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Improved Method in Breeding and Artificial Propagation for Chinese Giant Salamanders (*Andrias Davidianus*)

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Abstract

Context: The hatching rate for Chinese giant salamanders is only around 5% ~10%, and the average female parent can breed only 10 to 30 progenies. Therefore, how to improve the hatching rate of artificial breeding is currently one of hot research topics.

Aims: By optimizing the key technique such as artificial insemination and incubation, it could improve the hatching rate.

Methods: During the hatch period in Chinese giant salamanders, a series measures including living bait feeding, flow control, temperature control and light regulation, could promote parental development synchronization.

Key results: Two batches of artificial breeding for Chinese giant salamanders were studied in 2016, the results showed that all 19 tails of female salamanders were spawned and a total of about 9,157 eggs were collected. The survival rate of the fertilized eggs reached to 85.6% after 24 hours. The average hatching rate was 72% after hatching to 35 days.

Conclusion: This is far better than 5% to 10% average rate reported previously for artificial breeding of Chinese giant salamanders.

Implications: These measures help to ensure salamander maturation, male and female parent development synchronization, and improved the germination rate of artificial breeding of salamanders.

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Introduction

Chinese giant salamander (*Andrias davidianus*), commonly known as "living fossil"[1], is the largest living amphibian. It has been declared a Class II Protected Species by Chinese authorities, and it has been listed as Critically Endangered in the Chinese Red Book of Amphibians and Reptiles as well as Data Deficient on the IUCN Red List[2]. The researchers found that the unique species has a special value for scientific researches in the biological diversity, evolution, and sex determination mechanism. Also, it has potential value for sightseeing, usage for Chinese medicines, and for luxury food source. Populations of the species have sharply declined in both range and number since the 1960s because of the damage in eco-systems causing the habitat losses and hunting for the commercial food trade. Most local populations are on the verge of extinction and some are already extinct. According to the report of National Reserves that wild Chinese giant salamander biomass exists only about 40 thousand tails of wild population; survival status seems not optimistic[3].

A review on the development of Chinese giant salamander breeding showed that studies for Chinese giant salamander breeding, resources protection and artificial domestication had been conducted in China for 30 years. The domestic and foreign scholars have conducted in-depth studies and have achieved some success in anatomy, morphology, embryo development, and ecological protection. Artificial domestication technology for Chinese giant salamander also made a big progress. Licensed farmers for salamander domestication now exceeded ten thousands in China. However, in large scale breeding, technology is still lagging behind, the hatching rate for Chinese giant salamanders is only around 5% ~ 10%, and the average female

parent can breed only 10 to 30 progenies[4-6]. Therefore, how to improve the hatching rate of artificial breeding is currently one of hot research topics.

In study of salamander bionic and artificial propagation, we found some problems that directly related to low rate of artificial breeding[1]. reproductive period for male and female parents is difficult to be accurately determined[2]; the male could not extrude sperm, or gave no vital sperms[3]; the death of progenies could also increase because of broken eggs, or low quality of fertilized eggs in the late embryonic development (gill plate and tail bud stage)[7].

In this study, we adjusted some key factors in Chinese giant salamander development and reproduction; these include feed the parents with different food sources, temperature control, flow control and light regulation during the parental breeding. These measures help to ensure salam and ermaturation, male and female parent development synchronization, and improved the germination rate of artificial breeding of salamanders[9].

Materials and Methods

Parental Breeding

Parental selection: female parent in 8 years old, and male in 7 years old were selected. All the individuals were in good shape, strong physique, no trauma, and their body weight were between 5 kg to 8 kg.

Breeding sites: the study was conducted in Anhui Wanrun Chinese Giant Salamander Professional Cooperatives, where is located inside of Yuexi County in Anhui province. The area is the original inhabitation site of the wild Chinese giant salamanders, where the altitude is around 700 ~ 1200 m, the yearly water temperature is between 4 ~ 25°C with a total count of annually water temperature at 5900 ~ 6200°C •d.

Feed adjustment: in the period of breeding stage, feeding baits need to be diversified. These baits should include fresh fish, shrimp, loach, and animal liver; all food should be freshly prepared as possible. We generally feed once for every 2 to 3 days, with the feeding amount is about 3% ~ 5% of its body weight. In order to promote parent gonad development, vitamins E and C was also supplied during breeding stage from September to November, gave 50 mg of vitamins mixed with bait for each parental tail, feed once for each 2 weeks. In next summer cultivation and prenatal, in order to enhance the vitality of the sperm, apart from the normal food mentioned above, crabs and frogs were added in the food once a week, we believe that it can improve the rate of vital sperm and toughness of chalazae.

Light regulation: started from the beginning of spring 2016, followed the natural law of the moon phase change, we regularly manipulated the light intensity from natural light to a controlled light, this measure could help to promote gonadal maturation for Chinese giant salamander parents. From June to August, recruitment of natural light is about 1 h every day; the light intensity was controlled at 1500 ~ 2000 lx.

Water flow regulation: fish pool was made of water dropping style; the sound of water fall can not only help to stimulate gonadal development, but also to increase the amount of dissolved oxygen. When the water temperature rose to 16 degrees above, we gradually increased the flow rate to the fish pool. In order to promote the development of gonad salamander, in a month before breeding, we increased daily water flow stimulation up to 1h at the flow rate of 0.3 ~ 0.5 m^2/min.

Water temperature regulation: between June to August, the water temperature in fish pool was controlled at 16 ~ 22°C. The male Chinese giant salamander is usually lag behind in the course of development, by regulating the pool water temperature which was 1°C higher than that of the female pool, it can synchronize the male and female gonad development. If both male and female are in the same fish pool, the adjustment can also be done by incoming water flow, such as the indoor water temperature was higher than the outsource water, stimulated the female first, otherwise stimulated male first.

Artificial Inducing

To judge the sexual characteristics of Chinese giant salamander gonadal development, when the male cloacal apertureis red and swollen, it can have many white circles on the edge of the uplift; when the female abdomen swollen, it can be soft and elastic. Before breeding, select these good, more obvious features for gonadal development, and artificial reproduction. In order to easily collect the semen, stop feeding any bait in 10 days before collection.

Human chorionic gonadotropin (HCG) and luteinizing hormone releasing hormone analogue (LHRH-A2) were selected and mixed to use asoxytocin injection. The dosage for HCG is 300 ~ 1500 IU/kg and the dosage for LRH-A2 is 3 ~ 15 μg/kg, the amount of hormones can be adjusted based on the gonadal development and maturation. Injection was done on the back of Chinese giant salamander, needle depth was about 0.3 ~ 0.5 cm.

Artificial insemination

3 days after Oxytocin injection, the females were carefully inspected every 2 hours or so. When the female appeared chalazae, lightly squeeze the female stored cloacal orifice, if eggs found from squeeze, timely artificial insemination. After obtained salamander semen, examine the sperm semen under the microscope in order to choose the strong ones. Artificial insemination was carried out in the water by two people together, one gently holding salamander's abdomen; another holding egg slowly out with rhythm, and carefully put these eggs into a clean plastic basin. In order to facilitate the next step of artificial insemination, each basin generally put no more than 20 grains. After the prepared sperm was directly added into the eggs, some 0.6% saline was added and mixed fully with the sperm and eggs. Wait for 5 ~ 10 min, then add fresh water to the plastic basin, statically set for 30 min, replace with fresh water twice, it now completed the whole process of fertilization. The eggs kept in a small plastic basket suspended in the incubator pool, until the next stage of incubation.

Artificial incubation

The basic requirements for hatching fertilized eggs: the light intensity was controlled in the 3001x, the temperature was controlled in 19 ~ 20 °C, the water oxygen was higher than 5 $mg•L^{-1}$. Each day, carefully observe the status of the embryos,

eliminate the necrosis of the embryos, and prevent the disease infection into the healthy eggs. During the incubation period, the small plastic basket is pulled out pool every 6 h to prevent the local hypoxia during the development of fertilized eggs. Also, (point out what chemical you have used) was added to prevent the outbreak of the mold at this stage.

Results

The water quality in breeding pool for salamander parents

Water quality for Chinese giant salamander hatching pool was determined in 2016. Samples were taken once a week, data was statistically collected for water temperature, dissolved oxygen, pH value and organic oxygen consumption ($mg•L^{-1}$), the mean value of the specific data are shown in Table 1. Data showed that water quality in the hatching pool meet the requirements for Chinese giant salamander breeding.

Table 1: The water quality in breeding pool in 2015.

Project \ Month	1	2	3	4	5	6	7	8	9	10	11	12
water temperature (°C)	9.7	9.1	11.4	13.2	16.4	18.7	20.5	19.9	19.1	18.4	16.6	12.7
Dissolved oxygen ($mg•L^{-1}$)	6.4	6.2	5.9	5.7	5.4	5.6	5.5	5.1	5.4	5.7	5.3	5.9
Degree of acidity and alkalinity(pH)	7.2	7.3	7.2	7.4	7.5	7.3	7.4	7.3	7.4	7.5	7.3	7.4
Oxygen consumption of organic matte($mg•L^{-1}$)	3.5	3.2	3.1	3.9	3.7 ?	3.0	3.9 ?	3.2	3.8 ?	3.2	3.3	3.7

Artificial propagation

Two batches, with a total of 37 tails, of artificial reproduction were tested. The first batch was conducted on August 6, 2016, 9 male and 12 female were used for artificial reproduction. The second batch was conducted on August 16, 2016, 7 male and 9 female were used for artificial reproduction. In all experiments, we found that semen was easy to be extruded. When examined the sperm under microscope, we found that the sperm head looks like a pepper, completely dispersed, and the sperm moves spiral forward with string activity. After 4 days to 6 days of normal spawning period, all 19 tails of female salamander egged, oviposition rate was 100%, collected eggs counted for 9,157 grains. 24 h after the fertilization, the rate of fertilized eggs reaches 85.6%. After 35d ~ 38d of incubation, the average hatching rate was at 72%. The specific data was shown in Table 2 and Figure 1.

Table 2: The results of artificial spawning and hatching.

Date	Number (tail)	Average weight (Kg)	Oviposition amount (grain)	Fertilized egg (grain)	Fertility rate (%)	Incubation temperature (°C)	Emergence number (tail)	Hatching rate (%)
6th Aug.	♀8; ♂7	5.9	4185	3557	85.0	19 - 20.5	2987	71.4
16th Aug.	♀11; ♂9	7.1	4972	4286	86.2	19 - 20	3611	72.6

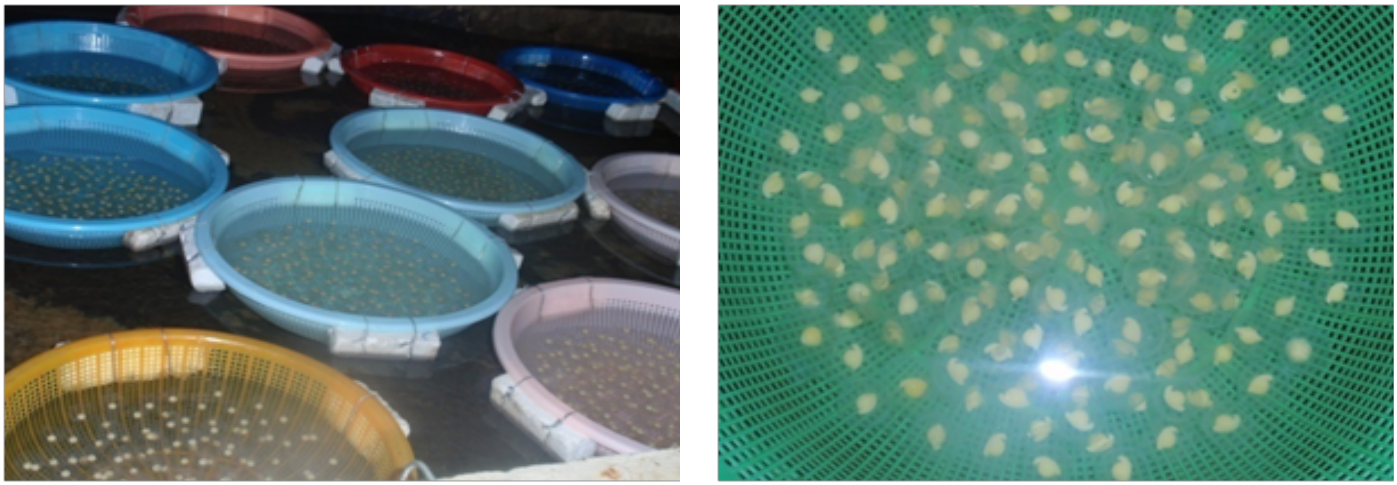

Figure1: Hatching the fertilized plots of Chinese giant salamander.

Discussion

Gonadal development synchronization

In matching the gonadal maturation of Chinese giant salamander, hypothalamus, pituitary and gonad were considered. These factors are usually affected by hormones regulation in both ways, so it can promote the synthesis of sex hormones and steroids, it can also help the sperm release and oocyte maturation[4]. Other factors that may affect the gonadal maturation are the living

environments in the hatching pool, such as pH value, water temperature, water contents, light source, algae, and the interactions between ecosystems and salamanders. As all these factors could affect the gland maturation, at present, the method of artificial reproduction combined the physiological conditions as well as the ecological conditions, preferably fit the natural hatching environment. In order to produce more offspring, many fishing farms blindly put as many parental salamanders as possible when breeding, regardless whether these gonads were mature or not. Most time, the semen was difficult to extrude from male parents, and viable sperm was very little. Some fishing farmers even killed the males for sperm, leading to the low efficiency of fertilization. We believe that immature sperm is the main reason for low fertilization rate, as male development usually lags behind female. In order to solve the problem, we raise the pool water temperature 1°C higher for males than that one for females. Other measurements for speeding the male maturation include increasing the water flow stimulation one hour per day in the month before breeding, injecting the oxytocin two days earlier than female, and so on. In our experience, these measures can effectively promoter the sperm maturation, and solve the problem of gonad development lag.

Most parents used for salamander reproduction come from wild brood stock in rivers. It takes time to adopt domestication in artificial environment. Most typical character for these wild salamanders is different in size, therefore the gonadal development may differ, and maturation time may not match. Regardless male or female, the injection of oxytocin can give good result only when gonad reaches certain maturity.

But in current technology, accurately predict the degree of stock maturity is difficult, so many fishing farmers took breeding blindly in batches,the quality of spawning eggs was low resulting in low rate of fertilization and offspring emerging. Therefore, the time to start oxytocin injection directly impacted on the rate of offspring emerges. According to our research experience based on a detailed record of annual reproduction cycle, we had established a temperature parameter from the calculation of annual water temperature. In Dabie Mountain Areas in Anhui province where average annual accumulated temperature of 6050°C •d, the best time for breeding should be in mid-August. One of the important measures to promote gonad development synchronization was to adjust the water temperature. The study found that the gonadal maturation for Chinese giant salamanders was different from the common fishes which normally give mature sperms at the end of year, while the mature eggs could last to next spring. In the contrast, the female Chinese giant salamanders usually produced mature eggs in late July, while the males gave mature sperm in mid-August. So when female eggs were ready for artificial spawning, the males have no mature sperms. Thus, it was important to synchronize gonad development from both male and female. To alter the fish pool temperature by adjusting water fell in the same breeding tank, it can effectively promote the development of salamander sexual gland. Use micro flow to stimulate the females if the incoming water temperature was lower than room temperature, or to stimulate the males vice versa. The temperature differed from inlet to outlet of micro flow should be equal or less than 1°C.

Breeding seedling rate

While average Chinese giant salamander weighing 5 to 8 kg can produce 500 to 1,000 eggs, most fishing farmers can only get 5% ~ 10% reproductive rate, about 10 to 30 seedlings produced for each salamander, that meant the increase of offspring reproduction rate had a big gap and a great potential. Most authors in previous studies had put artificial incubation technology and oxytocin dose as major research topics, others focused on the mechanism of giant salamander reproductive physiology and gonadal maturation mechanism[5,8]. While these studies on artificial breeding played a certain role, but it did not solve the problem of low reproduction rate. We found that the quality of eggs and sperms were directly related to low reproduction rate, as well as to the tail bud stage and the gill plate period prone to death. To improve the quality of eggs and sperms, in our study, other than adjust the water temperature in hatching pool, we fed the male giant salamanders with crabs, and fed the females with frogs which may enhance the sperm vitality and increase chalazae toughness for females.

In summary, the main reason for low rate of Chinese giant salamander breeding was that gonad development and gonadal maturation from male and female parents was not synchronized. By taken certain measurements such as feeding, controls of water flow, temperature and light source it can promote the parental development synchronization for Chinese giant salamanders. Combined with measurements like artificial insemination and incubation, the improved hatching rate of artificial breeding could be realized to produce tens of thousands tails of salamander seedlings.

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