Towards the Development of a Practical Method for Reversing the Sex (Male to Female) of Large Numbers of Ambystoma mexicanum

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INTRODUCTION

Sex reversal of amphibians has been attempted in many ways such as grafts,[1,4,5] direct injection of sex hormones,[1] and the introduction of hormones into the animals' aquarium water.[2,3] Those experiments have been carried out on a variety of species and with various degrees of success.[1]

The possibilities presented by induced sex reversal can be very intriguing to researchers working with maternal effects genes. If an efficient and highly effective procedure could be devised which would transform a genetically male animal into a physiologically functional female, the researcher could effectively double the number of affected female animals available for experimentation from a spawning of heterozygous parents. This paper presents preliminary data on a procedure which may be useful for sex reversal in Ambystoma mexicanum. Estradiol benzoate was employed as the reversal agent.

The process of inducing male of female sex reversal involves stimulating the cortex of the indifferent gonad while repressing the medulla at the same time. Estrogens introduced into the animals' aquarium water do this very well.[1,2,3] However, there are some pitfalls. Correct timing and proper dosages are essential. If the hormones are administered too late in the animals' life, then no reversal or incomplete reversal (resulting in "ovotestes") could result.[1] Too small a dosage of hormones could also result in ovotestes,[1,3] while too large a dosage could induce anomalous results

(e.g., using too much estrogen can induce genetic females to reverse to males).[1] This experiment employs a dosage found to be effective by Gallien[3] and attempts to determine the most effective, yet convenient, developmental stage during which the hormone should be administered.

METHODS AND MATERIALS

Chemicals: A stock solution of the estrogen (estradiol bezoate) in 95% ethanol was made to the concentration of 0.20 g estrogen per liter ethanol (100 µg estrogen/0.5 ml ethanol).

Animals: Seventy axolotls (A. mexicanum) which had just hatched were obtained from the Indiana University Axolotl Colony. Another set of 70 axolotls at Stage 28 was also obtained from the Axolotl Colony.

Treatment: The animals were divided into two series--the Hatchling Series, and the Stage 28 Series. Each series was further divided into three groups--Group I, untreated controls (10 axolotls); Group II, ethanol controls (30 axolotls); and Group III, experimental (30 axolotls). The jelly coats surrounding the Stage 28 embryos were removed to prevent their impeding the estrogen from reaching the embryos. This was accomplished by using sharp, pointed forceps to puncture and tear off the jelly coat.

The animals were kept in glass bowls, ten animals per bowl, with one liter of water per bowl. The water was changed daily, and after the change, each bowl of Group II animals received 0.5 ml of 95% ethanol (effective dose: 0.5 ml ethanol per 1.0 l water), while each bowl of Group III animals received 0.5 ml of the estradiol benzoate stock solution (effective dose: 0.5 ml 95% ethanol and 100 µg estradiol benzoate per 1.0 l water). This treatment was continued for 60 days. The Group I animals received no special treatment.

The water for the Hatchling Series was 50% Holtfretter's solution throughout the experiment. The water for the Stage 28 Series consisted of 20%

Holtfretter's solution until day 10, then 50% Holtfretter's solution for the rest of the experiment.

The Stage 28 Series suffered from a very high death rate. Hence a second set of 70 animals at Stage 28 was obtained from the Axolotl Colony, dejellied, divided, and treated just like the first set; except these animals were kept in 20% Steinberg's solution until day 6, then 20% Holtfretter's solution until day 10, then 50% Holtfretter's solution for the remainder of the experiment.

The Stage 28 Series animals were not fed until their yolk supplies were depleted (day 10). After day 10 they were fed brine shrimp (after each water change) until day 41. The Hatchling Series animals were fed brine shrimp (after each water change) from day 1 until day 41. From day 41 through the rest of the experiment, both series were fed pureed beef liver about 30 minutes prior to the water change.

After day 30 the animals were reallocated to glass bowls so that there were five axolotls per bowl, again with one liter of water per bowl.

After 60 days of treatment, 46 animals from the Hatchling Series (six from Group I, 20 from Group II, 20 from Group III) were anesthetized by adding MS-222 (ETHYL m-AMINOBENZOATE, METHANESULFONIC ACID SALT) to the water. Their gonads were removed, fixed in Bouin's fixative, sectioned (10 μm thick), stained with hematoxylin and eosin, and identified as male or female by their morphology.

The mean temperature of the laboratory where the animals were kept was 25°C with a range of 20°C to 26°C.

The schedule of care and treatment presented above is provided in Table I.

RESULTS

The six Hatchling Series Group I animals sampled were found to consist of

two females and four males.

The 20 Hatchling Series Group II animals sampled were found to consist of ten females and six males. The gonads of four animals could not be discerned on the prepared slides.

The 20 Hatchling Series Group III animals sampled were found to consist of 19 females and no males. The gonads of one animal could not be discerned.

These results, along with death rates within the various groups, are sumamrized in Table II.

DISCUSSION

The results indicate that the procedure outlined in this paper is very effective at a morphological level with hatchlings. All observed Group III axolotls were females, while the female to male ratio of the control groups was very close to 1:1. It can be concluded that Group III contains some sex-reversed males.

The Stage 28 Series animals were not sectioned primarily because of time constraints. It has been assumed that since the procedure worked (at a morphological level) with the Hatchling Series, it would produce similar results with the Stage 28 Series animals.

Practical considerations such as the time required to dejelly the Stage 28 embryos, the extra care these same embryos demanded, and their very high death rates indicate that treatment of hatchlings is very much preferred over treatment of Stage 28 embryos when sex reversal of a large number of animals is desired.

Although this experiment has demonstrated that the gonads of genetically male axolotls can be reversed, it has not proven that these animals are sexually functional or capable of spawning fertilizable eggs. Sexually functional reversed males can be identified because all of their offspring

will be male since the male in axolotls is the homogametic sex. It is possible that although the gonads have been reversed the accessory ducts and organs have not been reversed to a functional degree. This experiment also does not eliminate the possibility that the reversed males' ovaries will revert partially or completely back to testes as the animal matures. The test of sexual functionality will have to await the sexual maturation of the remaining Group III animals and of other axolotls currently undergoing sex reversal via the procedure presented in this paper. Nevertheless, this report represents a first step toward achieving large-scale sex reversal in the axolotl.

REFERENCES

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TABLE I. Care and Feeding Schedule of Axolotls Undergoing Sex Reversal

DAY OF ESTROGEN TREATMENT		1	5	10	15	20	25	30
Series								
Stage 28 (1st trial)	Water	/--20% Holtfretter's---//			50% Holtfretter's solution			
	Food	/---not fed		//	brine shrimp			
Stage 28 (2nd trial)	Water	/20% Steinberg's/		/20% Holtfretter's//	50% Holtfretter's solution			
	Food	/---not fed		//	brine shrimp			
Hatchling	Water	/50% Holtfretter's solution						
	Food	/brine shrimp						

DAY OF ESTROGEN TREATMENT		31	35	40	45	50	55	60
Series								
Stage 28 (1st trial)	Water	50% Holtfretter's solution						
	Food	---brine shrimp		//	pureed liver			
Stage 28 (2nd trial)	Water	50% Holtfretter's solution						
	Food	---brine shrimp		//	pureed liver			
Hatchling	Water	50% Holtfretter's solution						
	Food	---brine shrimp		//	pureed liver			

TABLE II. Number of Female Axolotls, Death Rates, and Probable Causes of Deaths in Various Groups and Series of Axolotls Undergoing Estrogen-induced Sex Reversal Experimentation.

SERIES	GROUP	TOTAL NUMBER OF ANIMALS AT BEGINNING OF EXPERIMENT	NUMBER OF ANIMALS SAMPLED	NUMBER OF FEMALES	% FEMALES	NUMBER OF DEATHS	DEATH RATE	CAUSE OF DEATH
Stage 28 (1st trial)	All	70	-	-	-	70	100%	Fungus[a]
Stage 28 (2nd trial)	I	10	0	-	-	4	40%	Fungus[a]
	II	30	0	-	-	15	50%	Fungus[a]
	III	30	0	-	-	10	33%	Fungus[a]
Hatchling	I	10	6	2	33%	0	0%	
	II	30	16[b]	10	63%	0	0%	
	III	30	19[b]	19	100%	2	7%	One was eaten by a sibling, the other jumped out of its bowl and dehydrated.

Notes: [a]The fungus was not identified. It tended to form around the mouth, cloaca, and in extreme cases the gills of the axolotls. It usually attached most of the animals of a single bowl, appearing overnight. It also tended to attack more than one bowl at a time.

[b]This number does not include the animals which were sacrificed but whose gonads could not be seen on the prepared slides.