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Effect of Water Hardness on Oocyte Quality and Embryo Development in the African Clawed Frog (*Xenopus laevis)*

Earl W. Godfrey, PhD[1,*] and George E Sanders, DVM[2,3]

Husbandry and health of the African clawed frog, *Xenopus laevis,* greatly influences the quality of oocytes produced. One factor affecting oocyte quality is the water conditions in which females are maintained. Dechlorination and adequate salt concentration are known to affect oocytes, but water hardness has not been considered an important factor in *Xenopus* husbandry by the research community. We found that, when females were kept in soft water or water with marine salts alone, even when it was cooled to 17 to 18°C, the quality of oocytes decreased; only 20 to 25% of resulting embryos developed to tailbud stages. Survival and normal development of embryos increased significantly within one month of addition to the laboratory housing water of salts that mimic conditions in African Rift Valley lakes. These salts greatly increased water hardness; development of embryos to tailbud stages remained high (50 to 70% on average) for more than a year after their addition to the water housing females. Water from South African ponds where *X. laevis* are collected, and from wells used by the major suppliers of *X. laevis*, also was moderately to very hard. Our results suggest that *X. laevis* is naturally adapted to hard water, and indicate that increasing general hardness during laboratory housing is more important for oocyte quality and embryo development than is increasing carbonate hardness (alkalinity) in the water used to house females.

The African clawed frog, *Xenopus laevis,* has been a common research animal for over a century, and its embryos are especially valuable for studies of early development (12, 15). Within the research community that uses *Xenopus* embryos, it has long been observed that quality of oocytes and embryos varies, depending on the health and husbandry conditions of the adult females producing the oocytes. Factors that affect oocyte quality include nutrition, light cycles, season of the year, water temperature, salinity (typically measured by conductivity), water contaminants or toxins (chlorine, ammonia, nitrate, nitrite), and disease (12, 16). Water quality, specifically hardness, has not been considered an important variable affecting oocyte quality and embryo development. In addition, water quality preferences of *Xenopus* in natural habitats have not been well characterized (15). However, adequate water hardness may be an important factor for maximizing oocyte quality and embryo development (3).

After moving the research laboratory of the first author (E.W.G.) from an area of moderately hard city water (Milwaukee, Wis.) to one of low municipal water hardness (Norfolk, Va.), we experienced increasing softness of *Xenopus* oocytes and early embryos. These embryos were easily deformed and often difficult to inject with a 10- to 20-µm tip needle; performing more advanced techniques, such as transgenesis by nuclear transplantation (5), proved even more difficult. This problem persisted for two years, even after the addition of sodium chloride or marine salts (1 g/L) to the water in which females were housed (16). Transfer of our adult frogs into a recirculating system in which the water was cooled to 17 to 18°C did not alleviate the poor oocyte quality, even after six months to a year. Suspecting that toxins were present in our dechlorinated tap water (which had been passed through an activated charcoal filter), we began to treat our city water source by reverse osmosis and deionization, with additional charcoal filtration, prior to addition of marine salts in the recirculating frog housing system. Subsequently, oocyte quality decreased even further, resulting in only 20 to 25% normal development of embryos to tailbud stages.

After contacting many investigators for suggestions and eliminating the possibility of infectious disease or other pathological changes by necropsy and histologic evaluation of females, we decided to test the effect of increasing water hardness in the housing of adult females on the quality of oocytes and the development of embryos derived from the oocytes. We found that housing females in hard water greatly improved the firmness of oocytes and early embryos and their survival and normal development. The most important factor seemed to be high concentration of calcium and magnesium ions (general hardness [GH]), rather than an increased concentration of carbonates and bicarbonates (carbonate hardness, alkalinity or KH). In many localities with a supply of soft water, oocyte and embryo quality may be improved by addition of fresh water salts to the water in which females live, to increase hardness and conductivity.

Materials and Methods

Animal husbandry and care. Adult female *X. laevis* (oocyte positive, 9+ cm long) were obtained from NASCO (Ft. Atkinson, Wis.). Frogs housed in static containers were transferred into

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[1]Department of Pathology and Anatomy, Eastern Virginia Medical School, P.O. Box 1980, Norfolk, Virginia 23501, [2]Department of Comparative Medicine, University of Washington School of Medicine, T-160 Health Science Center Box 357190, Seattle, Washington 98195-7190, and [3]Western Fisheries Research Center, 6505 NE 65th Street Seattle, Washington 98115.
[*]Corresponding author.

dechlorinated tap water (treated with AmQuel [0.1 ml/L; Kordon, Hayward, Calif.]) on arrival and kept at room temperature (21 to 22°C) in large opaque plastic containers (Rubbermaid, Winchester, Va.) containing four liters of water per frog (2 to 4 frogs/container). Animals were fed Frog Brittle (NASCO), and water was completely exchanged one to three hours after feeding, three times per week.

Most females were kept in the laboratory's recirculating housing unit (X-Mod, Marine Biotech, Inc., Beverly, Mass.). The system contained a total of about 100 females at the time of the static tank experiments in early 2002. A maximum of six female frogs was housed in each of 16 clear polycarbonate cages (23-L capacity each). Females were ovulated every six to 12 weeks, depending on our need for oocytes. Tap water entering the X-Mod was purified by reverse osmosis and deionization (to ≥ 17-megaOhm conductivity), dechlorinated by passage through three activated charcoal filters in series, and cooled to 16 to 17°C by a water-cooled condenser. Salts were added continuously to the X-Mod recirculating housing system, using a peristaltic pump to maintain constant conductivity of 2,000 to 2,100 µS, and 10 to 20% of the water was replaced daily. Temperature, conductivity, and pH were monitored and recorded daily, using a Hanna four-in-one meter (Hanna Instruments, Providence, R.I.) and a pH meter. The GH and KH values were determined weekly using the Tetratest GH/KH test kit (Tetra, Blacksburg, Va.). Ammonia (ionized and unionized), nitrate, and nitrite concentrations were monitored weekly, using a test kit (Aquarium Pharmaceuticals, Inc., Chalfont, Pa.).

Xenopus were maintained according to the *Guide for the Care and Use of Laboratory Animals* (7) and the United States Public Health Service Policy on the Humane Care and Use of Laboratory Animals. Their experimental use was approved by the Institutional Animal Care and Use Committee of Eastern Virginia Medical School. Frogs were euthanized by administration of an overdose of benzocaine (0.6 g/L, ≥ 10 min; Sigma Chemical Co., St. Louis, Mo.).

Testing of salt mixtures in static tanks at room temperature. To test various salt conditions, 15 newly purchased females were transferred into dechlorinated, deionized water containing the test salts within one week of arrival. Frogs were randomly assigned to groups of two to four per tank. The following salt mixtures (in dechlorinated, deionized tap water) were tested: Marine salts (MS; Marine Biotech, Inc., Beverly, Mass.), Cichlid Lake salts (CLS; Seachem, Inc., Stone Mountain, Ga.), CLS plus Equilibrium salts (ES; Seachem, Inc.), Marine salts plus ES, and dechlorinated municipal tap water alone. The pH of all solutions was adjusted to 7.5, using sodium bicarbonate (0.625 g/L, Sigma Chemical Co.) or alkaline buffer (Seachem, Inc.) containing carbonates and bicarbonates. The water in the static tanks was monitored for GH, KH, pH, and conductivity at two- to five-day intervals. Frogs were fed, and water was exchanged as previously described.

After four, 10, and 16 weeks of laboratory housing, ovulation was induced by injection of 700 IU of human chorionic gonadotropin (HCG; Sigma Chemical Co.) into the dorsal lymph sac of females. The next morning, oocytes were obtained in two clutches of 100 to 400 from each female by manual expression, and were fertilized with sperm from the testes of a freshly euthanized male frog (6). Embryos were de-jellied with 2% cysteine and incubated in 100-mm petri dishes (100 to 200 embryos/dish) in 10 ml of 0.1X modified Barth's saline (9) at room temperature. In some experiments, gentamicin (50 µg/ml; Mediatech, Inc., Herndon, Va.) was added to this medium to inhibit growth of suspected pathogenic bacteria. The embryos' medium was changed daily when fouled by lysed embryos, and developmentally abnormal or dead embryos were removed daily. The number of eggs fertilized and the number of embryos developing normally to tailbud were counted (stages 31–34 [8]) to calculate percentage of normally developing embryos. Oocytes of poor quality were soft and flattened, and/or had uneven pigment distribution in the animal pole. After fertilization, embryos derived from such eggs exhibited abnormal cleavage or gastrulation and subsequently died.

Table 1. Salt mixtures used to increase water hardness in recirculating system

12X Concentrated salts mixture	
MS	7.2 g/L
CLS	4.8 g/L
ES	0.8 g/L
12X Concentrated buffer	
Alkaline buffer (Seachem; increases KH)	4.4 g/L
Acid buffer (Seachem; use more if needed to reduce pH to 7.5)	3.9 g/L
Final concentrations of salt mixtures in recirculating *Xenopus* housing	
MS	0.6 g/L
CLS	0.4 g/L
ES	0.067 g/L
Alkaline buffer	0.36 g/L
Acid buffer	0.325 g/L

The concentrated salt solutions must be made up separately, because a precipitate forms if they are dissolved together. Tubing from the reservoirs containing these solutions is connected into a common tube, and the mixture is pumped into the reservoir of the recirculating system at a rate a sixth that of entering deionized water (a twelfth the rate if the two solutions are pumped separately). Resulting water should have pH of about 7.5 and conductivity of 2,000 to 2,100 µS. The MS can be varied to adjust total salt concentration (conductivity).
MS = marine salts; CLS = cichlid lake salts; ES = equilibrium salts.

Addition of salts to recirculating housing system. Initially, a 12-fold concentrated mixture of CLS (4.8 g/L) and marine salts (7.5 g/L), and a 12-fold concentrated mixture of alkaline buffer (4.4 g/L) and acid buffer (Seachem, Inc.; 3.9 g/L) were mixed separately. Tubing from the two containers was joined to produce a 1:1 mixture of salts and buffers. This solution was added continuously to the reservoir of the X-Mod recirculating housing system, using a peristaltic pump. Beginning 17 weeks later, ES (0.8 to 2.4 g/L) was added to the salt concentrate, with a corresponding decrease in marine salts (6 g/L), so that conductivity remained approximately 2,000 µS (Table 1).

Necropsy. To rule out potential infectious disease or reproductive pathological changes, one male and one female adult frog (apparently healthy) from the recirculating system were euthanized as previously described, and tissues were fixed in neutral-buffered 10% formalin. After routine histological processing, tissues were embedded in paraffin blocks, then 5-µm-thick sagittal sections were cut on a rotary microtome (American Optical, Buffalo, N.Y.) and transferred to glass slides (Fisher Scientific, Pittsburgh, Pa.). Slides were stained with hematoxylin and eosin (H&E) and were mounted with glass coverslips. Histological examination, using brightfield microscopy, was performed on sections from all major organs by a veterinary pathologist. One female frog that died after ovulation during the earlier period (2001) when egg quality and embryo development

Table 2. Effect of salt mixtures on embryo development and water general and carbonate hardnesses (GH and KH) in static housing system

Salt mixture	Hardness (ppm)		Normal embryos at tailbud stages (%)		
	GH	KH	4 wk	10 wk	16 wk
City tap water (dechlorinated)	62	79	43	...	...
MS (1 g/L in deionized water)	112	50	37	34	...
CLS (0.19 g/L)	104-142	74-99	<u>64</u>	14	30
CLS + ES (0.19 g/L + 0.375 g/L)	186-241	93-112	22	20	<u>69</u>
MS + ES (1 g/L + 0.375 g/L)	196	178	26	...	...

Newly purchased oocyte-positive females (three to four per container) were housed in water containing various salt mixtures. At the times indicated, oocytes from two to (usually) three females in each group were used for two fertilizations for each experiment. The percentage of embryos developing normally was determined by counting normal embryos at tailbud stages (approx. 48 h at 21 to 22°C). Ellipses (...) in table represent conditions that were not evaluated. Underlined values are the highest percentage of survival and normal development observed in these experiments.
See Table 1 for key.

were poor was similarly evaluated.

Water quality. We obtained samples of the water used to house frogs from two commercial *Xenopus* vendors in the United States (Xenopus Express, Plant City, Fla.; Xenopus I, Dexter, Mich.) and one in France (Xenopus Express, Vernassal, France). We also obtained water analysis results from another vendor (NASCO). Water samples collected in March 2003 (autumn) from five ponds in Western Cape Province, South Africa, from which *X. laevis* are commercially obtained, were provided (courtesy of Guy Pluck, Xenopus Express, Vernassal, France). Water from our laboratory's recirculating system, the three vendors, and the South African ponds was analyzed by ion chromatography (IC, Dionex Co., Sunnyvale, Calif.), an inductively coupled argon plasma machine (ICP, Thermo Jarrel Ash Co., Franklin, Mass.), and a pH meter (PHM 85 Precision pH Meter, Radiometer Co., Copenhagen, Denmark) in the Analytical Services Center, Department of Forestry, University of Washington (Seattle, Wash.). Ammonia, nitrate, and nitrite concentrations were measured with a water quality test kit (Hach Co., Loveland, Colo.). A total of 50 ml of water was provided per sample, and results were reported in parts per million (ppm = milligrams per liter).

Results

Static tank experiments. Normal development of embryos to tailbud stages was assessed after four, 10, and 16 weeks of housing females in various water conditions at room temperature. After four weeks, eggs from females kept in CLS produced embryos with the highest survival and normal development (64%; Table 2). After 10 weeks, this value decreased to only 14%, and the highest survival and normal development (34%) was seen using eggs from frogs housed in marine salts. However, after 16 weeks, the combination of CLS and ES gave the best results (69% normal development). Water containing CLS and ES also had the highest water hardness (GH and KH; Table 2). Females kept in the various solutions provided about the same number of oocytes.

Recirculating system. Because of the history of poor oocyte quality (20 to 25% normal development) from females housed in soft water in our recirculating system, results of the first static tank test were immediately applied to the water conditions of the X-Mod in February of 2002. Cichlid lake salts were added to increase GH, and the concentration of marine salts was decreased to maintain a constant conductivity (2,000 μS). Alkaline buffer, mixed with acid buffer to maintain a constant pH between 7.5 and 7.8, was introduced to increase KH. The GH increased from 125 to 185 ppm, but KH did not increase in response to this initial salt mixture (Fig. 1). One month after this change in salt composition, survival and normal development of embryos increased

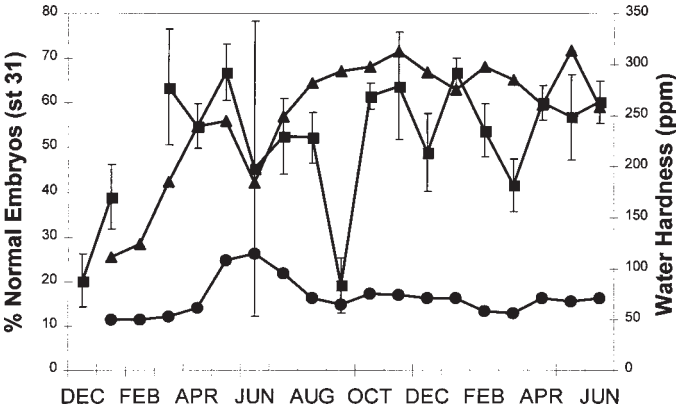

Figure 1. Effect of water hardness in recirculating housing system of female *Xenopus laevis* on survival and normal development of embryos derived from their oocytes. Water hardness was increased by addition of cichlid lake salts (CLS, beginning in February of 2002), of equilibrium salts (ES, June), and of alkaline and acid buffers (January), as described in *Materials and Methods* and *Results*. Blue squares represent the percentage of embryos that survived and developed normally to tailbud (stages 31–34). Purple triangles indicate general hardness (GH) values (ppm), and red circles indicate carbonate hardness (KH) values (ppm). Values shown for normal development are mean (± SEM, error bars) for embryos derived from eggs of individual females (n = 8 to 27) ovulated over the course of one month. Values for GH and KH are averages for each month of measurements made one to three times per week.

to 63% (in March of 2002), which was markedly better than the previous 20 to 40% in December of 2001 and January of 2002 (Fig. 1). One-cell-stage embryos had previously often been soft, flattened, and easily deformed, and in extreme cases, blastomeres did not adhere well to each other after the first several cleavages. Abnormal gastrulation and failure of neural tube closure had been common in these embryos. After the increase in water hardness of the housing of females providing the oocytes, one-cell embryos became firmer and easier to inject than under previous water conditions. They seemed to have a tougher vitelline envelope as well. Normal development of embryos to tailbud stages averaged 45 to 65% for an additional 15 months after this improvement, with the exception of September of 2002, when embryo survival and development were low.

To increase KH, the alkaline buffer concentration was increased from 0.2 g/L to 0.36 g/L in April of 2002, but no further increase in embryo survival and development resulted. Alkaline buffer contains carbonates and bicarbonates (1). Subsequently, ES was added to the mixture in a stepwise fashion in June of 2002 and was increased again in July of 2002 (range, 0.067 to 0.2 g/L) to further

Table 3. Water analysis of *Xenopus laevis* vendors, municipal water, and laboratory housing

Component	NASCO	X. Express	Xenopus I	X. Express France	Municipal	X-Mod
GH	340	213	445	214	39	250-300
KH	350	142	338	214	19	50-70
pH	7.4-7.6	8.1	8.0	7.5	7.4	7.4-8.0
Conductivity	490	280	256	425	131	~2,000
Calcium	64	46	94	54	10	33
Magnesium	41	4.7	29	8.5	3.4	36
Sodium	5.4	3.8	14.8	22	11	341
Sulfur	13	2.3	11.5	5.9	22	148
Bicarbonate	ND	0.23	0.22	0.44	ND	0.14
Ammonia	ND	0.01	ND	0.01	1	0
Nitrite	ND	0	ND	0	0	0
Nitrate	ND	ND	ND	ND	0	0

Elemental analysis values were determined by ion chromatography (IC) and the inductively coupled argon plasma (ICP) machine or with commercial test kits (*Materials and Methods*), or were provided by one supplier (NASCO). The GH and KH values (ppm or mg of $CaCO_3$/L), conductivity (μS), and concentrations of ammonia, nitrite, and nitrate were measured as described in *Materials and Methods*. Values for specific ions from NASCO are for water from nearby municipal wells. Norfolk, Va. municipal water data are from July of 2001, when oocyte quality severely decreased. The X-Mod values are from the period after hardness and development rate increased.
ND = Not determined.

Table 4. Analysis of water from South African collection ponds

Component	Pond 1	Pond 2	Pond 3	Pond 4	Pond 5
GH	134	178	374	249	89
KH	107	178	258	160	80
pH	7.4	7.5	7.2	7.5	6.9
Conductivity	419	637	1,550	1,002	294
Calcium	26	38	41	25	18
Magnesium	10	15	54	32	7.2
Sodium	45	72	243	147	28
Sulfur	2.8	4.4	10.8	1.7	2.3
Bicarbonate	0.25	0.42	0.59	0.38	0.16
Ammonia	0.02	0.27	0.16	0.11	0.003
Nitrite	0	0	0	0	0

Elemental analysis values were determined by IC and ICP or commercial test kits (*see Materials and Methods*).
Ponds: 1 = Eikendal Vineyards, Stellenbosch; 2 = Happy Vale, Stellenbosch; 3 = Klawervei Lower Dam, Koelenhof; 4 = Klawervei, Upper Dam, Koelenhof; and 5 = Vrede, Koelenhof—all located in Western Cape Province, South Africa.
See Table 3 for key.

increase hardness. Neither of these changes improved average embryo survival and development. Therefore, we have now reduced ES to a final concentration of 0.067 g/L, along with 0.625 g of marine salts/L and 0.4 g of CLS/L (Fig. 1). Our current recipe for the concentrated salt mixtures and the final concentrations is shown for convenience in Table 1.

Necropsy and histopathologic findings. Gross evaluation of the female and male frogs did not reveal significant lesions. However, on histological examination, mild to moderate tubular nephrocalcinosis was observed in both frogs. Presence of this lesion is not consistent with any infectious or other deleterious disease process and is typically seen in frogs from several colonies (11).

During the initial period of poor egg quality and low normal development (August through September of 2001), two females manifested clinical signs of "red leg syndrome" and subsequently died. One of these frogs was necropsied and evaluated histologically. The pathological diagnosis was bacterial dermatosepticemia; however, isolation of specific bacterial pathogens from the dead frogs was not attempted.

Analysis of laboratory housing water and that from *Xenopus* vendors. Analysis of water samples from four commercial vendors of *Xenopus*, and water from our recirculating system, indicated that GH was high in all samples, whereas KH varied (Table 3). Calcium and magnesium levels were correlated with GH, as expected. Both measures of hardness were low in our municipal water supply. Salinity, as measured by conductivity, was low in the water from the vendors, whereas we maintained a conductivity of ~2,000 μS in our recirculating system. Therefore, the parameter that was consistently high among these water sources was GH, rather than KH or salinity.

Analysis of water from South African ponds. Water was obtained from five ponds in South Africa, from which *Xenopus* are collected commercially. Analysis of these samples, collected in autumn after a hot, dry summer, is shown in Table 4. Water from four of five ponds was moderately to very hard, with GH values > 130 ppm. Interestingly, pond 3, which had the hardest water (GH = 374 ppm, KH = 258 ppm), also contained the largest number of frogs (10). Water from this pond closely resembled water from our laboratory housing in GH and salinity (Tables 3 and 4). Thus, it appears that *Xenopus* can thrive and develop better in moderately to very hard water, in the wild and in captivity.

Discussion

Many investigators maintain adult *X. laevis* in dechlorinated tap water or add sodium chloride or marine salts to water to increase conductivity (12, 16). Oocyte quality and embryo development vary between groups of eggs from different females and seasons. This variation is often attributed to differences between different individual frogs. Although there is general awareness in the research community that pH and salinity of water are important for oocyte production, water hardness has not traditionally been considered as important for egg quality or embryo development. Municipal water sources may vary tremendously in hardness and conductivity on the basis of the geography and

hydrology of specific regions.

One also has to take into account the health status (infections, metabolic, or congenital diseases), nutrition, and environment (population density) of frogs producing oocytes of poor quality (15). Since frogs in each group used for our experiments were housed under similar conditions (in either static or recirculating systems) and fed a commercial balanced diet, the potential ramifications of these variables were minimized. In addition, gross and histological evaluation of affected individuals from this colony during the period of water quality modification did not reveal significant lesions indicative of infectious, reproductive, or other pathologic changes. The presence of two moribund females, one of which was diagnosed with bacterial dermatosepticemia ("red leg syndrome") during the initial period of poor oocyte quality and embryo development, was most likely associated with trauma incurred during manual handling for oocyte expression. Disruption of the slime layer with subsequent wound colonization by opportunistic bacteria was the most probable cause of this condition (14). After the increase in water hardness in the recirculating system and normal development of embryos, clinical signs of disease or obvious gross lesions were not seen in either males or females.

Bacteria were not cultured from the media of embryos made from oocytes of poor quality that did not develop to tailbud stage. However, in some experiments, gentamicin was added to the embryos' medium to inhibit the growth of suspected pathogenic bacteria. Inclusion of gentamicin in the medium did not improve oocyte quality or normal development of embryos. Thus, either pathogenic bacteria were not present in the medium, or bacteria that were present were resistant to this antibiotic.

Initially we tested the effect of housing females in static tanks of water containing various salt mixtures and varying degrees of water hardness on survival and normal development of embryos after fertilization of their oocytes. Although the results were variable, they suggested that increased water hardness in tanks housing females increased survival and normal development of embryos derived from their oocytes. On the basis of these results, we increased GH in our recirculating system by adding CLS at a concentration that replicated the chemical content of water from Lake Tanganyika, a rift valley lake in Africa. Use of this salt mixture resulted in GH values > 175 ppm and greatly increased embryo survival and normal development within a month of its addition. The one-cell embryos were also firmer and easier to inject, possibly because of a stronger vitelline envelope. We interpret these results to indicate that increasing the calcium and magnesium contents of the water, possibly in combination with addition of other salts similar to those present in African rift lakes, contributed to the improvement of oogenesis by unknown mechanisms, resulting in better quality of oocytes and subsequent development of embryos. Although we still find some biological and seasonal variability (poorer quality oocytes in late summer, July to September), oocyte quality and the percentage of embryos surviving and developing have improved considerably since water hardness was increased, and this improvement has now persisted for 18 months.

By comparison, water hardness also affects the successful development of offspring from fertilized eggs in various species of fish. In Atlantic salmon (*Salmo salar*), brook trout (*Salvelinus fontinalis*), and rainbow trout (*Oncorhynchus mykiss*), high calcium concentration (which affects GH) of ≥ 522 mg/L in incubation water significantly decreased the survival rate of fertilized eggs (4). In silver carp (*Hypophthalmichthys rabilis*), the hatching rate of fertilized eggs was greatest at a water hardness of 300 to 500 mg of $CaCO_3$/L, but was significantly decreased at either 100 or 600 mg of $CaCO_3$/L (2). In striped bass (*Morone saxatilis*), there was a significant increase in the hatching rate of fertilized eggs when they were incubated in hard (200 mg/L), as opposed to soft (41 mg/L) water (13). Similar studies have not been published for amphibians, specifically for anurans like *X. laevis*. It seems logical to extrapolate from fish that the optimal water quality, particularly GH, for embryo survival and development should be within a similar range (e.g., 200 to 300 mg/L) for amphibians. However, the fish studies only describe the effect of water hardness on eggs after they have been released or removed from the ovaries of the female, and do not discuss the effect of water quality on the adult fish and the endogenous process of oogenesis. More research is needed on the effects of water hardness on oogenesis proper in aquatic amphibians and fish.

Interestingly, in our study, increasing KH by further addition of another fresh water salt product, ES, in addition to CLS, did not further increase normal rates of embryo development. This mixture increased GH (to 300 ppm) and KH (to 100 ppm). Our data correlate well with the experiments done on salmon and trout eggs, which suggest that there may be a degree of hardness at which the beneficial effects of increased water hardness in fish ends (4). Determining this potential upper and the exact lower beneficial water hardness for *Xenopus* will require further experimental investigation.

Water samples from our recirculating system and from holding tanks at three commercial facilities in the United States and one in France were analyzed by IC and ICP. General hardness in all samples was in the range of 150 to 300 ppm, whereas KH varied from 70 to 350 ppm. Hardness (GH and KH) was lower in our municipal water supply, consistent with our observations of poor oocyte quality and low normal development when females were housed in water from this source. The water analysis results are consistent with our finding that increasing GH in the housing of females providing oocytes resulted in greater survival and normal development of embryos.

Water from South African ponds that serve as commercial sources of *X. laevis* was also moderately to very hard. Interestingly, the pond that contained the largest number of frogs observed by the commercial vendor also had the hardest water and the highest conductivity of any of the ponds. In fact, water from this pond was harder (GH and KH) than that in our laboratory housing, but the conductivity (salinity) of these two water sources was similar. Thus, it appears that *Xenopus* can thrive and develop better in moderately to very hard water, in the wild and in captivity.

The common features of all the water sources evaluated that resulted in high survival and normal development of *Xenopus* embryos are a high concentration of calcium and a high GH value. Thus, we conclude that water hardness should be an important consideration in housing *Xenopus* females to be used for oocyte production.

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