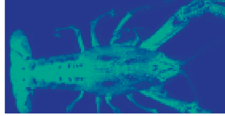
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Effects of a nonsteroidal aromatase inhibitor on gonadal differentiation of bluegill sunfish *Lepomis macrochirus*

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Abstract

In the present study, the efficacy of Letrozole, a potent nonsteroidal aromatase inhibitor (AI), on gonadal sex differentiation and sex reversal was examined in bluegill sunfish (*Lepomis macrochirus*). In Experiment 1, using AI diet treatments (50, 150, 250 and 500 mg kg^{-1}) from 30 to 90 days posthatch (dph), AI interrupted ovarian cavity formation at a dose of 500 mg kg^{1} diet and one intersex fish was identified in this group. The proportions of males in all the treated groups were significantly higher than those in the control group. In Experiment 2, using AI immersion treatments (250, 500 and 1000 μg L^{-1}) during 30–50 dph, the treated groups of 500 and 1000 μg L^{-1} produced significantly more males than the control and 250 μg L^{-1} groups. Histological examination revealed no differences in ovary or testis tissue between control and AI-treated fish. There were no significant differences detected in body weight and length among the AI treated and control groups ($P > 0.05$) for both experiments. The results from these two experiments suggest that inhibition of aromatase activity by AI could influence sex differentiation in bluegill sunfish.

Keywords: letrozole, aromatase inhibitor, masculinization, sex differentiation, *Lepomis macrochirus*

Introduction

It has been demonstrated in a variety of fish species that phenotypic sex differentiation can be influenced by the administration of exogenous oestrogens or androgens during sex differentiation, transforming genetically male or female individuals into the opposite gender (Pandian & Sheela 1995; Nakamura, Kobayashi, Chang & Nagahama 1998; Strüssmann & Nakamura 2002). Yamamoto (1969) postulated that oestrogens act as female inducers and androgens function as male inducers. The hormonal balance between oestrogens and androgens appears to be crucial in the process of sexual differentiation in developing gonads. This balance relies on the availability and activity of the steroid-synthesizing enzymes, and in particular on the cytochrome P450 aromatase complex (P450arom). The importance of P450arom in gonadal differentiation of fish has been demonstrated in several studies that used treatments of aromatase inhibitors (AIs) (Kwon, Haghpanah, Kogson-Hurtado, McAndrew, Penman 2000; Afonso, Wassermann & Oloveira 2001). These studies demonstrated that AIs can inhibit the aromatase enzyme activity by catalysing the biosynthesis of oestradiol-17β (E_2) from its precursor testosterone, and can result in reduced oestrogen production (Steele, Mellor, Sawyer, Wasvary & Browne 1987).

In the earlier experiments, different aromatase inhibitors failed to masculinize gonads, disrupted ovarian differentiation or induced only slight gonadal masculinization (Pieau, Girondot, Desvages, Dorizzi, Richard-Mercier & Zaborski 1994). However, more conclusive results have been obtained with two nonsteroidal aromatase inhibitors: Fadrozole (CGS 16949) and Letrozole (CGS 20267). Treatments with

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these compounds have successfully masculinized many nonmammalian animals including teleost fish, such as chinook salmon *Oncorhynchus tshawytscha* (Piferrer, Zanuy, Carrillo, Solar, Devlin & Donaldson 1994), Nile tilapia *Oreochromis niloticus* (Kwon *et al.* 2000), zebrafish *Danio rerio* (Fenske & Segner 2004) and golden rabbitfish *Siganus guttatus* (Komatsu, Nakamura & Nakamura 2006). Moreover, Letrozole was reported to be more potent than Fadrozole in masculinizing gonads in turtles (Dorizzi, Richard-Mercier, Desvages, Girondor & Pieau 1994). As for its clinical use, Letrozole is capable of inhibiting 98–99% of aromatase activity and reducing serum concentrations of oestrone and E_2 to below the limit of detection in human patients (Smith 1999). Letrozole has been demonstrated to induce sex inversion in the protogynous red spotted grouper *Epinephelus akaara* (Li, Liu & Lin 2005) and inhibit oocyte growth of Japanese medaka *Oryzias latipes* (Sun, Zha, Spear &Wang 2007).

The bluegill sunfish *Lepomis macrochirus* is currently recognized as one of the most valuable North American recreational fish. Moreover, it has become an economically important and high-value aquaculture species in several states in the United States and in other countries (Ehlinger 1989). Studies have shown that bluegill possess the inherent capacity to grow to food market sizes substantially faster than hybrid bluegill (Hayward & Wang 2002), and male bluegill, in particular, appear to hold the greatest potential for the food market due to their more rapid growth capacity relative to females (Hayward & Wang 2006). These findings suggest that monosex male culture holds considerable potential as a method to increase the efficiency and profitability of bluegill aquaculture by improving growth rates. Among the reports on masculinization in bluegill sunfish, initial attempts by oral administration of 17α-methyltestosterone (MT) were unsuccessful (Chew & Stanley 1973). Al-Ablani (1997) reported that 93% males were produced by a 1000 μg L^{-1} trenbolone acetate (TBA) immersion treatment. Al-Ablani and Phelps (2002) found a predominance of intersex fish and a reduction in the survival of both males and females in the oral administration treatments of TBA and MT. Predominant male populations were produced by MT immersions with dimethyl sulphoxide as a penetration enhancer (Arslan & Phelps 2003). These studies on the masculinization of the bluegill sunfish were all focused on the effects of sex steroids.

In the present study, the efficacy of a potent non-steroidal aromatase inhibitor (Letrozole) to masculinize bluegill sunfish was examined by administering the compound in the diet and immersion at different dosages during the critical period of sexual differentiation. The objectives were to determine (1) whether inhibition of aromatase activity by AI could influence sex differentiation of bluegill sunfish and (2) whether it could be used as an alternative method to produce monosex male populations in bluegill sunfish.

Materials and methods

Fry production

Eight male and four female bluegill broodfish were selected from the Ohio State University South Center's Aquaculture Wet Lab. They were stocked in two tanks at a ratio of two females to four males per tank with flow-through well water. Two plastic bowls ($R = 15$ cm, depth $= 10$ cm), containing a small amount of gravel, were placed in each tank as spawning nests. The artificial nests were lifted out of the tank and checked for eggs each morning. Nests with eggs were placed at the bottom of aerated 400 L tanks with flow-through well water for incubation at 24–26 °C for 30–36 h. The newly hatched larvae were reared in the 400-L round tanks with flow-through well water at 24–26 °C for 4 weeks before the experiment. Before Letrozole treatment, fry were gradually weaned from brine shrimp to a dry diet (larval AP micro-feed).

AI administration

In Experiment 1, the desired quantities of Letrozole were dissolved in 400 mL 95% ethanol kg^{-1} feed, and then thoroughly mixed with commercial fish feed using an electric mixer to achieve final concentrations of 50, 150, 250 and 500 mg kg^{-1}. For the control, 95% ethanol without the AI was mixed with the feed. The feed was dried overnight under a fume hood to allow the ethanol to evaporate before use. When the fry were 30 days posthatch (dph), with 0.93 ± 0.08 cm mean total length and 0.23 ± 0.06 mg mean body weight, 120 fish were randomly assigned to each of 25 L round tanks. The control plus four treatment groups led to a total of five groups, each having three replicates. The fry used in all the treatments (50, 150, 250 and 500 mg kg^{-1} AI) and controls were from the same batch. The fry in all groups received their ration of AI feed five times a day and mortality was monitored daily in each experimental group from 30 to 90 dph. After completion of AI administration, the fish were

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fed four times daily with a normal commercial diet. Twenty fish were sampled from each tank for body weight and total length at 90 and 150 dph. All fish were sacrificed at 210 dph for gonad samples and measured to examine the growth of fish after AI diet treatments.

For Experiment 2, the same batch of fry for Experiment 1 was used and immersions were provided to the fry in their rearing tanks (25 L). The AI was dissolved in 95% ethanol to preparte appropriate stock solutions. Before the addition of appropriate amount of AI, water flow to the tanks was turned off and the water level was reduced to 10 L. The various AI stock solutions were poured into tanks to prepare concentrations of 250, 500 and 1000 $\mu g\,L^{-1}$. The same amount of ethanol was added to the control group. From 30 to 50 dph, fry were immersed in each AI solution on five occasions for 8 h a day at 5-day intervals between immersion treatments. At the end of each immersion period, water flow was turned back on. The fish were fed five times daily with a normal commercial diet. Twenty fish were sampled from each tank for body weight and total length at 50 dph. After completion of AI immersion, the fish were fed four times daily with a normal commercial diet until sacrificed for gonad samples at 210 dph. The body weight and total length of sacrificed fish were also measured.

During these two experiments, the daily mean ($\pm$ SD) water temperature and dissolved oxygen concentrations were 24.1 $\pm$ 2.4 °C and 6.6 $\pm$ 0.7 $mg\,L^{-1}$, respectively, and the water flow rate was approximately 0.5 $L\,min^{-1}$.

Evaluation of gonadal development

In the present study, histological examination of the gonads was performed using the criteria described for gonadal sex differentiation in bluegill sunfish (Gao, Wang, Rapp, O'Bryant, Wallat, Wang, Yao, Tiu & MacDonald 2009). At the beginning of AI treatments (30 dph), six fish were randomly sampled and examined to ensure that the gonads were sexually undifferentiated.

In Experiment 1, twelve fish from each group were randomly sampled and examined on 90 dph. At 210 dph, fish gonads ($n = 20–31$) from each group were dissected and sexed through microscopic examination of gonadal tissue using the gonadal squash method (Guerrero & Shelton 1974). In Experiment 2, six fish from each group were randomly sampled and examined on 50 dph. At 210 dph, fish gonads ($n = 22–27$) from each group were dissected and sexed through microscopic examination of gonadal tissue using the gonadal squash method (Guerrero *et al.* 1974). In addition, for both experiments, a single gonad from each fish was submitted for histological analysis to confirm the results of the squash method. For each fish, at least five cross sections (4–6 μm) covering different portions of the gonad were cut using a Reichert–Jung 820-II microtome, stained with Mayer's haematoxylin and eosin phloxine B solution following the routine procedure for light microscopy and then examined and microphotographed to determine their phenotypic sex.

Data analysis

Differences in the mean responses among treatments in survival and growth, and alteration in the sex ratios and ratios of gonads with or without the ovarian cavity were determined with one-way ANOVA using SAS program (SAS Institute, 1988, Carry, NC, USA).

Results

Experiment 1

Based on the histological examination conducted at the beginning of the treatment, the gonads were sexually undifferentiated and consisted of sporadically distributed germ cells, a surrounding sex cord-like structure and outer somatic cells (Fig. 1). The ratios of gonads with or without the complete ovarian cavity after the completion of the AI diet treatment (90 dph) are shown in Fig. 2. Fifty-eight per cent of all control fish possessed ovarian gonads with complete ovarian cavities (OC, ovarian cavity; Fig. 3a) while others showed testes structures (Fig. 3b). The percentages of gonads that possessed the ovarian cavity were 58%, 50%, 50% and 42% in the 50, 150, 250 and 500 $mg\,kg^{-1}$ AI diet groups respectively. Some gonads in the AI diet groups showed no trace of ovarian cavity formation in the ovary (Fig. 3c) and some gonads showed testes structures (Fig. 3d). In contrast, the 500 $mg\,kg^{-1}$ AI diet group showed a higher ratio of gonads with no evidence of ovarian cavity formation compared with the control group ($P < 0.05$) and one intersex gonad showing oocytes in the middle of testicular tissue was also identified in this group (Fig. 3d).

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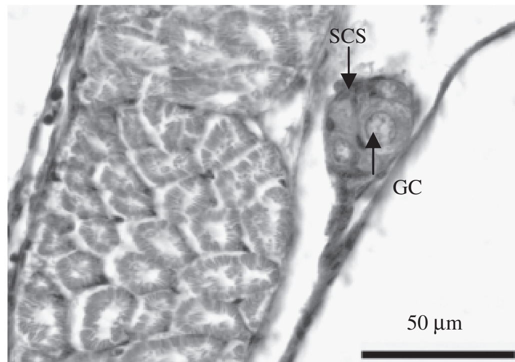

Figure 1 Undifferentiated gonad of a juvenile bluegill sunfish immediately before the onset of AI administration. GC, germ cell; SCS, sex cord-like structure.

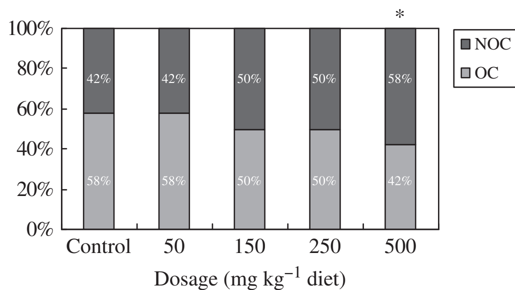

Figure 2 Ratios of gonads with no ovarian cavity (NOC) and ovarian cavity (OC) in the bluegill sunfish at 90 dph in Experiment 1 with various AI diet treatments (mg kg $^{-1}$ dosage). *Indicates a significant deviation towards the NOC gonads or testis compared with the control group ($P<0.05$).

At 210 dph, the proportion of males in all AI diet treatment groups increased significantly when compared with the control group (Table 1). The proportion of males increased as AI diet dosages increased and the 500 mg kg $^{-1}$ AI diet treatment had the highest proportion (70%) of males. All the ovaries from the AI diet-treated groups were histologically similar to those of the control group (Fig. 4a and b). All the testes in the AI diet-treated groups were similar to those in the control group (Fig. 4c and d).

The survival rates in all the experimental groups were between 40% and 45% and no significant differences in survival ($P>0.05$) were detected among groups. No signs of toxicity or behavioural differences between treatment groups and control fish were observed during and after the treatment. The mean body weights and total lengths of the experimental fish are presented in Table 2. There were no significant differences among the AI-treated and control groups at the terminal day of AI diet treatment ($P>0.05$). After AI treatment, the body weight and total length still did not exhibit significant differences among the AI-treated and control groups ($P>0.05$) at 210 dph.

Experiment 2

Based on the histological examination conducted at the beginning of the treatment, the gonads of the Experiment 2 fish group were also sexually undifferentiated at the beginning of the AI immersion treatments. After AI immersion treatments at 50 dph, there were some gonads with initial ovarian cavity formation indicated by the presence of two elongated aggregations of somatic cells in the proximal and distal portions of the gonads. The sex ratios at 210 dph in each experimental group are shown in Table 3. Although the male proportion was 41% and 44% in the control and 250 μg L $^{-1}$ in the AI immersion groups, respectively, the gonads from the 500 and 1000 μg L $^{-1}$ AI immersion groups showed a significant bias towards male gonads. All the ovaries and testes from the AI immersion groups were histologically indistinguishable from those of the control group.

The survival rates in all experimental groups were between 40% and 50%, and a relationship between administration duration and survival was not observed. The mean body weight and total length of the experimental fish are presented in Table 4. At the terminal day of AI immersion treatment (50 dph), there were no significant differences among the AI-treated and control groups ($P>0.05$). After AI treatment, the body weight and total length still did not exhibit significant differences among the AI-treated and the control groups at 210 dph ($P>0.05$).

Discussion

The results of the present study demonstrated that the aromatase inhibitor Letrozole induced masculinization in the bluegill sunfish through either diet or immersion administration during the sexual differentiation period. The sensitivity of gonadal differentiation to the influence of AI increased with the increasing dosages, suggesting that Letrozole treatment efficacy was dose dependent. In the present study, although 100% masculinization through the AI treatments was not induced, the male proportion significantly increased when compared with the con-

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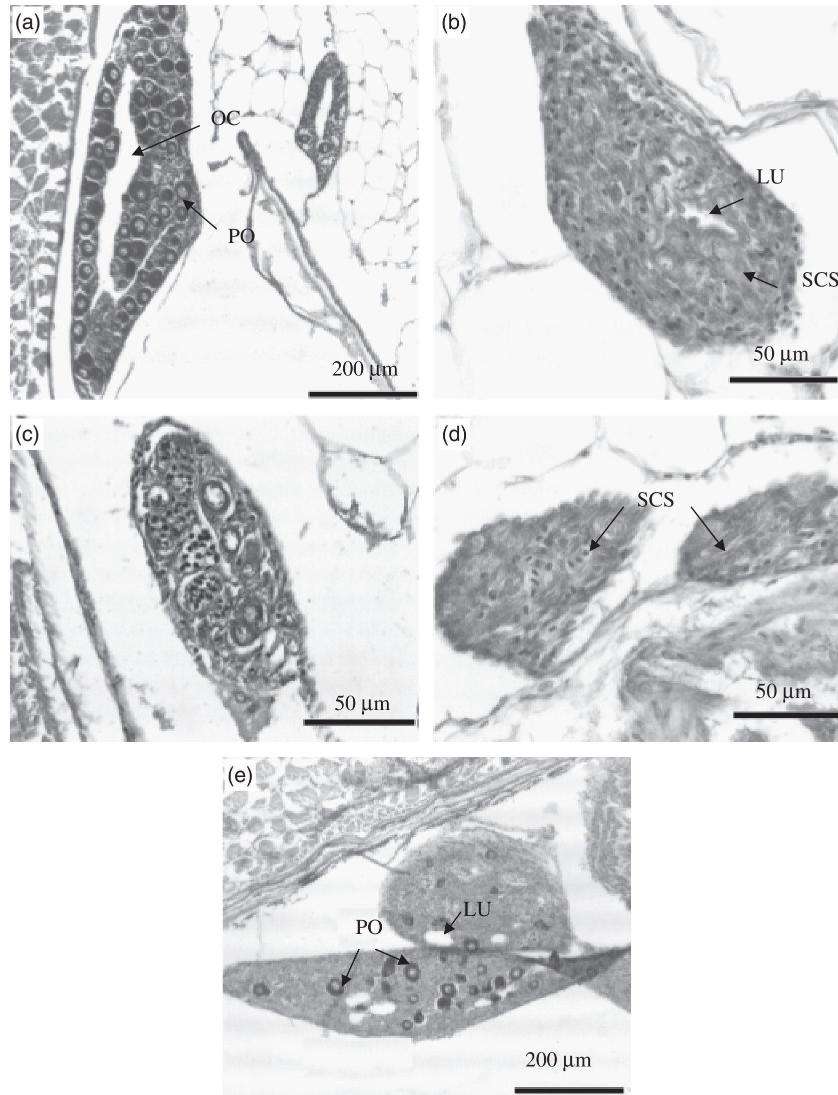

Figure 3 Histological structures of the bluegill sunfish gonads at 90 dph in Experiment 1. (a) Ovary from the control group showing the complete ovarian cavity (OC) and peri-nucleolus oocytes (PO); (b) Testis from the control group showing Lumen (LU); (c) Ovary without an ovarian cavity from the AI diet (250 mg kg $^{-1}$)-treated group; (d) Testis from the AI diet (500 mg kg $^{-1}$)-treated group; (e) Ovotestis from the AI diet (500 mg kg $^{-1}$)-treated group, showing peri-nucleous oocytes in the middle of testicular tissue. LU, Lumen; OC, ovarian cavity; PO, peri-nucleolus oocytes; SCS, sex cord-like structures.

trol group, even at the lowest diet dose (50 mg kg $^{-1}$). Piferrer (2001) indicated that the most important factors to be considered for inducing sex reversal are the onset time of treatment, the duration, the drug, and the dose used. Based on our previous studies on bluegill sunfish gonadal sex differentiation, we believe that the critical period of sex determination occurs between 1.32 and 1.60 cm total length (between ~ 30 and ~ 60 dph) when sexually undifferentiated gonads are more responsive to the action of exogenous steroids (Gao *et al.* 2009). The timing of AI treatments in the present study was matched to this sex differentiation period. Therefore, our results showed that the dosages used in this study may not be effective enough to induce 100% masculinization.

In AI diet treatment groups observed immediately at the conclusion of the AI administration period (90 dph), the numbers of gonads with complete ovarian cavities in the 500 mg kg $^{-1}$ Letrozole diet group were significantly less than in the control group.

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Table 1 Sex ratios of the bluegill sunfish fed diets containing different dosages of AI from 30 to 90 dph

AI dosage (mg kg^{-1})	*N*	Males (%)	Females (%)	*P*-value
Control	31	39[a]	61	0.028
50	29	59[b]	41	0.072
150	20	65[b]	35	0.003
250	20	65[b]	35	0.003
500	26	70[b]	30	0.002

The different superscript letters indicate significant (χ^2-test, $P<0.05$) differences in proportion of males among the groups. *P*-values indicate differences from the theoretical 50:50 sex ratio (χ^2-test).

Moreover, a testis with some oocytes was also observed in the same diet group, giving it the appearance of having developed from an ovary. These results suggest that the AI inhibits or retards ovarian development. Afonso *et al.* (2001) also found a similar intersex gonad in the Nile tilapia *O. niloticus* that received 50 mg kg^{-1} AI for 30 days. Komatsu *et al.* (2006) found that a testis from the golden rabbitfish *S. guttatus* that was fed a 500 mg kg^{-1} AI diet for 30 days had indentations on both lateral sides, giving it the appearance of having developed from a gonad with an incomplete ovarian cavity. This diet treat-

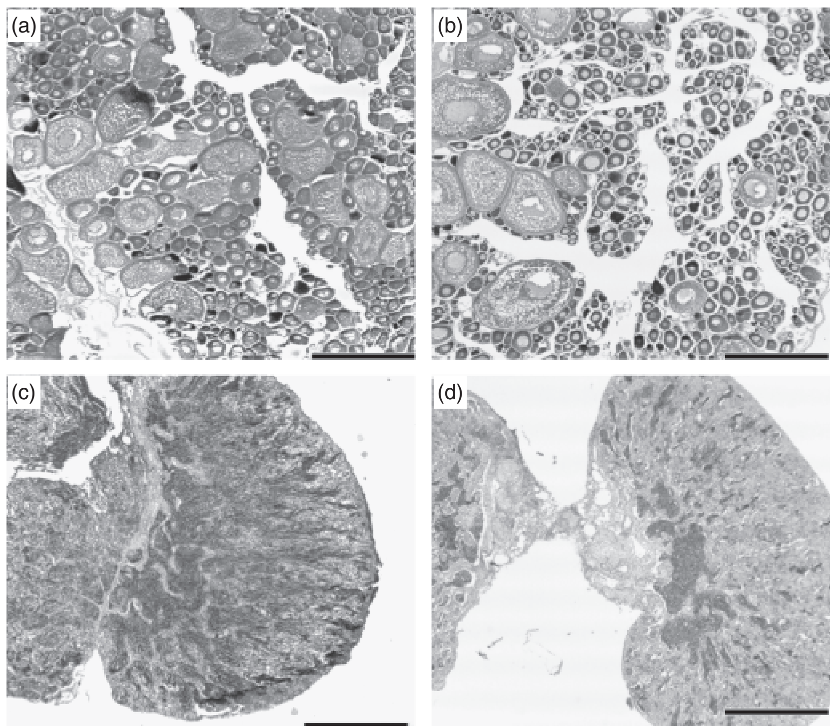

Figure 4 Histological structures of the bluegill sunfish gonads at 210 dph in Experiment 1. (a) Ovary from the control group; (b) Ovary from the AI diet (500 mg kg^{-1})-treated group; (c) Testis from the control group; (d) Testis from the AI diet (500 mg kg^{-1})-treated group. Scale bar = 500 μm.

Table 2 Effects of feeding diets containing different dosages of AI from on growth of bluegill

	90 dph		150 dph		210 dph	
AI dosage (mg kg^{-1})	BW (g, ± SD)	TL (cm, ± SD)	BW (g, ± SD)	TL (cm, ± SD)	BW (g, ± SD)	TL (cm, ± SD)
0 (control)	0.06 ± 0.01	1.57 ± 0.44	2.23 ± 1.32	4.99 ± 0.96	7.90 ± 4.40	7.73 ± 1.22
50	0.07 ± 0.01	1.59 ± 0.36	2.39 ± 1.75	5.00 ± 1.24	8.23 ± 4.37	7.93 ± 1.18
150	0.07 ± 0.01	1.66 ± 0.35	2.30 ± 1.67	4.84 ± 1.41	10.43 ± 5.13	8.43 ± 1.02
250	0.08 ± 0.01	1.74 ± 0.32	2.53 ± 1.53	5.11 ± 1.18	8.75 ± 4.30	8.03 ± 1.16
500	0.09 ± 0.01	1.80 ± 0.58	2.30 ± 1.43	4.78 ± 1.12	8.51 ± 4.10	7.80 ± 1.07

No significant differences were detected among the groups (ANOVA, $P>0.05$).

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Table 3 Sex ratios of the bluegill sunfish immersed into different concentrations of AI solutions for 8 h day^{-1} on 30, 35, 40, 45, and 50 dph

AI dosage (μg L^{-1})	*N*	Males (%)	Females (%)	*P*-value
Control	22	41^{a}	59	0.072
250	27	44^{a}	56	0.230
500	24	67^{b}	33	0.001
1000	24	75^{b}	25	0.001

The different superscript letters indicate significant (χ^2-test, $P<0.05$) differences in proportion of males among the groups. *P*-values indicate differences from the theoretical 50:50 sex ratio (χ^2-test).

Table 4 Effects of periodic immersions of fry into different concentrations of AI solutions on growth of bluegill sunfish

AI dosage	50 dph		210 dph	
(μg L^{-1})	BW (g, ± SD)	BL (cm, ± SD)	BW (g, ± SD)	BL (cm, ± SD)
0 (control)	0.02 ± 0.02	1.32 ± 0.24	8.87 ± 4.96	8.02 ± 1.47
250	0.03 ± 0.03	1.43 ± 0.28	10.39 ± 4.81	8.19 ± 1.74
500	0.03 ± 0.02	1.39 ± 0.34	12.28 ± 3.27	8.89 ± 1.40
1000	0.03 ± 0.04	1.42 ± 0.30	12.65 ± 4.15	8.58 ± 1.40

No significant differences were detected among the groups (ANOVA, $P>0.05$).

ment also produced 67% males, providing further evidence that AI may inhibit or retard ovarian cavity formation (Komatsu *et al.* 2006). The data presented from our study add to the body of evidence on the role of sex steroids and aromatase in sex determination and differentiation in fish. Previous studies on bluegill sunfish sex determination have focused on the effects of a variety of steroids on this process. The effects of aromatase inhibitors have not been studied before in the bluegill sunfish, but the successful sex reversal of gonads by AI has been well documented in fish, amphibians, reptiles and birds. As for teleosts, immersion treatment with AI (10 mg L^{-1}) for only 2 h, when the gonads were bipotent, caused 22% genetic females to develop into normal males in the *O. tshawytscha* (Piferrer *et al.* 1994). In Japanese flounder (*Paralichthys olivaceus*), 100 mg kg^{-1} AI administered for 70 days, starting 30 dph, produced 100% males (Kitano, Takamune, Nagahama & Abe 2000). In the Nile tilapia *O. niloticus*, Kwon *et al.* (2000) reported that 500 mg kg^{-1} AI diet administration from 7 to 37 dph induced 96% males and Afonso *et al.* (2001) reported 100% males were produced by 30 days of 75 and 100 mg kg^{-1} AI diet treatment from 7 dph. In the zebrafish *D. rerio*, 100% males were induced by 100 and 1000 mg kg^{-1} AI diet treatment from 15 dph to 40 dph (Uchida, Yamashita, Kitano & Iguchi 2004), and 500 mg kg^{-1} AI diet treatment for 90 days produced 100% males in the golden rabbitfish *S. guttatus* (Komatsu *et al.* 2006). However, some uncertainties still remain. Kawahara and Yamashita (2000) reported that medaka eggs incubated with an AI resulted in no abnormal sex ratios. In a more recent study (Kuhl & Brouwer 2006), the results demonstrated that co-exposure to dichlorodiphenyltrichloroethane and AIs did suppress aromatase activity but did not prevent sex reversal. Therefore, Kuhl *et al.* (2006) suggested that altered aromatase activity levels were not a requirement for sex inversion in the medaka. Thus, whether aromatase activity levels are involved in sex inversion or oestrogens are involved in ovarian differentiation of teleost species remains controversial. In the present study, AI induced masculinization of the bluegill sunfish, which suggests that aromatase inhibition by AI occurred in this species and is correlated with testis development.

Piferrer (2001) postulated two possibilities regarding the effects of steroids on growth, wherein one shows an increase in growth at a particular effective dose, and in the other case there is either no effect or there is a reduction in growth. In our present study, the growth of the fish was not significantly affected by the diet or immersion treatment of nonsteroidal aromatase inhibitor. Kwon *et al.* (2000) also reported that AI had no significant effects on the growth of Nile tilapia *O. niloticus*.

In summary, the present study demonstrated that it is possible to influence bluegill sex differentiation in favour of males by suppressing aromatase activity through oral or immersion administration of the aromatase inhibitor Letrozole. The results support the hypothesis that sex steroids are natural sex inducers and aromatization can direct the differentiation of the gonad.

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