longer than usual to raise the fry (Jul-a-dung & Komanpririn, 2007; Vinarukwong et al., 2018). The 3 cement tanks in each treatment were set as control groups (AC1, AC2, AC3) and the three cement tanks were set as the treatment groups (AH1, AH2, AH3). The 20,000 fish fries were used for each pond. The fish were fed 4 times daily. In the first week, the fish feed contained hormones and was administered at the rate of 30% of the body weight, and in the second and third weeks, the feed was administered at rates of 20% and 15% of the body weight, respectively based on Ruangsri (2020). In April and June, the hormonal treatments of both fish were administered for 21 d. In November, the hormonal treatment of both fish ended on day 28 due to the fish consuming less feed. The farmer extends the hormonal treatment time routinely during the cool period. During the hormonal treatment, there was no water drainage process. The initial weight and length of the fry were not measured prior to rearing due to their extremely small size. Therefore, we did not intervene in the rearing process, which may cause the death of fish fry. When the hormonal treatment ended, the farmer moved the fish fry to the bigger tanks and reared the fish with a regular diet. Before the new batch of the hormonal treatment began, the water in all tanks was drained into the earth ponds for 30 d and the cement tanks were washed and sun-dried for 2 weeks. After ceasing hormonal treatment, the fish were reared with a regular diet for 1 month. To evaluate the sex of fish subjected to hormonal sex induction, 100 fish were randomly sampled from each experimental pond per rearing batch. The fish were put in containers filled with crushed ice to euthanize them. We randomly selected 10 fish fries of each pond (total was 30 fish fries from control tanks and 30 fish fries from hormonal tanks) to measure weight and length of each batch, then the fish were kept in plastic bags and stored at 4°C. The fish samples were sent to the Pathum Thani Aquatic Animal Genetic Research and Development Center, Pathum Thani Province, Department of Fisheries, Thailand to identify the sex of the fish. The sex characteristics of fish samples were determined using acetocarmine dying and identifying the sex under a microscope by the experts at the center. The fish fries in each tank were collected three times including after the last meal of hormonal treatment and 24 h. after hormonal withdrawal. The fish samples comprised the *A. testudineus* control group (AC) and hormonal treatments (AH). The fries were caught at the same time by a net and 50 g (around 12–16 fish fries) of fish fries in each tank was put in containers filled with ice to euthanize. The fries were weighted and their body length was measured. Then, the fish samples were kept in plastic bags and stored at 20°C until they were analyzed for the hormone residues using the LC-MS/MS at the laboratory of the Bureau of Quality and Safety of Food Department of Medical Sciences, Ministry of Public Health, Thailand.

5. Fish sample preparation

The fish samples were prepared based on Hoga et al. (2018); Kuanha et al. (2018). The fat content in fish tissue was removed twice using liquid/liquid extraction. Briefly, 50 g of fish were thawed at room temperature and pulverized by grinders until the fish sample was ground thoroughly. Then, 2 g of homogenized fish samples were added to the screw cap centrifuge. Next, 10 mL of MeOH was added to the fish samples and shaken for 10 min. Thereafter, the well-mixed fish samples with MeOH were centrifuged at 6000 rpm for 10 min at 4°C. The supernatant that contained MeOH was removed and stored in centrifugal tubes. Then, 5 mL of MeOH was added to the fish samples again and the process was repeated. The MeOH that was collected from two extractions was added to 10 mL of hexane saturated with acetonitrile and centrifuged at 6000 rpm for 10 min at 4°C. The hexane saturated with acetonitrile was discarded and the remaining MeOH was removed to the new tube and dried under the nitrogen evaporator (11220-A, Organomation Associates, Inc., USA) until the volume of MeOH remained at 50 µL. Then, 5 mL of deionized water was added and mixed with a vortex mixer.

6. 17β-estradiol analysis

Analysis of 17β-estradiol was performed based on Hoga et al. (2018); Kuanha et al. (2018). The solid phase extraction (SPE) cartridges were preactivated and conditioned with 5 mL of MeOH and 10 mL of deionized water respectively. The extracted MeOH was added to the prepared SPE cartridges. SPE cartridges were eluted with 10 mL of acetonitrile and the solvent of collected fractions was dried using a nitrogen evaporator (11220-A, Organomation Associates, Inc., USA). The remaining solution in the tubes was dissolved using 1 mL of 0.1 formic acid: MeOH (65:35, v/v). The extractions derived in this process were 1 g/mL of concentrated extractions. The concentrated extractions were percolated using a PVDF 0.45 µm micro-spin filter and stored in amber bottles. The E2 residue in extractions of fish

samples was analyzed by LC-MS/MS (Xevo TQ-XS ACQUITY UPLC I-Class, Agilent, USA). The LC-MS/MS was prepared using a column zorbex SB C-18 and the applied mobile phase was the mixing of H2O: MeOH: acetonitrile (3:1; v/v).

7. Quality control

Quality control of samples and the analytical process was conducted by spiking blank fish samples (prepared from healthy *A. testudineus* with determined levels of hormone residues by LC-MS/MS) with standard hormones at concentrations of 2.0, 6.0, 10.0, and 15.0 µg/kg and adding the internal standard (DES or D8) at concentration 6.0 µg/kg. After that, the blank fish sample containing standard hormone and internal standard was analyzed in 10 replications. The results were evaluated for the recovery percentage and relative standard deviation (% RSD). The obtained recovery percentage and % RSD were acceptable as shown in Table 1. Limits of detection (LOD) and limits of quantification (LOQs) were determined based on Kuanha et al. (2018). The LOD and LOQ values were 1 µg/kg and 2 µg/kg respectively and the linearity working range was 2.1–100 µg/kg which showed a correlation coefficient of 0.9919–0.9996.

Table 1 The recovery percentages of spiked hormones in blank samples

Hormones	Spike concentration (ng/g)	Found (ng/g)	RSD (%)	% Recovery
17β -estradiol	10.00	11.51±1.02	1.06	115.10

8. Water quality measurement

The water parameters including, pH, total dissolved solids (TDS), electrical conductivity (EC), dissolved oxygen (DO), and temperature were measured in situ once per week by a portable water measurement YSI 600 (YSI Incorporated, USA). The obtained data on water parameters in rearing tanks were measured in the control group (AC) and the hormonal treatment group (AH). The water in each tank was brought once per week to analyze the ammonia (NH3) levels at the Environmental Center of Suan Dusit University laboratory in Bangkok, Thailand. However, our previous study reported that the accumulation of E2 in the water of all rearing tanks was undetectable (Deki et al., 2023). Therefore, the relationship between hormone levels in water and the residues of hormones in fish was not considered.

9. Data representation and Statistical analysis

The obtained data were expressed as the average ± standard deviation. The obtained data were summarized as tank-level means ± standard deviation, because tanks represented the true experimental units in this study. Individual fish within a tank were considered subsamples and were not treated as independent observations. Therefore, statistical comparisons of survival rate, mean length, and mean weight between the control (AC) and hormonal treatment (AH) groups were performed using one-way ANOVA, with treatment as the fixed factor (n = 3 tanks/group). Significant differences in the data between the survival rate, length, and weight of the hormonal and control groups were tested by Nest ANOVA. Prior to analysis, the assumptions of normality and homogeneity of variance were tested. The normality of residuals was assessed using the Shapiro–Wilk test, and homogeneity of variance was evaluated using Levene's test. Statistical analysis was performed by using R program (Version 4.3, The R foundation for Statistical Computing, Austria)**.** All assumptions were met. Statistical significance was accepted at $p<0.05$.

Results and discussion

1. The water quality index

The parameters of water in the fish rearing tanks (3 control tanks and 3 hormonal tanks) that were measured 3 times during April, June, and November are shown in Table 2. The ammonia level in all cement tanks ranged from 1.03±0.01 to 1.21±0.01 mg/L, which did not exceed the standard level for aquaculture.

Table 2 The water quality of the fish-rearing tanks

Treatment	NH_3 (mg/L)	pH	TDS (mg/L)	Temp (°C)	EC (ms/cm)	DO (mg/L)
April						
AC	1.20±0.04	8.51±0.01	0.49±0.02	26.66±0.71	0.78±0.02	7.58±1.29
AH	1.21±0.01	8.65±0.03	0.48±0.01	27.08±0.10	0.77±0.01	7.92±0.30
June						
AC	1.03±0.01	8.45±0.05	0.64±0.03	29.48±0.71	1.05±0.05	5.44±0.11
AH	1.05±0.03	8.43±0.01	0.62±0.04	29.42±0.11	1.03±0.07	5.05±0.41
November						
AC	1.16±0.01	8.53±0.01	0.54±0.03	24.81±0.15	0.86±0.03	6.43±0.10
AO	1.19±0.03	8.52±0.03	0.55±0.04	26.20±0.17	0.98±0.02	5.54±0.32

Remark: All data were represented as average ± SD, n = 3

2. Growth and survival rate

In this study, the length and weight of the fish were measured after the hormonal treatment ended. The 30 fish were selected from the rearing tanks in the same time to measure weight and length. The weights and lengths of *A. testudineus* are shown in Fig 1 and Table 3. The growth of fish fry was acceptable which similar to a study of Jul-a-dung & Komanpririn (2007). There were significant differences in the length and weight of fish between the hormonal treatment group and the control group ($p<0.05$).

Fig. 1 The *A. testudineus* fries, the left panel represents the fries obtaining from control tanks and the right panel represents the fries obtaining from hormonal treatment tanks

Table 3 The average survival rate, length, and weight of *A. testudineus*

Treatment	Survival rate (%)	Length (cm)	Weight (g)
April			
AC	21.66±1.42[a]	3.23±1.12[a]	3.43±0.92[a]
AH	22.55±1.44[a]	3.80±0.30[b]	4.10±0.17[b]
June			
AC	20.83±1.41[a]	3.30±0.82[a]	3.50±0.70[a]
AH	22.50±2.50[a]	3.83±0.55[b]	4.16±0.20[b]
November			
AC	16.66±1.40[a]	3.30±0.70[a]	3.33±0.68[a]
AH	21.25±3.17[a]	4.00±0.20[b]	4.20±0.26[b]

Remark: All data were represented as average ± SD, n for measuring weight and length was 10 fish fries/tank. Different letters within the same column indicate statistically significant differences ($p<0.05$)

The obtained data of this study showed that the lengths and weights of the fish in the hormonal treatment group were greater than those of the control group indicating that E2 can induce the female fish fry and the bigger size of the fish was observed. However, compared to a study by Ruangsri (2020)

the previous study exhibited better growth and survival rates. After culturing the fish fry for 21 d, the weights of *A. testudineus* from Ruangsri (2020) study ranged from 4.44±0.43–5.02±0.06 g and the length of the fish ranged from 5.28±0.08–6.16±0.01 cm.

3. Sex ratio

After the hormonal treatment was completed for each batch of fish rearing, the fish were caught and sent to identify their sexes. The oocyte and spermatocyte of *A. testudineus* are shown in Fig 2. This study revealed that *A. testudineus* showed a high percentage of female fish as shown in table 4. The control groups showed a variation of the ratio of fish sex. In this study, the survival rates of *A. testudineus* were notably low, necessitating the stocking of 20,000 fry to compensate for losses. This contrasts with findings by Ruangsri (2020), who reported significantly higher survival rates ranging from 80.89%±1.83% to 82.38%±0.38%. Observations from the farm suggest that cannibalism was a primary contributing factor; specifically, size disparities likely resulted in smaller *A. testudineus* fry becoming prey for larger individuals. This hypothesis aligns with Mustakim (2020), who characterized wild *A. testudineus* in lake and swamp habitats as carnivorous. Therefore, the low survival rate observed in this study may be attributed to aggressive behavioral traits and predation among the fry. However, it is important to note that there were no statistically significant differences in survival rates between the hormonal treatment groups and the control group ($p>0.05$). In Thailand, the administration of hormones via feed is recognized as an effective method for sex reversal in fish (Jul-a-dung & Komanpririn, 2007; Ruangsri, 2020). In the November trial, a 100% female population was observed in the hormonal treatment group. These results suggest that prolonged dietary exposure to the hormone is highly effective for inducing female mono-sex populations.

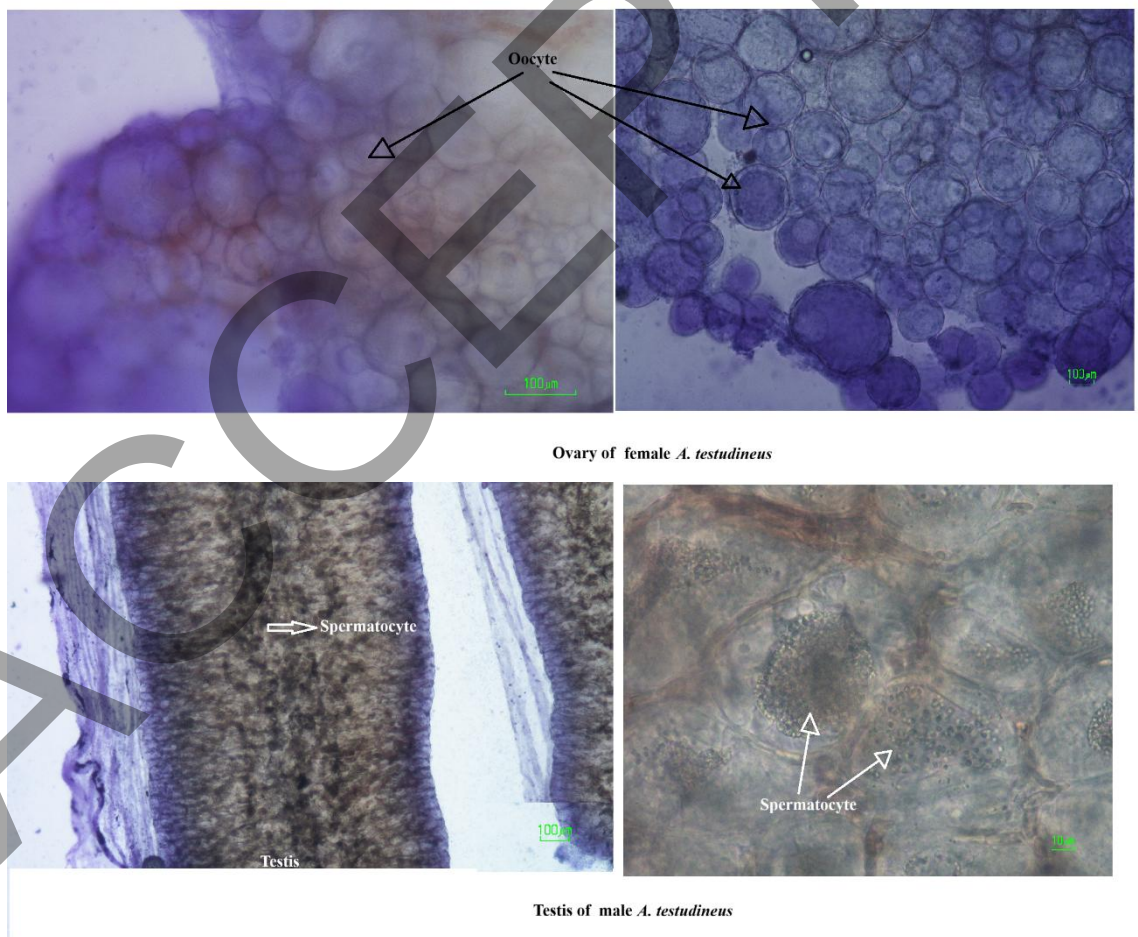

Fig. 2 The reproductive feature of *A. testudineus*. The upper panel is the reproductive organ of female fish and the lower panel is reproductive organ of male fish

Table 4 The sex ratio of control groups and hormonal treatment groups of A. *testudineus*

Treatments	Sex	
	Male (%)	Female (%)
April		
AC	10.00±1.00	90.00±1.00
AH	4.00±1.00	96.00±1.00
June		
AC	14.66±1.53	85.33±1.52
AH	3.66±1.16	95.67±1.15
November		
AC	23.33±1.53	76.67±1.52
AH	00.00±0.00	100.00±0.00

4. Hormone residues in fish

The results of the hormone residues in fish are shown in Table 5. The residues of E2 were not found in the control groups. The values of E2 found in *A. testudineus* on the last day of hormonal feeding in samples collected from the three rearing tanks ranged from undetectable (ND) to 1575.0 ng/g. There were large variations among the obtained hormone levels in each fish sample. The hormone residues in fish in the rearing tanks in the same cultured period revealed high variation.

The highest residue values of E2 were found in the fish sample collected in November. In this month, the rearing period of climbing perch was 28 d, which was longer than the others. In November, the low water temperature as shown in Table 2 affected the feed consumption of the fish. The farmer indicated that climbing perch consumes less feed during the cold period. This phenomenon can affect the growth and survival rates of fish. Therefore, the farmer extended the hormonal treatment period to ensure the maximum number of female *A. testudineus* fry. Therefore, climbing perch fry reared in November consumed fish feed containing hormones longer than other batches which may lead to higher hormone residues in fish tissue. However, there were no observed hormone residues in *A. testudineus* samples after ceasing the feed containing hormones for 24 h.

The level of hormone residue in some of the fish samples could not be detected while it was observed in some of the fish samples in the hormonal treatment tanks. In this study, we applied the rearing pattern following the farmer's practice. The *A. testudineus* farming in this study used the larger rearing tanks and raised the fish in the open air compared to several studies that reared fish in laboratory units and used lower fish density. Therefore, some factors such as the weather each day, and feeding may affect the obtained hormone residue data.

However, the elevated hormone residues observed in fish collected in November may be a reflection of the lower survival rate. High hormone levels are known to adversely affect the survival and growth of fish (Ruangsri, 2020). The relatively low survival rate of *A. testudineus* fry in this study appears to be related to the high levels of hormone residues found in the samples. Specifically, the surviving *A. testudineus* fry likely consumed more feed due to the reduced stocking density and lower competition in the rearing tanks, resulting in increased hormonal intake. Consequently, the remaining fish faced a higher risk of exposure to hormones from both the water and the feed. Moreover, hormone residues in the environment and biological tissues exhibit bioaccumulation, a process where substances accumulate in organisms at higher trophic levels (Liu et al., 2024). Therefore, if surviving fish are exposed to increased hormone levels via their diet and environment, it will likely result in greater accumulation in the tissues of the climbing perch.

The results of this study differ from those reported by Vinarukwong et al. (2018), who found that mestanolone residues in tilapia ranged from 0.856 to 3.198 ng/g at 24, 48, and 72 h post-withdrawal, becoming undetectable after 5 d. However, these discrepancies may be attributed to methodological differences; Vinarukwong et al. (2018) administered mestanolone via an immersion technique at 80 mg/kg for 15 consecutive days, whereas the current study utilized hormone-treated feed administered for at least 21 to 28 d. Although immersion techniques offer the advantage of a shorter treatment duration, they are generally less favored by farmers as they often require higher quantities of hormones compared to oral administration. Furthermore, farmers have indicated that immersion is less practical for on-farm operations and likely increases hatchery unit costs. Moreover, the administration of hormones for sex

induction should be performed under strict safety guidelines and supervised by veterinarians or aquatic animal health specialists with expertise in appropriate dosage and timing. This professional oversight is crucial to help farmers mitigate adverse impacts on both the fish and the environment (Khatun et al., 2024).

The absence of detectable hormone residues in fish samples after hormone withdrawal may be attributed to physiological clearance mechanisms, as fish excrete excess hormones to maintain homeostasis (Hoga et al., 2018; Ruangsri, 2020). While residues in this study appeared higher than those reported by Ruangsri (2020), this discrepancy is likely due to differences in sampling timelines. Ruangsri (2020) observed E2 residues ranging from 3.9 to 7.7 ng/kg in *A. testudineus* fry after a 28-d withdrawal period. Because that study did not investigate residues at the 24-h which could show lower levels of hormone residues in *A. testudineus* when compared to this research. However, Ruangsri (2020) did not investigate the hormone in fish after 24 h of withdrawal, resulting in the observed lower hormone residues when compared to this study. The lower levels reported by Ruangsri (2020) reflect the extended withdrawal period rather than a fundamental difference in uptake.

The application of hormones for sex induction in fish production presents both advantages and disadvantages. On one hand, hormone-induced monosex culture significantly enhances production efficiency, as the selected sex typically exhibits superior growth and feed conversion rates. On the other hand, hormonal induction can negatively impact fish health and reproductive fertility. Furthermore, although hormones are rapidly eliminated from fish tissue, the excreted residues enter the aquatic environment, posing potential risks to the ecosystem (Pandian & Sheela, 1995; Hoga et al., 2018).

Discontinuing hormone administration at the appropriate time is a necessary procedure to minimize hormone contamination in the fish. Continued exposure to hormones up to the harvest stage will result in hormone residues accumulating in the fish tissue. A study by Khatun et al. (2024) reported that Rui (*Labeo rohita*), Catla (*Catla catla*), and Tilapia (*Oreochromis niloticus*) samples collected from farms and markets were found to be contaminated with testosterone range from 2.1 to 16.96 µg/kg and progesterone range from 31.47 to 731.57 µg/kg respectively. Furthermore, the presence of more than one type of hormone was detected within the same fish species. Hormone contamination in fish sold in markets or harvested from farms reflects farm management practices, particularly the failure to adhere to appropriate withdrawal periods for sex-determining hormones. The presence of hormone residues in market fish highlights the potential of the hormone contaminated in environment particularly in water column and sediment.

Table 5 The residues of E2 in *A. testudineus* on the last day of hormonal treatment and after ceasing hormonal treatment for 24 h

Treatments	**Residue of 17β-estradiol (ng/g)**					
	Last day of hormonal treatment			**24 h after hormone withdrawal**		
	April	**June**	**November**	**April**	**June**	**November**
AC1	ND	ND	ND	ND	ND	ND
AC2	ND	ND	ND	ND	ND	ND
AC3	ND	ND	ND	ND	ND	ND
AH1	ND	424.32	ND	ND	ND	ND
AH2	ND	ND	27.65	ND	ND	ND
AH3	233.26	ND	1575.00	ND	ND	ND

Remark: ND means undetectable (<LOQ), The LOD and LOQ values were 1 µg/kg and 2 µg/kg respectively. The residual hormone in each pond derived from 50 g of fish samples which approximately contained 12–16 fish fries per sample

Conclusion

The results of this study indicate that using fish feed containing E2 for the female mono-sex induction of *A. testudineus* is a viable approach for Thai farmers. Although high residues of E2 were detected in fish on the final day of hormonal treatment, these residues were undetectable after 24 h of hormone withdrawal. These findings demonstrate that adhering to an appropriate withdrawal period significantly minimizes residual accumulation in fish tissue. Therefore, if the fish are subsequently reared on a hormone-free diet, residue levels at the time of consumption are likely to be negligible. However,

despite the undetectable hormone levels reported in the water during this study, monitoring residues in water and sediment receiving effluent from fish farms is recommended, as previous studies have documented the accumulation of steroid hormones in water sources near agricultural operations. Furthermore, continued research into the ecological and human health risks associated with hormone exposure via the food chain is essential for the effective surveillance of endocrine-disrupting chemicals (EDCs).

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Conflicts of 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.

Declaration of generative artificial intelligence in the writing process

During the preparation of this manuscript, the authors used Google Gemini for enhancing language clarity and improving grammar. The final content was reviewed and edited by the authors, who take full responsibility for the accuracy and integrity of the publication.

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