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Induction of diploid gynogenesis in *Micropterus salmoides* using irradiated heterogeneous sperm from *Siniperca chuatsi*

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A R T I C L E I N F O

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A B S T R A C T

The artificial induction of gynogenesis in fish breeding can be used to produce monosex populations. *Micropterus salmoides* (largemouth bass) is an important freshwater aquaculture fish, and the females grow faster than the males. In this study, we aimed to determine the best way to artificially induce largemouth bass spawning and identify suitable heterologous sperm donors to induce gynogenetic largemouth bass by suppressing extrusion of the second polar body in fertilized eggs. With respect to the artificial induction of spawning in LB, four groups were tested for hormone types. The results showed that a single intramuscular injection of the hormone combination HCG (2000 IU/kg) + LHRH-A2 (20 μg/kg) + DOM (5 mg/kg) into female largemouth bass could yield desirable spawning results, and the mean number of eggs per female in kg was 28,780 ± 4574. With respect to suitable sperm donors, largemouth bass eggs were fertilized with sperm from five fish species, and it was found that mandarin fish (*Siniperca Chuatsi*) sperm can activate the development of largemouth bass eggs, but most of the embryos died during development, which indicated that mandarin fish are the potential of suitable heterologous sperm donors for gynogenetic experiments with largemouth bass. The suitable optimal induction conditions were detected for chromosome doubling. The results indicated that the most effective method for promoting gynogenesis in largemouth bass was to expose inseminated eggs to a temperature of 3 °C for 15 min, beginning 2 min after fertilization. In addition, the DNA content, karyotype and morphological characteristics of the gynogenetic offspring were consistent with those of the largemouth bass. Examination of sex-specific markers and artificial propagation of the gynogenetic largemouth bass revealed that all the tested fishes were female, indicating female homogamety (XX) in the largemouth bass. In summary, in this study, we established an improved artificial spawning technique for largemouth bass and successfully induced diploid gynogenetic largemouth bass using irradiated heterogeneous sperm from mandarin fish. Our study lays a foundation for the mono-sex breeding of largemouth bass.

1. Introduction

Micropterus salmoides (largemouth bass) belongs to the genus *Micropterus* of Centrarchidae, Perciformes, which is native to the Mississippi River system in North America. Largemouth bass was introduced into China in 1983 and is favored by fish farmers and consumers because of its rapid growth, short aquaculture cycle, tasty meat and lack of intramuscular bones. The total production of largemouth bass in 2020 was approximately 621,300 tons (FAO, 2022). However, due to the rapid expansion of the cultivation scale, the degradation of germplasm resources in the process of cultivation is becoming increasingly serious, which manifests as reductions in the growth rate and disease resistance (Deng et al., 2011).

Several aspects of largemouth bass breeding, including selective

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breeding, hybridization and sex control, have been reported. Regarding selective breeding, based on four selective breeding populations of northern largemouth bass in China, the new varieties of the largemouth bass "Youlu No. 1" and "Youlu No. 3" were bred (Gui et al., 2018). In cross-breeding, most of the studies involving largemouth bass as parents were between the northern subspecies and the Florida subspecies (Kleinsasser et al., 1990; Neal and Noble, 2002). However, there have been few studies on distant hybridization with largemouth bass as parents, and there are no reports on the cross-breeding and heterosis utilization of largemouth bass (Li et al., 2020). In addition, sex reversal via oral administration of sex steroids has been used to produce mono-sex populations in largemouth bass (Arslan et al., 2009; Garrett, 1989).

As an important breeding technology, artificially induced gynogenesis refers to the use of inactivated sperm to activate eggs, in which the development of zygotes is controlled by maternally derived genetic material (Gong et al., 2019). Artificially induced gynogenesis has been successful in >100 fish species. Some artificial gynogenetic offspring show superior traits, including fast growth rates (Wang et al., 2022, Wu et al., 2021, Yun et al., 1983), hypoxia tolerance (Gong et al., 2020; Fu et al., 2022; Wang et al., 2023), strong resistance to diseases (Slierendrecht et al., 2001) and better cold tolerance (Li et al., 2023). Largemouth bass show sexual dimorphism, with females growing faster than males. Thus, it is of great significance to breed the all female largemouth bass populations by using gynogenesis breeding technology.

Researchers have suggested that the sex determination of largemouth bass is female homogamety (XX/XY) (Du et al., 2021; Wen et al., 2022). The artificial induction of gynogenesis and detection of the offspring sex ratio can confirm the sex determination of fish, but artificial induction of gynogenesis is lacking for largemouth bass. The difficulties in inducing gynogenesis in largemouth bass are mostly existed in two aspects: First, the egg development of female largemouth bass is not synchronized, and they spawn multiple times during the breeding season, which makes it difficult to obtain sufficient numbers of eggs by artificially induced spawning; second, it is difficult to find suitable heterogeneous sperm for inducing gynogenesis, as using close relatives often yields hybrid offspring, and using overly distant relatives prevents the activation of egg development.

In this study, we established an improved artificial spawning technique for largemouth bass and successfully induced diploid gynogenetic largemouth bass using irradiated heterogeneous sperm from *Siniperca chuatsi* (mandarin fish). This is the first report of diploid gynogenetic largemouth bass to be successfully induced by inactivated sperm from mandarin fish. Our results lay a foundation for the mono-sex breeding of largemouth bass. By combining gynogenesis and hormonal reversal, a large scale of all-female largemouth bass can be produced by hybridizing female largemouth bass and hormonally reversing male gynogenetic largemouth bass.

2. Materials and methods

2.1. Ethical statement

Fish work was performed in strict accordance with the recommendations in the Guidelines for the Care and Use of Laboratory Animals of the National Advisory Committee for Laboratory Animal Research in China and approved by the Animal Care Committee of Hunan Normal University (Permit Number: 4236).

2.2. Source of samples

The experimental fish were obtained from *Hunan Yuelu Mountain Science and Technology Co., Ltd., Amed at Aquatic Breeding*. Largemouth bass and *Siniperca Chuatsi* were housed for 2 years at an appropriate density in a 0.067-ha earthen pond. The fish were fed small feeder fish, including crucian carp (*Carassius auratus*), *Rhodeus sinensis*, silver carp (*Hypophthalmichthys molitrix*), and *Pseudorasbora parva*. The food consumption was approximately 4% of the body weight per day. Green sunfish (*Lepomis auratus*), common carp (*Cyprinus carpio*) and blunt snout bream (*Megalobrama amblycephala*) were raised for 2 years at an appropriate density in an open pool. Channel catfish (*Ictalurus punctatus*) were raised for 5 years at an appropriate density in an open pool. These fish were fed artificial fodder (crude protein >40%).

2.3. Artificial induction of largemouth bass spawning

Sexually mature largemouth bass were segregated by sex, and 200 females and 200 males were selected. Ten males and ten females were randomly paired as a group and placed in separate indoor aquaria (300 *L*), resulting in a total of 20 groups of largemouth bass. When the water temperature reached 18 °C, spawning was artificially induced in the female largemouth bass. The water quality for spawning in aquaria was controlled and maintained. The water temperature range was 18–20 °C, the pH range was 7.0–8.5, the dissolved oxygen (DO) concentration was >5.0 mg/L, and the ammonia–nitrogen concentration was <0.01 mg/L. To obtain desirable spawning results, we established four artificial propagation groups based on the injection of different types of hormones. Each experimental group consisted of ten males and ten females that were paired randomly, and each experiment was repeated five times. Group 1 included female largemouth bass given only a single intramuscular injection of HCG (2000 IU/kg of body weight); Group 2 included female largemouth bass given only a single intramuscular injection of LHRH-A2 (20 μg/kg of body weight); Group 3 included female largemouth bass given only a single intramuscular injection of DOM (5 mg/kg of body weight); and Group 4 included female largemouth bass given a single intramuscular injection of the hormone combination HCG (2000 IU/kg) + LHRH-A2 (20 μg/kg) + DOM (5 mg/kg). Largemouth bass males were given a single intramuscular injection of the hormone combination of HCG (800 IU/kg of body weight) and LHRH-A2 (10 μg/kg of body weight). When spawning behavior was observed in the aquarium, the female largemouth bass were fished, and the eggs were immediately extruded. The female largemouth bass weight, the response rate, the range of the latent period (h), the total weight of the eggs released (g), the number of eggs in 1 g before fertilization, and the total number of eggs collected from each group were recorded. Unfertilized eggs collected from each female were weighed to the nearest 1 g and placed in a petri dish to count the number of stripped eggs/g. Subsequently, eggs from each group were weighed and counted.

2.4. Identification of suitable sperm donors and UV irradiation

Female largemouth bass (n = 10) were randomly selected. After artificial spawning, the eggs of the ten females were extruded, pooled and evenly mixed. Eggs from the sample pool were divided into six equal parts and artificially fertilized with the sperm of male largemouth bass (control), green sunfish (*Lepomis auratus*), mandarin fish (*Siniperca chuatsi*), common carp (*Cyprinus carpio*), blunt snout bream (*Megalobrama amblycephala*) and channel catfish (*Ictalurus punctatus*). The milt was collected into clean tubes from mandarin fish, common carp, blunt snout bream and green sunfish using gentle abdominal pressure; however, the semen of channel catfish could not be obtained through abdominal pressure. Thus, collecting the channel catfish milt was obtained by dissecting the male fish, extracting the testes, and grinding them. The sperm concentration was determined by counting spermatozoa in a hemocytometer at 400× magnification. A precise volume of sperm comprising 250,000 spermatozoa per egg was used for fertilization (Betsy et al., 2019). The fertilized eggs were spread well on glass culture dishes (2000–3000 eggs/dish), which were then placed on a wooden shelf for incubation at a water temperature of approximately 18–19 °C. The fertilization rate, number of hatchlings and number of morphologically normal larvae were recorded for each cross. All experiments were repeated three times.

The milt of the mandarin fish was stripped and then diluted with

Hank's solution (1:10–20). After dilution, the concentration of spermatozoa in semen was approximately 10^8 per milliliter. Subsequently, 4 mL of dilute sperm was poured into clean, cold glass culture dishes (15 cm) on ice and exposed to UV irradiation (darkroom) (two UV bulbs, 15 W, 254 nm), with a total UV dosage in the range of 3000–3600 mJ/cm^2 (Gong et al., 2019). The glass culture dishes were churned with shaking tables to maintain a uniform circular distribution. Sperm viability was observed under a microscope at 1-min intervals to monitor irradiation, and irradiation ceased when approximately 80% of the sperm were inactive. The irradiated sperm were collected in black glass tubes, which were stored at 4 °C.

2.5. Cold shock treatment to promote DNA diploidization

Spawning was artificially induced in female largemouth bass (n = 30), and mature eggs were extruded, pooled and evenly mixed. Subsequently, the eggs from the sample pool were divided equally into three equal parts. Largemouth bass eggs were fertilized with UV-irradiated mandarin fish sperm and subjected to cold shocks of different temperature. To obtain desirable cold shock results, we performed three experiments according to previous studies (Gong et al., 2019; Wang et al., 2023; Wu et al., 2023). First, four different cold shock temperatures (1, 3, 5, and 7 °C) were tested, with a shock duration of 15 min applied 2 min after insemination. Largemouth bass♀ × mandarin fish♂ were used as controls. Second, 2 min after insemination, the fertilized eggs were treated in water at 3 °C for different durations (5, 10, 15, 20 and 25 min). Third, at 1, 2, 3, 4, and 5 min after insemination, the fertilized eggs underwent cold shock in water at 3 °C for 15 min. The eggs used in the above three experiments were consistent with those evenly divided from the sample pool. In each experiment, the eggs were evenly divided again based on the parameter settings. All experiments were repeated three times.

In every group, approximately 2000 fertilized ova were chosen for detection of the fertilization rate and hatching rate. The stages of embryonic development were observed and photographed under a microscope. Surviving fry were transferred to earthen ponds for further culture.

2.6. Morphological traits

At the age of 1 year, 20 gynogenetic largemouth bass, 20 ordinary largemouth bass and 20 mandarin fish were randomly selected for morphological examination. The numbers of abdominal, dorsal and anal fin rays and the numbers of upper lateral scales, lateral scales, and lower lateral scales were analyzed. The measurable traits included body length (BL), whole length (WL), body width (BW), head length (HL), head width (HW), tail length (TL), and tail width (TW). Moreover, the means ± standard deviations of the ratios of body length to body width (BL/BW), whole length to body length (WL/BL), head length to head width (HL/HW), body length to head length (BL/HL), body width to head width (BW/HW), and tail length to tail width (TL/TW) were calculated.

2.7. Measurement of DNA content and preparation of chromosome spreads

The ploidy level of the gynogenetic largemouth bass was determined by measuring the mean DNA content and detecting chromosome numbers. The erythrocyte DNA contents of the gynogenetic largemouth bass, largemouth bass and mandarin fish were measured using a flow cytometer (cell counter/analyzer, Partec). The method is described in a previously published paper (Liu et al., 2001; Xiao et al., 2014). The difference in mean DNA content between gynogenetic largemouth bass and largemouth bass (mandarin fish) was tested by the χ^2 test using SPSS Statistics 18.0.

Chromosomal preparations were obtained from peripheral blood cell cultures of 10 gynogenetic largemouth bass and 10 largemouth bass individuals at the age of 1 year. The chromosomes were prepared in accordance with a previous study (Xiao et al., 2014). The karyotype was analyzed under a microscope. For each fish type, 200 metaphase spreads of chromosomes from 10 individuals were counted and analyzed for further determination of ploidy.

2.8. Sex identification

To determine the sex of the gynogenetic largemouth bass, nine 3-month-old gynogenetic largemouth bass were randomly selected (unknown sex), and one male and one female largemouth bass was used as a control. Sex-specific bands from largemouth bass controls and gynogenetic largemouth bass were amplified according to sex-specific primers (forward: GACTCAGGTCCGACACTTTCAT, reverse: AAGCC-TACCCTGGCAAGCAACT) reported in a previous study (Wen et al., 2022). The PCR reactions were performed according to previous studies (Wen et al., 2022). The thermal programme consisted of an initial denaturation step at 94 °C for 30 s followed by 35 cycles of 94 °C for 30 s, 56 °C for 60 s, and 72 °C for 30 s and a final extension step at 72 °C for 5 min. The amplification products were separated on a 1.5% agarose gel using TBE buffer.

2.9. Statistical analysis

Statistical analysis was conducted using SPSS Statistics 18.0. Regarding the metrological data, a one-way ANOVA with homogeneity of variance test and multiple comparisons was used. A statistically significant difference was represented by $P < 0.05$. Regarding the attribute data, the raw data were subjected to normality tests using the Shapiro-Wilk test prior to conducting a one-way ANOVA.

3. Results

3.1. Artificial induction of spawning in largemouth bass

The respective response rates for group 1(HCG), group 2 (LHRH-A2), group 3 (DOM) and group 4 (HCG + LHRH-A2 + DOM) were 60%, 50%, 30% and 90%, with mean responses of 11.2, 13.1, 5.8 and 40.9 g of eggs/kg female largemouth bass, respectively (Table 1). The mean latent periods for group 1, group 2, group 3 and group 4 were 38.4, 35.7, 39.6 and 35.8 h, respectively. The mean numbers of eggs collected from the group 1, group 2, group 3 and group 4 treatments were 8241, 9420, 4352 and 28,780, respectively. There were significantly more eggs per female in kg in the group 4 treatment than in the group 1, group 2 and group 3 treatments ($P < 0.05$) (Table 1).

3.2. Suitable sperm donors

The fertilization rate and survival of hybrid embryos obtained after the fertilization of largemouth bass eggs with the sperm of five different species are given in Table 2. The control group (largemouth bass♀ × largemouth bass♂) had high fertilization rates (88.3% ± 8.2%) and high hatching rates (81.7% ± 5.7%), and most of the hatched larvae (89.5%) were morphologically normal. The sperm from common carp, blunt snout bream and channel catfish failed to fertilize the largemouth bass eggs. The fertilization rates of the largemouth bass♀ × mandarin fish♂ and largemouth bass♀ × green sunfish♂ cross groups were 78.4% ± 9.1% and 88.4% ± 6.8%, respectively. However, fertilization of largemouth bass eggs with mandarin fish sperm usually resulted in mortality during embryonic development, and a few hatched larvae (only 3 fry) were morphologically abnormal and died within several days after hatching. A higher hatching rate (77% ± 7.4%) was observed for the largemouth bass♀ × green sunfish♂ cross group, and most of the hatched larvae (82.7%) were morphologically normal.

Table 1
Ovulation response of largemouth bass females to three hormone treatments and parameters measured during the breeding season. Tests evaluated the use of hormonal agents for artificial propagation. The mean values (± SDs) are presented.

Parameters	HCG	LHRH-A2	DOM	HCG + LHRH-A2 + DOM
Mean weight of females (g)	1184 ± 42	1211 ± 50	1204 ± 28	1196 ± 60
Response rate (%)	60	50	30	90
Range of latent period (h)	35.2–46.8	30.2–39.4	34.6–44.1	34.5–38.2
Mean latent period (h)	38.4 ± 4.2	35.7 ± 7.8	39.6 ± 8.6	35.8 ± 4.7
Total weight of eggs released (g)	80	79.3	21	440.5
Mean weight of eggs per female (g)	13.3 ± 3.4	15.9 ± 2.4	7 ± 1.8	48.9 ± 7.4
Number of eggs in 1 g before fertilization	618 ± 45	594 ± 31	622 ± 28	588 ± 55
Total number of eggs collected	49,446	47,100	13,056	259,020
Number of eggs collected per female (mean)	8241 ± 4532	9420 ± 3364	4352 ± 2641	28,780 ± 4574
Number of eggs collected per female (range)	3200–24,300	4860–16,700	2740–6840	9324–38,750

3.3. Optimization of cold-shock parameters

Cold shock was effective in suppressing extrusion of the second polar body in eggs and resulted in morphologically normal diploid gynogenetic larvae. These larvae were viable and swim normally after several days, in contrast to abnormal larvae (having haploid syndrome), which soon died. The most effective cold shock temperature was approximately 3 °C. In this shock temperature range, the hatching rate was 2.3% ± 0.86%, and 76.5% of the hatched larvae (26 normal larvae from 34 hatchlings) were normal (Table S1). The temperatures of approximately 5 and 7 °C were not sufficiently low to suppress extrusion of the second polar body, and only 3 normal larvae and 1 normal larva from hatchlings were observed, respectively (Table S1). The temperature of approximately 1 °C was too low and only 3 normal larvae from hatchlings were observed (Table S1). No normal larvae developed in the control gynogenetic or hybrid progeny obtained without shock application or in the hybrid progeny subjected to a cold shock of approximately 3 °C (Table S1).

The most effective cold shock duration was approximately 15 min. At this duration, the highest hatching rate (2.6% ± 0.64%) and the largest number of normal larvae (25 normal larvae from 35 hatchlings) were observed after cold shock at 2 min after fertilization (Table S2). Cold shock durations of approximately 10 and 5 min were not sufficient to suppress extrusion of the second polar body, and only 6 and 1 normal larvae from hatchlings were observed, respectively (Table S2). Cold shock application approximately 20 and 25 min after fertilization decreased the number of normal larvae among hatchings (15 and 4 normal larvae, respectively) (Table S2).

Cold shock applied at 2 and 3 min after fertilization resulted in similar hatching rates (2.6% ± 0.62% and 2.6% ± 0.85%, respectively). However, there were more normal larvae among hatchlings at 2 min after insemination (28 normal larvae from 41 hatchlings) than at 3 min after insemination (16 normal larvae from 35 hatchlings). The application of cold shock at 4 min after fertilization yielded only one normal larva. Cold shock applied at 5 min was ineffective for suppressing extrusion of the second polar body in eggs and did not result in the appearance of normal larvae (Table S3).

3.4. Development of embryos

Largemouth bass embryonic development consists of the cleavage stage, blastocyst stage, gastrula stage, neurocoele stage, organogenesis stage and hatching stage. The incubation times of common largemouth bass and gynogenetic largemouth bass were 46 h and 51 h, respectively. The timing of embryo development at each stage is shown in Table S4. The developmental morphology of each stage is shown in Fig. 1.

3.5. Production of gynogenetic largemouth bass and their morphological traits

Based on the developmental time of the largemouth bass embryo, we could determine that it took approximately one hour from fertilization to the first cleavage. Thus, after cold shock at approximately 3 °C for approximately 15 min at 2 min after fertilization, normal fry were produced via meiotic gynogenesis. The morphological traits of the gynogenetic largemouth bass are shown in Fig. 2. The countable and measurable traits of the gynogenetic largemouth bass and control largemouth bass are presented in Table S5 and Table 3, respectively. Gynogenetic largemouth bass had the same appearance and almost the same measurable and countable traits as largemouth bass did ($P > 0.05$); therefore, the two groups were indistinguishable based on their appearance.

3.6. Measurement of DNA content and examination of chromosome number

The DNA content of the largemouth bass was used as the control, and the DNA content of the gynogenetic largemouth bass was obtained. The mean DNA content of the gynogenetic largemouth bass was equal ($P > 0.05$) to that of the largemouth bass.

The results for the chromosomes of the gynogenetic largemouth bass and largemouth bass at metaphase and their karyotypes are shown in

Table 2
Fertilization and hatching rates of largemouth bass eggs after fertilization with sperm of difference fish species.

Cross[a]	Number of eggs	Number of fertilized eggs	Fertilization rate(%)	Hatchlings		Number of normal larvae
				Number	percent(%)	
LB♀ × LB♂	1863	1654	88.3 ± 8.2	1352	81.7 ± 5.7	1210
LB♀ × GS♂	2144	1896	88.4 ± 6.8	1460	77 ± 7.4	1357
LB♀ × MD♂	1974	1547	78.4 ± 9.1	3	0.2 ± 0.06	0
LB♀ × CC♂	2013	0	0	0	0	0
LB♀ × BSB♂	1947	0	0	0	0	0
LB♀ × CA♂	2081	0	0	0	0	0

[a] LB = largemouth bass, GS = Green sunfish, MD = mandarin fish, CC = common carp, BSB = blunt snout bream, CA = channel catfish.

Fig. 1. Embryo development of gynogenetic largemouth bass. 1: blastoderm stage; 2: 2-cell stage; 3: 4-cell stage; 4: 8-cell stage; 5: 16-cell stage; 6: 32-cell stage; 7: morula stage; 8: blastula stage; 9: early gastrula stage; 10: mid-gastrula stage; 11: later gastrula stage; 12: early neurula stage; 13: later neurula stage; 14: somite stage; 15: muscular effect stage; 16: hatching stage; 17: 24 h after hatching stage; 18: 48 h after hatching stage; 19: 72 h after hatching stage.

Fig. 3. In terms of the largemouth bass, 87% of the chromosomes had a karyotype formula of 2 m + 2st + 42 t at metaphase. The karyotype formula of 2 m + 2st + 42 t accounted for approximately 90% of the chromosomes in the gynogenetic largemouth bass. The above results indicated that the chromosome numbers and karyotypes of the gynogenetic largemouth bass and largemouth bass were identical.

3.7. Sexual identification of gynogenetic largemouth bass

The sex-specific markers of the largemouth bass were used to identify sex in the gynogenetic largemouth bass. The agarose gel results showed that the male control had two bright bands at approximately 500 bp and 900 bp, while the female control and gynogenetic largemouth bass had only one band at approximately 900 bp (Fig. 4). Therefore, all the randomly selected gynogenetic largemouth bass individuals were female.

4. Discussion

In natural waters, largemouth bass normally spawn from spring to early summer at water temperatures of 15–24 °C (Jackson, 1979). Artificially induced spawning of largemouth bass has been applied in many studies, such as those involving distant hybridization (Gomelsky et al., 2004) and the induction of triploidy (Neal et al., 2004). However, due to asynchronous egg development in largemouth bass, the number of eggs released from each female after artificial propagation is limited, making it difficult to meet the needs of gynogenesis. In this study, we optimized the types and doses of spawning hormones used for artificial propagation of largemouth bass to obtain more mature eggs. The results showed that a single injection of the hormone combination of HCG (2000 IU/kg) + LHRH-A2 (20 μg/kg) + DOM (5 μg/kg) was effective at inducing ovulation in largemouth bass. The average number of eggs per female in kg and latent periods were 28,780 and 35.8 h, respectively. The latency period in our study was consistent with that in other studies, ranging from 34.5 to 38.2 h (Sharma et al., 2021).

In general, the sperm that induces gynogenesis can be either homologous sperm or heterologous sperm. The induction effect of heterologous sperm is often better than that of homologous sperm, as homologous sperm induction often leads to self-mating offspring, which are difficult to distinguish from gynogenetic offspring (Guan et al., 2022). In this study, sperm from 5 fish species were used to fertilize largemouth bass eggs, and the results showed that mandarin fish sperm were most suitable for the induction of gynogenesis in largemouth bass. The spermatozoa of mandarin fish can fertilize the eggs of the

Fig. 2. The appearance of gynogenetic largemouth bass. (A) The appearance of gynogenetic largemouth bass at 3 months of age. (B) The appearance of gynogenetic largemouth bass at 1 year of age. Bars = 1 cm.

Table 3
Comparison of measurable traits among the largemouth bass, mandarin fish and gynogenetic largemouth bass.

Fish type[a]	WL/BL	BL/BW	BL/HL	HL/HW	TL/TW	BW/HW
LB	1.13 ± 0.03	3.21 ± 0.13	3.50 ± 0.04	0.99 ± 0.05	1.86 ± 0.13	1.07 ± 0.03
MD	1.19 ± 0.03	2.78 ± 0.13	2.68 ± 0.14	1.53 ± 0.11	1.67 ± 0.08	1.50 ± 0.06
GLB	1.11 ± 0.04	3.22 ± 0.26	3.48 ± 0.05	1.01 ± 0.22	1.84 ± 0.14	1.05 ± 0.03

[a] LB = largemouth bass, MD = mandarin fish, GLB = gynogenetic largemouth bass.

largemouth bass, but the large-scale embryos will die in the later stage of development, and the few hybrid larvae that are produced will die soon after hatching. In this case, when using the inactivated sperm of mandarin fish to induce gynogenesis in largemouth bass, the surviving diploid offspring obtained should all be gynogenetic offspring.

With respect to chromosome doubling during gynogenesis, temperature shock is more convenient and effective than other methods. During this process, weaker embryos die, and surviving individuals have greater vitality and disease resistance (Mao et al., 2020). The shock start time, shock temperature, and shock duration are three important factors for the success of chromosome doubling during gynogenesis. Different fish require different shock conditions for gynogenesis. For largemouth bass, the highest survival rate occurred after exposure to approximately 3 °C water for approximately 15 min, which was initiated 2 min after activation. Cold shock at temperatures below 2 °C or a cold-shock duration that exceeded 15 min affected egg development and led to a low survival rate. The highest survival rate for meiotic gynogenesis in mandarin fishes occurred after exposure to 4–6 °C water for 20–25 min, which was initiated 2 min after activation (Wu et al., 2023). Similarly, a high percentage of gynogenetic *Epinephelus fuscoguttatus* could be induced by cold shock at 5–7 °C for 10 min, which was initiated 6 min after activation (Zhang et al., 2021). Therefore, for different fish types, the best treatment for gynogenesis should be explored.

Artificial induction gynogenesis, an effective method for checking the sex determination in fish species, has been successfully applied to

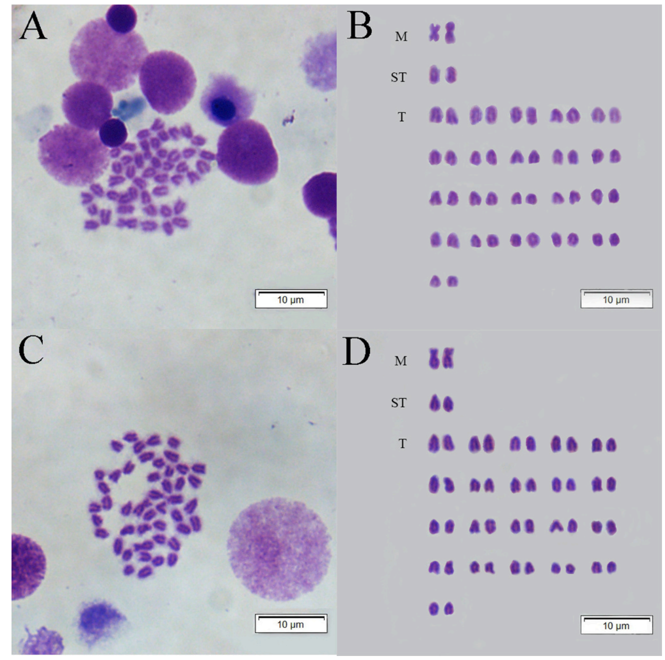

Fig. 3. Chromosomes at metaphase and karyotype in ordinary largemouth bass and gynogenetic largemouth bass. (A) The 46 chromosomes of the ordinary largemouth bass; (B) the karyotype of the ordinary largemouth bass: 2 m + 2st + 42 t; (C) the 46 chromosomes of the gynogenetic largemouth bass; and (D) the karyotype of the gynogenetic largemouth bass: 2 m + 2st + 42 t.

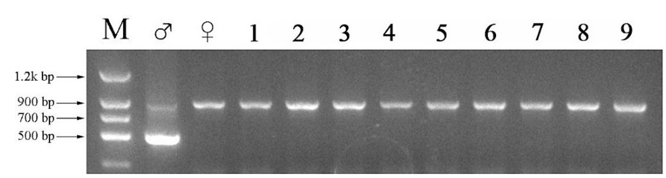

Fig. 4. PCR validation of sex-specific markers presented in agarose gels with 1 male (♂), 1 female control (♀) and 9 gynogenetic largemouth bass (1–9).

fish species, including *Opsariichthys bidens* (Guan et al., 2022), *Cynoglossus semilaevis* (Chen et al., 2009), *Scophthalmus maximus* (Castro et al., 2003), and *Carassius cuvieri* (Yuan-Dong et al., 2006). In largemouth bass, Glennon and his colleagues identified the sex determination mechanism of largemouth bass as female heterogamety (ZZ/ZW) by detecting the sex ratio of gynogenetic offspring (Glennon et al., 2012). However, researchers have suggested that the sex determination of largemouth bass is female homogamety (XX/XY) (Du et al., 2021; Wen et al., 2022). In this study, sex-specific marker and artificial propagation analyses revealed that all the examined gynogenetic largemouth bass individuals were female, which suggests that the sex determination system in largemouth bass is female homogamety (XX/XY).

Studies have shown that female largemouth bass typically grow faster than males (Sabo-Attwood et al., 2004). Therefore, some researchers have conducted exploratory experiments in the hope of obtaining a high proportion of single-sex groups to improve aquaculture efficiency (Garrett, 1989; Arslan et al., 2009). In this study, we utilized gynogenesis technology to improve the germplasm of largemouth bass and obtained thousands of gynogenetic largemouth bass. However, the number of gynogenetic largemouth bass is relatively small and cannot be directly applied to aquaculture. Thus, by combining artificial induction of gynogenesis and sex reversal, large number of all-female largemouth bass can be obtained by crossing female largemouth bass and functional sex reversal male gynogenetic largemouth bass.

In summary, an improved artificial spawning technique for largemouth bass was established, through which sufficient largemouth bass eggs can be obtained by a single-injection. Meiotic gynogenesis was induced in largemouth bass eggs using UV-irradiated spermatozoa from mandarin fish, after which the female parent DNA was duplicated with an approximately 15 min cold shock at approximately 3 °C starting 2 min after insemination; in this case, a population of gynogenetic largemouth bass was successfully obtained. The morphological characteristics, DNA content, chromosome number and karyotype of the gynogenetic largemouth bass and largemouth bass were compared and analyzed, and the results confirmed that the surviving offspring obtained were gynogenetic largemouth bass. In addition, sex-specific marker and artificial propagation analyses revealed that the sex determination system in largemouth bass is female homogamety (XX/XY). Our study provides a foundation for the mono-sex breeding of largemouth bass, and by combining gynogenesis and sex reversal, all-female largemouth bass can be obtained on a large scale.

CRediT authorship contribution statement

Haitao Zhong: Validation, Investigation. **Yu Sun:** Validation, Investigation. **Mingli Liu:** Investigation. **Hong Chen:** Methodology. **Pengfei Yu:** Methodology. **Chang Wu:** Validation. **Xinyan Zhu:** Visualization. **Xueyan Wang:** Visualization. **Yilin Wu:** Data curation. **Na Tang:** Formal analysis. **Siyu Wu:** Visualization. **Shi Wang:** Visualization. **Ming Wen:** Visualization. **Fangzhou Hu:** Writing – original draft, Supervision, Methodology, Funding acquisition. **Chun Zhang:** Supervision, Funding acquisition. **Shaojun Liu:** Supervision, Resources, Funding acquisition, Conceptualization.

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

No data was used for the research described in the article.

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Appendix A. Supplementary data

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