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Salinity Tolerance of Eggs of *Buergeria japonica* (Amphibia, Anura) Inhabiting Coastal Areas

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***Buergeria japonica* is one of a few frogs that breed in coastal areas. To understand why this species can breed in coastal areas, I tested the salinity tolerance of eggs of *B. japonica* collected from a coastal area of Okinawa Island, Japan. All eggs hatched within four days after oviposition. At 0‰ salinity (control), over 94% of eggs hatched normally, and even at 1‰ salinity over 85% of eggs hatched. Survival rate of eggs was low at 2, 3, and 4‰, and no eggs hatched at 5‰ salinity. These results indicate that low salinity, close to pure water, is necessary for successful egg development, even for populations of *B. japonica* that breed in coastal areas. Future studies are necessary to examine whether females of *B. japonica* breeding in coastal areas select appropriate oviposition sites where the environmental salinity level is sufficiently low for eggs.**

Key words: salinity tolerance, amphibian eggs, *Buergeria japonica*, coastal area, egg mortality

INTRODUCTION

Coastal areas are considered a risky habitat for amphibians because adults and tadpoles have poor osmoregulatory ability at high salinity levels (Boutilier *et al*., 1992). In particular, high salinity leads to high mortality of eggs (Duellman and Trueb, 1986), decreasing the probability of successful breeding and development (Beebee, 1985; Uchiyama *et al*., 1990a; Viertel, 1999). Accordingly, few amphibian species breed in coastal areas (Balinsky, 1981). Only a few frog species can inhabit coastal as well as inland areas. *Buergeria japonica* is a ground-dwelling rhacophorid frog distributed on most islands of the Ryukyu Archipelago of Japan and on Taiwan. In the breeding season, the frogs retreat to places such as the rocky banks of streams during the day and lay eggs at night. This species has been reported to lay eggs occasionally in the mouths of streams, no more than 100 m from the sea (Maeda and Matsui, 1989). Haramura (2004) reported that the salinity of oviposition sites in coastal populations of *B. japonica* on Okinawa Island is continuously 2‰ or less despite their exposure to the sea breeze. However, no information is available on the salinity tolerance of eggs of *B. japonica.* To fully understand the adaptation of *B. japonica* to the coastal environment, we must determine the upper salinity-tolerance limit of the eggs. In the present study, I experimentally tested the survival rate of eggs of *B. japonica* at several salinity levels.

METHODS

Experiments were conducted in September 2006, which is late in the breeding season of *B. japonica*. To obtain eggs, amplexed pairs found in the water or alongside streams were captured between 2100 and 2400 h in an area on the coast where a study on oviposition site selection by *B. japonica* has been underway (from the shoreline up to approximately 230 m inland; Okinawa Island, Japan; 26°50'N, 128°17'E). Each pair was placed separately into a plastic cup and taken to the Yona Experimental Forest Research Station, University of the Ryukyus, approximately 6 km from the field study site. Females deposited egg masses by the morning after capture, and the salinity-tolerance experiment started within 8 hours of egg deposition. Eggs of *B. japonica* are small (1.2–1.4 mm diameter); average clutch size is approximately 450, and no foam nest is constructed. Because a pilot experiment revealed that all eggs die at salinities greater than 5‰ (Haramura, unpublished data), the present experiment was conducted at six salinity levels (0‰=control, 1‰, 2‰, 3‰, 4‰, and 5‰). Salinity was adjusted with tap water and NaCl (control=0‰, no NaCl; 1‰, 0.70g NaCL/700ml; 2‰, 1.40g/700ml; 3‰, 2.10g/700ml; 4‰, 2.81g/700ml; 5‰, 3.51g/700ml). Before the experiment, tap water was left for a few days in an open container to evaporate chloride. The salinity of seawater in the study area is approximately 33‰.

The egg mass of each female was divided into six portions of 20 eggs each. Each portion was then selected randomly, placed in a plastic cup containing 700 ml of water at one of the salinity levels, and left to develop at ambient temperature. Eggs were completely immersed in the solution. Room temperature was almost the same as the outside air temperature during the experiment because I kept windows open. The number of live and dead eggs, water temperature, and pH were recorded daily during the experiment. Water was renewed daily, and dead eggs were removed. Eggs were assumed to be dead when egg development stopped or the embryo was infected with fungus (Gosner and Black, 1957). Embryos were scored as alive if they completely escaped from their membrane. Any deformities in hatchling larvae were recorded. I maintained eggs in another cup with tap water to examine the rate of normal larval development. Five eggs were removed every six hours and fixed with formalin. The stages of larval development in tap water (0‰ salinity level) followed the classification of Gosner (1960), and are shown in Table 1. Each experimental treatment was continued until all eggs either hatched or died. The trial at each salinity level was replicated six times, using six egg masses laid by six females. Immediately after the termination of each trial, embryos were

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Table 1. Larval development of *Buergeria japonica* in tap water (water temperature 27.4–31.5°C). Development stage follows that described by Gosner (1960). Experiment started within 8 hours of egg deposition.

Time after fertilization	Start	6h	12h	18h	24h	30h	36h	42h	48h	54h	60h	66h	72h	84h	96h	108h	120h	132h	144h
Gosner stage	8–9	12	15–16	18	19	20	20–21	21–22	21–22	22	22–23	22–23	23	23–24	24	24–25	25	25	25

released at the sites where their parents were collected. I used a total of 720 eggs in the experiment.

The Friedman test was used to compare the survival rate of eggs among salinity levels, water temperatures, and pH levels during the first two days of the experiment. The egg survival rate was compared across treatments for the entire duration of the experimnet. Because of small sample sizes due to the death of eggs in later days of the experiment, water temperature and pH were examined only for the first two days. To examine where differences resided among salinity levels, I performed Steel-Dwass post-hoc tests.

RESULTS

The overall ranges of experimental water temperature and pH were 27.2°C–28.7°C and 8.1–8.9, respectively. There was no significant difference in water temperature

Table 2. Mean±SE of water temperature (WT) and pH at six salinity levels (0‰–5‰) throughout the experiment. Figures in parentheses indicate sample size.

		Exposed day				
Salinity level		Start	1 day	2 day	3 day	4 day
0‰ (control)	WT	28.0±0.1	27.5±0.1^{acd}	27.8±0.1	28.3±0.1	28.3
	pH	8.5±0.04^{e}	8.7±0.1	8.8±0.1	8.5±0.04	8.8
		(6)	(6)	(6)	(6)	(1)
1‰	WT	27.9±0.03	27.4±0.1^{ab}	27.7±0.1	28.2±0.1	–
	pH	8.4±0.03^{efghi}	8.4±0.02^{jkl}	8.4±0.05	8.3±0.03	–
		(6)	(6)	(6)	(6)	–
2‰	WT	27.9±0.03	27.4±0.1^{ab}	27.7±0.1	28.2±0.1	–
	pH	8.3±0.02^{f}	8.4±0.03^{mno}	8.3±0.03	8.4±0.1	–
		(6)	(6)	(6)	(3)	–
3‰	WT	27.9±0.04	27.4±0.1^{c}	27.9±0.2	28.3	–
	pH	8.3±0.01^{g}	8.3±0.01^{jm}	8.4±0.1	8.3	–
		(6)	(6)	(3)	(1)	–
4‰	WT	27.9±0.04	27.4±0.1^{c}	27.9±0.1	28.2	–
	pH	8.3±0.04^{h}	8.3±0.02^{kn}	8.3±0.1	8.3	–
		(6)	(6)	(3)	(1)	–
5‰	WT	28.1±0.1	27.5±0.1^{d}	28.2	28.5	–
	pH	8.3±0.02^{i}	8.3±0.02^{lo}	8.5	8.5	–
		(6)	(6)	(1)	(1)	–

The same letter indicates not significant by post hoc test (Steel-Dwass) at the 0.05 level.

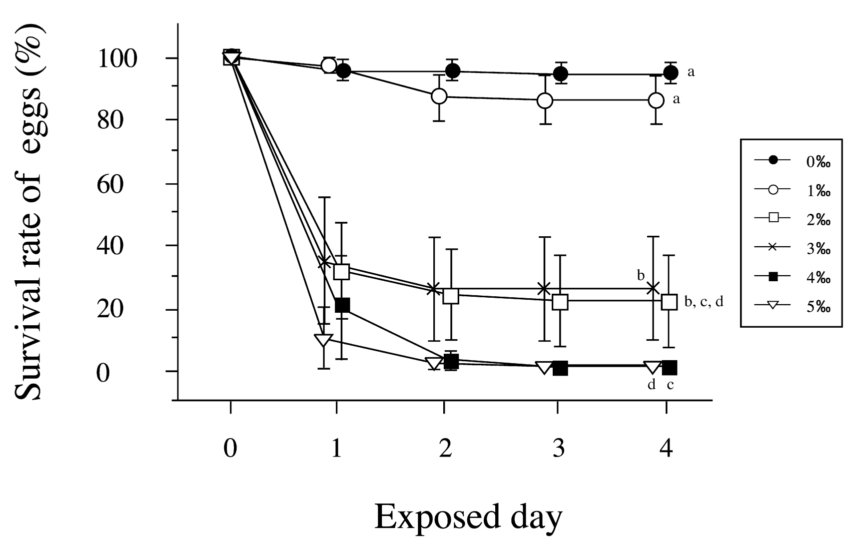

Fig. 1. Effects of salt exposure time on egg survival rates. Six replicates of twenty eggs each were placed in each salinity level (0–5‰) at the start of experiment. Means±SE are shown. The same letter indicates not significant by post-hoc test (Steel-Dwass) at the 0.05 level.

among the six salinity levels on the starting day (Friedman test, df=5, χ^2=10.64, P>0.05), but there were significant differences 1 day after (Friedman test, df=5, χ^2=13.31, P<0.05). There were significant differences in pH among the six salinity levels on both the starting day and 1 day after (Friedman test; starting day, df=5, χ^2=15.33, P<0.01; one day after, df=5, χ^2=18.45, P<0.01; Table 2). Approximately 94% of eggs at 0‰ salinity (control) developed and hatched by the fourth day; at 1‰ salinity, approximately 85% of eggs hatched. At 2‰ and 3‰ salinity, the hatching rate of eggs was lower than 30%. At 5‰ salinity, no eggs developed to hatching. At 0‰ salinity, hatching was observed on the second day (48 h) after fertilization. Salinity level significantly affected egg survival, which was high at 0‰ and 1‰ salinity and low at 4‰ and 5‰ (Friedman test, df=5, χ^2=21.05, P<0.001; see Fig. 1). Deformities in hatchling larvae were observed at elevated salinities (1‰, one hatchling; 2‰, two; 3‰, one), but no deformities were observed at 0‰.

DISCUSSION

Because few amphibian species breed in coastal areas (Balinsky, 1981), there have been few studies on the survival rate of amphibian eggs under saline conditions. The amphibian species most intensively studied with regard to egg development in saline conditions is the crab-eating frog (*Rana cancrivora*), which lives in brackish water such as mangrove swamps. Adults and tadpoles of *R. cancrivora* have a wide range of salinity tolerance, from freshwater to 80% seawater (26.4‰ salinity; Gordon *et al*., 1961; Gordon and Tucker, 1965, 1968), but eggs fail to develop even in 10% seawater (approximately 3.3‰; Uchiyama *et al*., 1990a). In *Bufo calamita,* which sometimes lives in areas frequently inundated with seawater, Beebee (1985) reported that eggs are killed by 15–20% seawater (approximately 4.95–6.6‰ salinity). The present study showed that most eggs of *B. japonica* survived and hatched at 1‰ salinity. However, most eggs failed to hatch at 2 and 3‰, and all eggs died at 5‰. These results indicate that low salinity, close to pure water, is necessary for successful egg development, even though the population of *B. japonica* studied here breeds in a coastal area.

Low pH in ambient water (approximately pH 3.6–4.6) also interrupts the embryonic development of eggs (Gosner and Black, 1957; Dunson and Connell, 1982; Freda and Dunson, 1985; Tyler-Jones *et al*., 1989; Beattie *et al*., 1992; Picker *et al*., 1993). Although pH values measured on the starting day and 1 day after were different among salinity levels, there were no low values (pH 8.1–8.9). Therefore, the differences in egg mortality among salinity levels could not be attributed to differences in pH. Differences in pH among the experimental treatments may simply have been caused by the differences in salinity. Water temperature 1 day after showed significant differences among the experimental treatments; the water temperature at 0‰ and 5‰ salinity was approximately 0.1°C higher than that at other salinity levels (see Table 2). The cups containing the 0‰ and 5‰ salinity treatments were positioned in the outmost rows of cups, and their slightly higher water temperature may have been caused by this positional difference. At 0‰ salinity (=control), more than 94% of eggs hatched by the fourth day, and there were no hatching failures or deformities in the hatchling larvae. Deformities in hatching larvae were observed in the salinity treatments. This was likely the result of elevated salinity; deformities in hatching larvae due to elevated salinity have been reported in other studies (Gordon and Tucker, 1965; Bebee, 1985).

The low salinity tolerance of amphibian eggs may induce deliberate oviposition behavior by females that breed in coastal areas. There has been no study concerning the oviposition behavior of amphibians inhabiting coastal areas; therefore, it is unknown how these females avoid high salinity for the successful development of their eggs. According to my research on *B. japonica* inhabiting a coastal area, Okinawa, Japan, the salinity at coastal oviposition sites is 2‰ or less (Haramura, 2004). Females move from a broad area upstream to a stream mouth and lay eggs in several masses (Haramura, 2005). In addition, an experiment on oviposition site selection showed that females are able to distinguish salinity levels in order to avoid high salinity for egg laying sites, by discriminating salinity levels higher than 2‰ (Haramura, submitted data). Several studies have shown salinity tolerance for adult frogs (Gordon *et al*., 1961; Gordon, 1962; Schmidt-Nielsen and Lee, 1962; Uchiyama *et al*., 1990b). It is unknown how well adult *B. japonica* are able to tolerate high salinity, but at my field study site, both males and females were observed to remain and court in high-salinity areas influenced by saltwater ripples at high tide. I hypothesize that females of *B. japonica* breeding in coastal areas select appropriate oviposition sites where the salinity is low enough for eggs to develop successfully. More information is needed on oviposition behavior to understand amphibian adaptations to coastal environments.

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