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Original Article

Effects of an Endocrine Disruptor, Methoxychlor, on the Cutaneous Water Absorption in Japanese Tree-frogs

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Global decline of population size and species in amphibians during the last few decades has been discussed among environmentalists and biologists for possible causes [1]. Although various factors such as chemical or physical agents affecting environmental integrity have been considered as possible casuses for the decline, no decisive evidence has ever appeared [2]. Adult anuran amphibians usually do not drink water orally, but intake it mainly through a part of their ventral skin. We have previously shown that Japanese tree-frogs show sexual dimorphism in water absorption in the ventral skin. We examined the influence of an insecticide, methoxychlor, which has been known as an endodrine disruptor in modifying sexual integrity of animals, on the cutaneous water absorption of Japanese tree-frogs. After being kept for one month in water containing methoxychlor, male tree-frogs showed apparent depression of the cutaneous water absorption rate at concentration of 10^{-6} M or higher, but the agent had no effects on the females up to 10^{-5} M. When frogs were brought into a dehydrated environment after being kept in water containing methoxychlor for one month, all animals, males and females, showed a marked depression of the water absorption rate even at a low concentration of 10^{-9} M. These results suggest one possible reason for the severe decline in the amphibian population during 1960s when the agent had been widely used [3], may be due to disability of the water absorption caused by the endocrine disruptors.

Key Words: Enviroment, Insecticide, Endocrine disruptor, Frogs, Skin, Water uptake

Introduction

During the last several decades, worldwide amphibian declines have been recognized and discussed among both biologists and environmentalists. There have been various reports of possible causes of the decline, such as chemical contaminants, climatic changes and parasitic hazards [4, 5]. The water most frogs usually consume is from shallow still sources, such as ponds, rice paddies and irrigation ditches, which are usually contaminated with insecticides and herbicides, industrial and domestic chemicals. Since most of anuran amphibians are literally semi-

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aquatic and intake water they need through the skin, water intake is involuntary and dependant on the blood osmolarity. We intended to see if any water pollutants in the ambient water affect the physiology and water economy of the Japanese tree-frogs. Anuran amphibians usually intake water mainly through a portion of ventral skin, so called "pelvic patch" [6]. We have reported that the cutaneous water absorption in Japanese tree-frogs is controlled by two independent systems on the pelvic patch. One appears only when animals thrive in a long period of drought, and is activated by the adenylyl cyclase-Na^+, K^+-ATPase system through adrenalin β or vasotosin receptor. The other works under the ordinary wet environment and is inhibited by angiotensin II [7, 8]. The former is the "enhanced" system and the latter, the "ordinary" system. We also showed that Japanese tree-frogs exhibit sexual dimorphism under the ordinary wet condition, that is, the water absorption rate in male frogs is much greater than that of females [9]. It has been shown that some of insecticides and herbicides in environmental water cause serious endocrine disorders on the sexual integrity of aquatic animals [10]. The insecticide, methoxycholor, which was used throughout the world as an agricultural chemical for pest control, is known to be an endocrine disruptor which may modify sexual integrity of animals. Although it was banned decades ago, residual effects of the agent in water supplies still cause various problems for animals, especially in male animals becoming feminized [11-13]. In this experiment, we tried to observe if the agent has any effects on Japanese tree-frogs, which show sexual difference in their water absorption system.

Material and Methods

Tree-frogs were collected from a local field and kept in a laboratory until used. The laboratory was conditioned for temperature (25 ℃) and illumination (photoperiod of 12 h light (3000 lx) and 10 h dark daily, with one hour's gradual change of lightness in each transition phase). Animals used in the experiments were fed *ad libitum* with crickets (*Grillus dimacrata*) and supplied water containing designated concentrations of methoxychlor freely for the experimental period. Methoxychlor was administered by Prof. Taisen Iguchi, Okazaki National Research Institutes of Japan. The highest concentration of methoxychlor used in this experiment was 10^{-5} M, to mirror natural conditions of water where the insecticide was once used and may remain in the environment. Referring to the direction supplied by the manufacturer, we estimated the remaining concentration of the agent was at most 10^{-5} M. After being kept in methoxychlor tainted water for one month, frogs were double pithed and skinned and their skins immediately set on a device to measure the water flow. A detailed description on the device was appears elsewhere [7]. Some of animals that were kept one month in the experimental water were further used for dehydration experiments. Dehydration was carried out in a plastic chamber with humidity and temperature controlled until animals lost 35% to 40% of their initial body weight. For histological tracing of cells through which water is transported, a vital stain, neutral red, was dissolved in the water to be taken by frogs.

Each experiment was carried out with at least five animals and repeated twice. Significance of the results among experimental groups was estimated by the

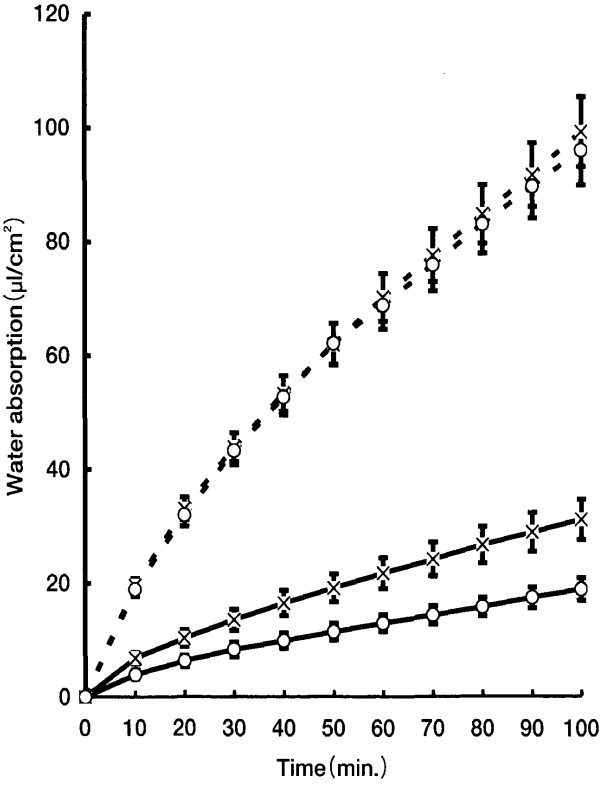

Fig. 1 Cutaneous water absorption in Japanese tree-frogs under ordinary wet (solid line) or dehydrated (dotted lines) conditions. The ordinate indicates cumulative water flow through the skin in 100 minutes (the abscissa). Open circles show female frogs and crosses mark males. Under dehydrated circumstance, the tree-frogs absorb almost five times more water than that under the ordinary wet condition. Under ordinary wet condition (solid lines), male frogs (x) absorb significantly more water than females (○).

Student's t-test.

Results

Japanese tree-frogs exhibit two types of water absorption systems in the ventral skin, that is, the ordinary system and the enhanced one (Fig. 1). The ordinary system is always active irrelevant to the environmental condition (humid or dry), and suppressed by protein kinase C which is activated by angiotensin II [8]. The enhanced one appears only under dehydrated conditions and is activated by the protein kinase A through adrenalin or vasotocin receptor [7]. Furthermore, there is sex based difference in the ordinary water absorption system in Japanese tree-frogs, that is, males intake significantly more water than females per unit period (Fig. 1).

When the Japnanese tree-frogs were allowed to absorb water containing various concentrations of the endocrine disruptor, methoxychlor, freely, the water absorption rate in the male frogs was significantly depressed by a concentration of 10^{-5}M (Fig. 2). There were, however, no marked effects observed on the female frogs. The effect of methoxychlor on the cutaneous water absorption of male tree-frogs depended roughly on the concentration of the agent (Fig. 3). Under the ordinary wet condition, the water absorption rate of male frogs were significantly affected by 10^{-6} M or higher concentration of the agent, but in females the water absorption rate showed no marked effects with concentrations up to 10^{-5} M (Fig. 3).

On the other hand, in the enhanced water absorption system which appeared under the dehydrated condition was strongly affected by methoxychlor even with a concentration of 10^{-9} M; ten thousandth less than that effective in the ordinary system (Fig. 4).

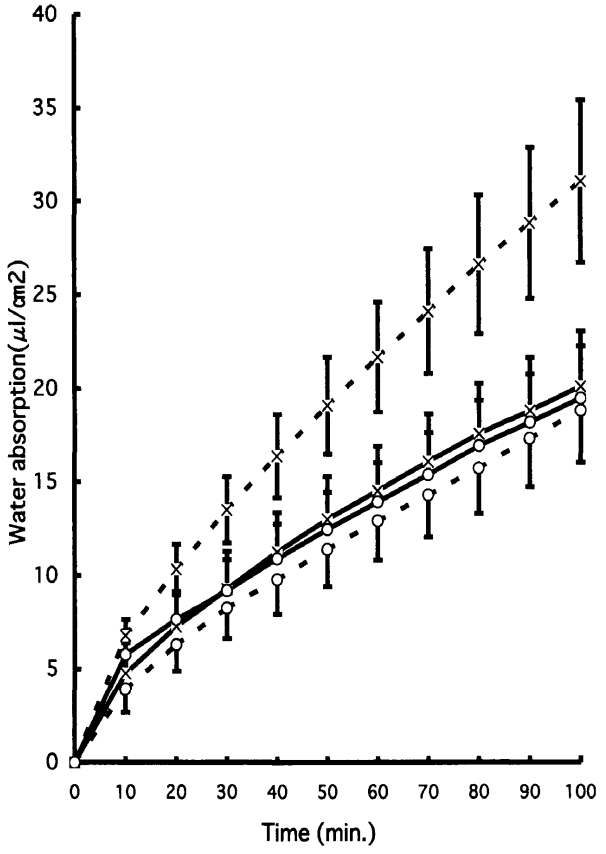

Fig. 2 Effect of 10^{-5} M methoxychlor on the cutaneous water absorption in Japanese tree-frogs after being kept for one month in the experimental water. The water absorption rate in male frogs was decreased by 30% during 100 min experiment (dotted lines). However, there were no notable effects observed in females even in this highest concentration of the agent (solid lines).
Control: cross marks, Methoxychlor: open circles

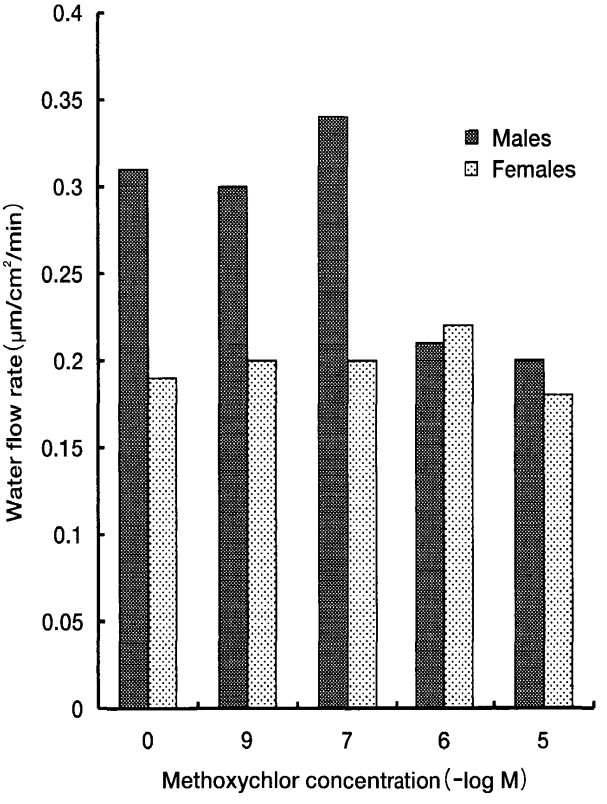

Fig. 3 Effects of various concentration of methoxychlor on the cutaneous water absorption in Japanese tree-frogs being kept for one month in the experimental water. In male frogs, 10^{-6} M or higher concentrations of the agent suppressed the water absorption clearly, while in females no significant effects were observed.

Kept in water containing 0.1% ethanol, the vehicle of methoxychlor, showed no effects on water absorption rates in the both systems of the both sexes (not shown). The vehicle also showed no effects on the dehydration response followed after the methoxychlor treatment.

By tracing of the water pathway through the skin with a vital stain, neutral red, it showed that cells through which water was absorbed were significantly decreased in number after the methoxychlor treatment for one month (Fig. 5).

Discussion

Global amphibian declines have been reported and discussed among biologists for the last several decades [1-3]. However, worldwide declines were mostly observed since 1960s during which time chemical agents were widely used to control pests and weeds in agriculture, fishery, domestic and industrial fields [3]. Since then, many works on the possible causes for the decrease in population size and/or in number of amphibian species have been reported. Various factors including water-polluting chemicals, climatic changes and parasitic organisms have been listed as possible causes of the anuran decrease throughout the world [4, 5]. Since amphibians lay eggs, develop in water, and spend a large portion of their life in water, the quality of the ambient water might directly affect the lives of these animals [6, 14]. Because rice paddies and irrigation ponds are main nurseries for

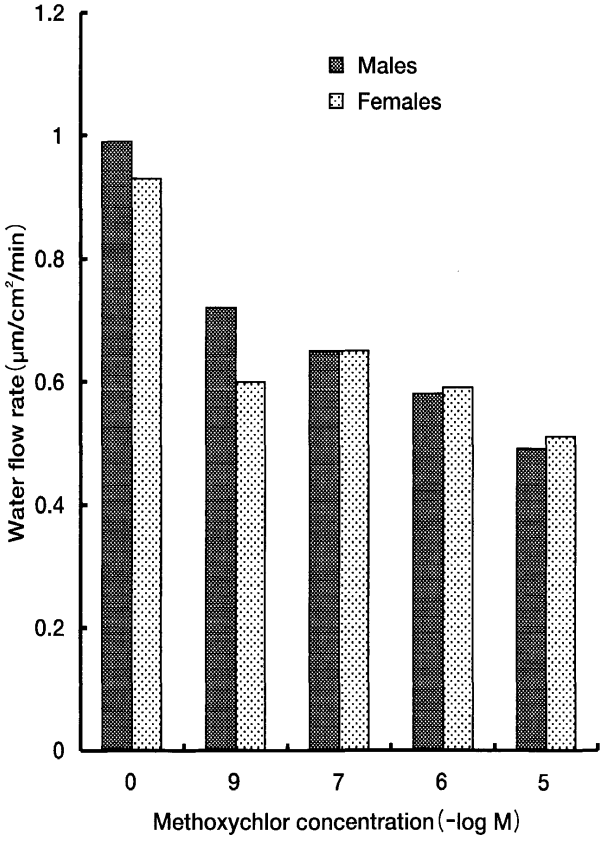

Fig. 4 Cutaneous water absorption rate under dehydrated condition in Japanese tree-frogs after being kept for one month in water with various concentrations of methoxychlor. Under the dehydrated circumstance, water absorption in tree-frogs of the both sexes were affected concentration wise up to 10^{-9} M of the agent.

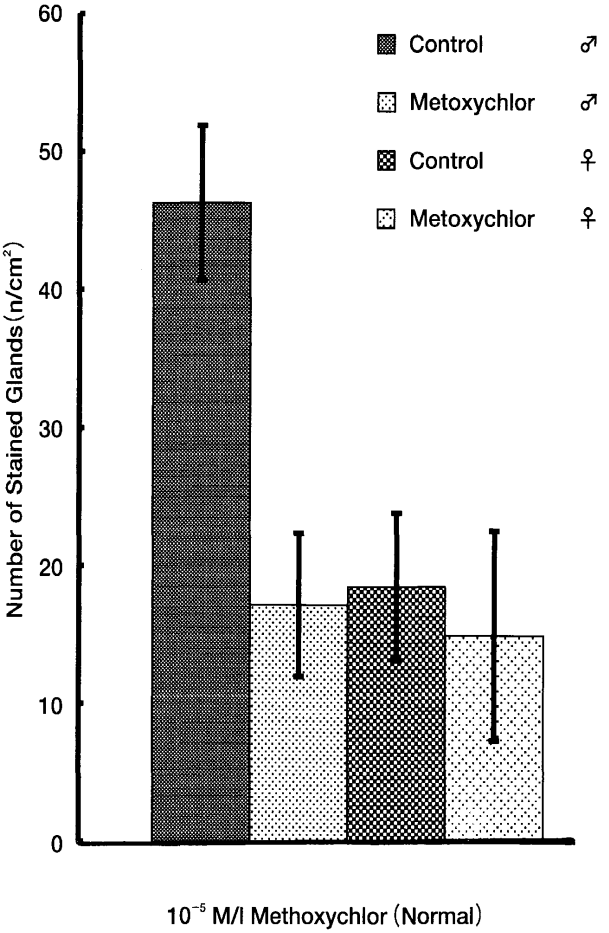

Fig. 5 Number of water transporting glands (granular glands) traced with neutral-red stain in Japanese tree-frogs being kept for one month in 10^{-5} M methoxy-chlor water. In male frogs, number of the glands was markedly reduced after the methoxychlor treatment. In females, no significant changes were observed. These morphological observations are in perfect accordance with results of water absorption measurements (see Fig. 2).

most frogs in Japan, insecticides and/or herbicides applied to the fields during the rice cultivation seems to be one of possible agents that might negatively affect anuran population in this country. As has been reported previously, Japanese tree-frogs have two types of water absorbing systems in their ventral skin [7, 8]. The one, the ordinary system, works under any conditions, but with low absorption rate, the other, the enhanced one, works under the dehydrated environment, and with an accelerated absorption rate. Furthermore, in the ordinary water absorption system, there is the sexual difference between males and females. Males absorbed water more readily than females. This sexual difference in the water absorption rate would suggest a possibility of endocrine disruptors, such as methoxychlor that feminize males in various species [12, 13], may also disturb the water absorption system of frogs, especially in male tree-frogs. Present results show the agent clearly suppresses water intake of male frogs under ordinary wet circumstances by a concentration comparable to that remaining in the environment after the agricultural uses. After keeping frogs for one month in the methoxychlor water, the enhanced water absorption system was severely affected in both sexes even by one to ten thousandth less concentration than the ordinary system (Figs. 4 and 5). This means tree-frogs that have spent a certain period in contaminated water with methoxychlor would not take water properly and if dry weather (drought) comes afterward, it would make their survival difficult. How this insecticide causes the ordinary water absorption system of Japanese tree-frogs to become disabled is not clear. However, vital staining of the skin shows stained granular mucous glands in which the ordinary water absorption takes place (not published) was decreased in number (Fig. 5). Since the activity of mucous glands are controlled by prolactin or sex hormones, mathoxychlor, the feminizing agent, may disturb the water absorption via these hormones.

Since amphibian decline in this country seems partly to be due to the decrease of habitat by expansion of human activity and endocrine disruptors, such as insecticides that affect the internal integrity of animals, such decline would effect the regional or global fauna leading to further destruction of the ecosystem.

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