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Housing and Husbandry of *Xenopus laevis* Affect the Quality of Oocytes for Heterologous Expression Studies

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To assess the effect of *Xenopus* husbandry on oocyte quality for membrane transport physiology experiments, we compared a recirculating-water housing system with a static-water system in a 23-mo study. Two groups of frogs (n = 8) were maintained separately for the entire study: one group was housed in a multiinvestigator centrally managed *Xenopus* facility, which consists of 33 tanks placed on a shared and recirculating water system; the other group was housed in a satellite facility used by a single investigator and consisting of static tanks placed in a dedicated cold-room. The activity of a heterologously expressed membrane transporter was assessed every 4 to 5 wk for a total of 23 mo. Activity of the mouse cotransporter NKCC1 was assessed through isotopic ^{86}Rb influx measurements under 2 experimental conditions: stimulation of cotransporter by coinjection of regulatory kinases and by exposure to a hypertonic solution. The results showed a significant difference in the level of ion fluxes under these 2 experimental conditions between the 2 groups of oocytes. During the entire period, oocytes isolated from frogs maintained in the static facility demonstrated consistently robust NKCC1 function, whereas oocytes isolated from frogs maintained in the recirculating facility showed inconsistent and weaker cotransporter function. Furthermore, the oocytes isolated from frogs maintained in the recirculating facility showed significant deterioration during the summer months (April to August), a seasonal variation that was muted in frog oocytes maintained in the static facility.

The *Xenopus laevis* frog is native to wetlands, ponds, and lakes of subSaharan Africa. The *Xenopus* frog has been used as a source of protein, an aphrodisiac, and a fertility medicine[25] and as a laboratory research animal. During the 1940s, injection of a woman's urine into female frogs to produce eggs was used worldwide as a pregnancy test.[25] In a reflection on the centennial of the *Journal of Biological Chemistry,* Donald D Brown stated that "The *Xenopus laevis* egg and its possibilities for experimental manipulation were introduced to modern biology in the late 1950s by John Gurdon."[1] Isolated frog eggs were used first for nuclei transfer and then for studies on the control of gene expression or transcription and mRNA translation. In addition, oocytes from *Xenopus laevis* have been used extensively for the expression of foreign proteins after microinjection of cRNA into the cytoplasm or of cDNA into the nucleus (for reviews, see references 1 and 21). During the 1980s and 1990s, oocytes were used extensively for expression cloning. This method allowed the isolation and identification of the mRNAs encoding for membrane transport proteins. Typically, mRNA was isolated from tissues rich in a specific transporter or channel. This mRNA was microinjected into *Xenopus laevis* oocytes, and specific features of that transporter or channel were sought functionally. If the oocytes demonstrated functional expression, a plasmid cDNA library was generated, cRNA was transcribed from a subset of the library, and tested for function. When the function was identified, smaller subsets were tested until a single functional clone was identified. This method first was used for cloning of the Na^+-dependent glucose cotransporter in 1987.[12] After this initial success, a variety of membrane transporters,[24] channels,[15,16] and receptors[23,27] were cloned by using this method, including the cation–chloride cotransporters that our laboratory uses in its research.[5,6,7]

The relatively large size (1.0 to 1.3 mm) of the frog oocyte makes it easy to manipulate and provides many experimental advantages. It is an easy cell to use for direct injection of cRNA, cDNA, and proteins; for electrophysiology experiments; and for assessing tracer ion fluxes.[4] Furthermore, the *Xenopus laevis* oocyte is very active in gene transcription and protein synthesis. One advantage critical for our particular studies is the low expression or absence of expression of K^+ transport molecules, resulting in a low background level of K^+ uptake. Therefore, in contrast to mammalian cells, heterologous expression of transport proteins in *Xenopus* oocytes yields signals that are almost entirely due to the function of the foreign protein.

There are many factors that affect oogenesis and the quality of oocytes.[11,14,26] One of the key challenges identified early on by many laboratories and discussed in a 1990 review is the poorly understood seasonal variation in oocyte quality.[21] Variation in oocyte quality has been reported anecdotally even when room temperature and light:dark cycles are controlled tightly. In the mid1990s, expression cloning in *Xenopus laevis* oocytes was used to identify the thiazide-sensitive NaCl cotransporter,[8] the ATP-sensitive K^+ channel,[15] and the Ca^{2+} receptor.[2] Those investigators determined that keeping the frogs in aquaria with static water at a cooler and constant temperature in a modified cold-room minimized this seasonal variation.[12] Although published data are lacking, this belief generally has been accepted by many researchers who use the *Xenopus laevis* oocyte model system.

We have been using an environmental cold room as a static facility to maintain our frog colonies for the past decade,

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performing experiments throughout the year with consistently high-quality oocytes. Three years ago, our institution purchased and installed in its central housing facility a recirculating *Xenopus* aquatic housing system. Our laboratory isolates frog oocytes on a weekly basis to study the molecular physiology of the NKCC1 cotransporter, which we cloned in 1994.[3] Prior to moving our frogs to the new recirculating facility, we decided to conduct a long-term study to determine whether the functional activity of the heterologously expressed cotransporter differed between oocytes isolated from frogs maintained in the recirculating and static facilities. We measured cotransporter activity every 4 to 6 wk over a 2-y period and found a profound difference in the functional expression of the cotransporter in oocytes isolated from the 2 facilities. We report here differences in several quantitative metrics between the recirculating and static facilities and discuss how these differences may account for the observed variations in our functional expression studies of NKCC1.

Materials and Methods

Animals. For this study, all 16 oocyte–positive *Xenopus laevis* pigmented female frogs were lab-bred under stringent care (Nasco, Fort Atkinson, WI). Frogs were packaged in cardboard boxes with damp grass to minimize stress associated with overnight shipping. On arrival, the frogs were examined by a veterinarian for abnormalities, skin lesions, and parasites and then randomly were assigned into 2 equivalent groups. Each frog was placed individually in a moist container, measured, weighed, and photographed. Pigmented frogs possess unique markings on their back, allowing for animal identification without any need for branding, tagging, or tattooing (Figure 1). Two frogs were each placed into off-system static containers (12.5 L water in a 27-L aquarium) for a 3-wk quarantine period consisting of a 4-d levamisole treatment (12 mg/L), followed by a 10-d period off the drug and a second 4-d levamisole treatment. Levamisole was used to prevent any possible worm infestation into the recirculating facility. During the quarantine period, frogs were fed approximately 4 pellets of Frog Brittle (Nasco) twice per week. After quarantine, one group was placed in a tank in the recirculating water facility (managed by the Division of Animal Care, Vanderbilt University, Nashville, TN), and the other group was placed in a tank in an investigator-managed static water facility. All procedures and housing details were approved by the Vanderbilt University Animal Care and Use Committee.

Facilities. The recirculating facility consists of a continuous recirculating water system (X-Mod System, Marine Biotech, Beverly, MA). Water in the system passes through a series of particulate and carbon filters, exposure to UV light (dose rate, 30,000 $\mu W/cm^2$), and then return to the tanks with a flow rate of 1.15 L/min (Figure 2 A). The total volume of the recirculating water system is 2887.9 L with approximately 10% of the water replaced daily with fresh dechlorinated water. The recirculating facility uses municipal water, which is filtered and dechlorinated (NBW Series Residential Backwash Filters, Cuno Water Treatment, Churubusco, IN). The city water flows through a sand-filled particulate filter followed by an activated carbon filter, which removes chlorine. The dechlorinated water is stored in a 435.3-L reservoir. The temperature is kept at 17.7 °C and a pH range of 6.8 to 7.2. Cichlid lake, marine, and equilibrium salts at a ratio of 39.5:59.5:1 are added to the water to achieve a conductivity of 1000 µS. The total air exchange for the recirculating facility is 16.75 air changes hourly. Frogs were housed in a 23-L tank (2.9 L per frog) containing a polyvinyl chloride pipe for environmental enrichment (Figure 2 B and C). Frogs were fed (Frog Brittle, Nasco) on Mondays, Wednesdays, and Fridays with as much food as they would eat in 5 min.

The static facility consists of an environmental chamber (cold room) kept at 16 °C and with fluorescent lights on 12:12-h on:off cycle. The frogs were housed in a 54-L glass aquarium (3.4 L per frog). Similar to that in the recirculating facility, polyvinyl chloride pipe (diameter, 15 cm; length, 30 cm) was cut in half longitudinally and placed in the tank to provide environmental enrichment. We had all the edges of the plastic pipe polished to prevent any possibility of the frogs causing injury to themselves by abrasion (Figure 2 D and E). Frogs were fed (1 g per frog; Frog Brittle, Nasco) on Mondays and Thursdays; the aquarium was drained, cleaned, and refilled with clean dechlorinated water on Tuesdays and Fridays (including holidays). No cleaning or disinfection agents were used on the aquaria. For cleaning of the aquaria, frogs were held temporarily in a 20-L bucket (containing 10 L water). The aquaria were emptied, rinsed with regular tap water, scrubbed with a soft bristle brush to remove any deposits, rinsed again with regular tap water, and then wiped down before refilling with dechlorinated tap water from a 240-L storage tank (Figure 2 F). Typically, the container was refilled with unfiltered tap water on the day of cleaning and allowed to dechlorinate by air exchange. Because of the Tuesday–Friday cleaning schedule, the water was used 3 to 4 d after equilibration in the room.

Water-quality tests. The room and tank temperatures and conductivity in the recirculating system were recorded daily. The pH, ammonia, nitrate, alkalinity, and water hardness were tested (Fresh Water Aquaculture Test Kit, LaMotte, Chestertown, MD) once each week. The room and tank temperatures of the static facility were monitored and recorded daily. The conductivity of the water in the static facility did not require monitoring, because salt was never added to the frog aquaria. To ensure that our static facility water quality tests were comparable to those of the recirculating facility, we also measured the pH, ammonia, nitrate, alkalinity, and water hardness (Fresh Water Aquaculture Test Kit, LaMotte) in that system every day for a 3-wk period.

Surgery. After an acclimation period of 6 wk, the study started. On week 1 (January 2008), a frog from each facility was brought to the laboratory, anesthetized with unbuffered tricaine (1.7 g/L), and a small 4- to 6-mm incision was made on the left lower abdomen by using a disposable sterile scalpel. Ovarian lobes were externalized with sterile curved forceps, removed with sharp scissors, and placed in a 10-cm plastic culture dish containing modified L15 solution (250 mL Leibovitz L15 Ringer [Invitrogen, Carlsbad, CA], 200 mL deionized water, 952 mg HEPES (acid form), 400 µL 50 mg/mL gentamycin [Invitrogen]; pH 7.0; 195 to 200 mOsM). The solution was filtered by using a 500-mL, disposable, 0.2-µm, surfactant-free cellulose acetate filter (Nalgene, Fisher Scientific, Pittsburgh, PA), and the dish containing ovarian lobes was kept on ice. The wound was sutured with 3 to 4 stitches by using 4-0 silk braided surgical suture (DemeTech, Miami, FL; 18-mm needle, 3/8 circle, reverse cutting). After the frogs recovered from anesthesia in shallow water (specific to their husbandry), they were returned to their respective facilities. Animals were observed daily (including weekends and holidays) for any signs of distress as a result of the surgery. Experiments were performed every 4 to 6 wk; after all frogs had been used once, repeat surgeries were performed in the same order but with an incision on the right lower abdomen. Once all frogs had been operated a second time, a third round of surgeries was performed again on the lower left abdomen, and the frogs were euthanized after deep anesthesia.

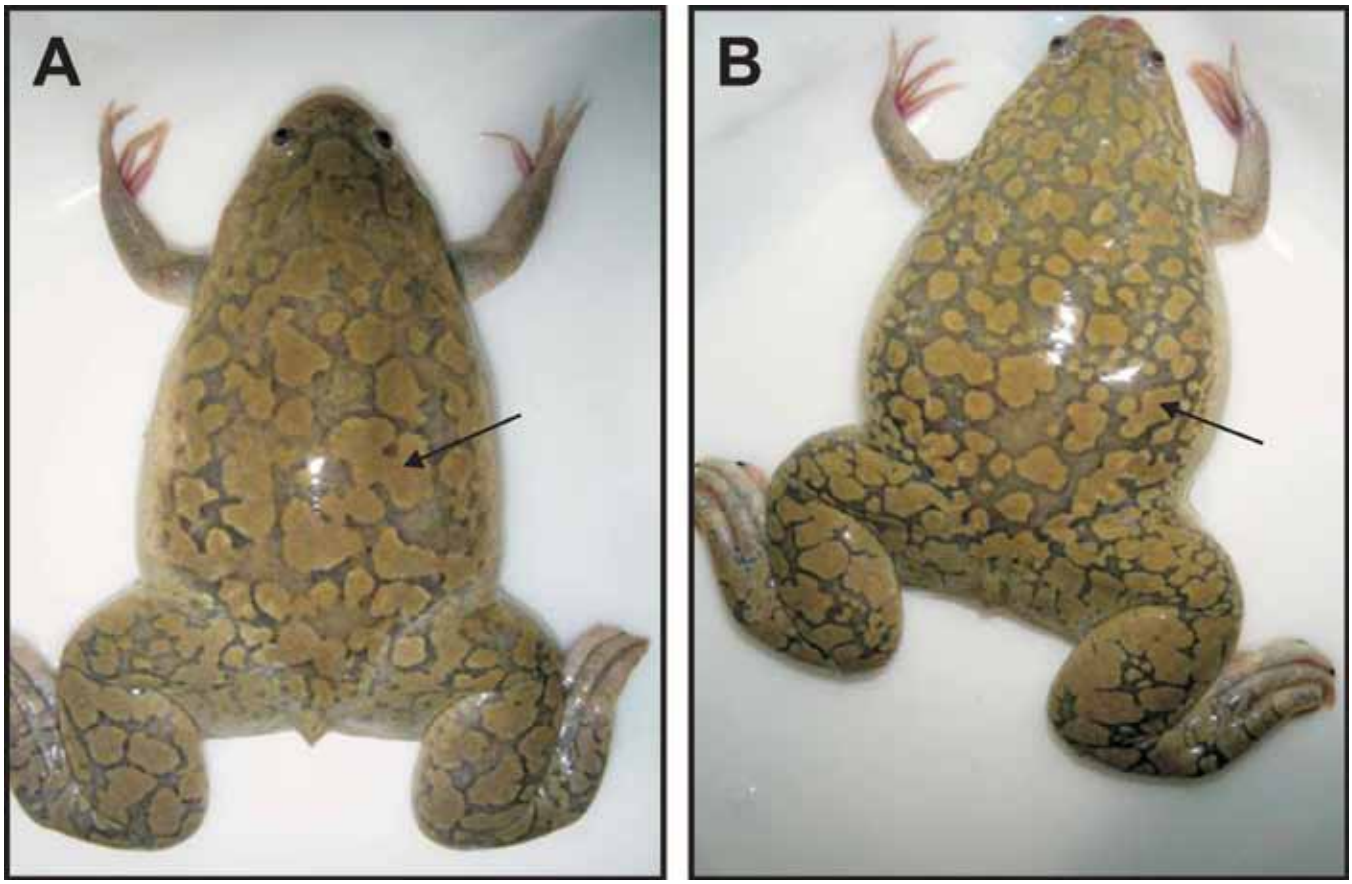

Figure 1. Identification of *Xenopus laevis* frogs based on markings. Photograph of 2 frogs, showing unique markings on their back. Arrows point to specific pigmentation patterns used to identify the frogs from one another.

Oocytes. Frog eggs (stage V to VI; n = 20 to 25) were defolliculated manually and maintained at 16 °C in modified L15 solution. The day after isolation, the oocytes were washed with fresh solution and counted, and their quality was assessed according to the colors of the light and dark poles by using a scale of 1 to 4 (4, light pole was pure ivory and dark pole was pure brown; 3, shaded ivory and pure brown; 2, shaded ivory and light brown; 1, shaded ivory and mottled brown). Dying oocytes were discarded.

cRNA transcription. DNA fragments encoding the mouse cotransporter NKCC1,[3] mouse SPAK,[19] and mouse WNK4[6] were inserted individually into pBF, an amphibian expression vector (kindly provided by Dr Bernd Falker, University of Tubingen). Each construct was transformed into *E. coli* and grown in Luria–Bertani broth containing 0.1 mg/mL ampicillin. The DNA was isolated and purified from the bacterial culture by using a plasmid midiprep kit (Qiagen, Valencia, CA) and quantitated by measuring DNA absorbance at 260 nm. DNA (20 µg) was linearized overnight with *Mlu*I and purified by using a purification kit (QIAquick for PCR, Qiagen). Linearized DNA (2.5 µg) was transcribed into cRNA (mMessage mMachine SP6 Transcription System, Ambion, Austin, TX). cRNA quality was verified by gel electrophoresis (1% agarose, 0.693% formaldehyde) and quantitated by measurement of absorbance at 260 nm.

cRNA injection. The day after isolation (day 2), oocytes were injected by using a 10-µL digital microdispenser (Drummond Scientific, Broomall, PA) fitted with pulled glass capillary tubes. The capillary tubes were pulled by using a horizontal pipette puller (Sutter Instruments, Novato, CA) and cooked overnight at 170 °C under vacuum. After the tip was broken under the dissecting microscope, the tube was backfilled with mineral oil, and placed on the microdispenser. The microdispenser was fitted on a right-handed Kite manual micromanipulator (World Precision Instruments, Sarasota, FL). The cRNA solution (5 µL) was placed in the cap of a 1.5-mL Eppendorf tube and slowly aspirated into the glass pipette. Each oocyte was injected with 50 nL RNAse-free water containing 15 ng mouse NKCC1 cRNA. The day after injection of the cotransporter (day 3) oocytes were washed, and some were injected with 50 nL RNAse-free water containing 10 ng mouse SPAK and WNK4.

Unidirectional K^+ influx. Groups of 20 to 25 oocytes placed in a 35-mm dish were washed once with 3 mL isosmotic saline (96 mM NaCl, 4 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES; pH 7.4) and preincubated for 15 min in 1 mL isosmotic saline containing 1 mM ouabain (to inhibit K^+ uptake through the Na^+–K^+ pump). The solution was then aspirated and replaced with 1 mL isosmotic flux solution containing 5 µCi ^{86}Rb. The hydration sphere of Rb^+ is similar to K^+ and therefore makes a good substitute because it is transported using the K^+ transport site of many transporters, including NKCC1 (for reviews, see references 9 and 20); Rb^+ is used instead of K^+ because its isotope, $^{86}Rb^+$, has a much longer half-life than does ^{42}K. Two 5-µL aliquots of flux solution were sampled at the beginning of each ^{86}Rb-uptake period and used as standards. After 1 h, the radioactive solution was removed, and the oocytes were washed 4 times with 3 mL ice-cold isosmotic solution. Single oocytes were transferred into glass vials, lysed for 1 h with 200 µL 0.25 N NaOH, and neutralized with 100 µL glacial acetic acid, and ^{86}Rb tracer activity was measured by β-scintillation

Figure 2. Images of recirculating and static frog facilities. Photographs of (A) pump and filtration water system, (B) tank racks, and (C) individual frog tank of the recirculating water frog facility. Photographs of (D, E) frog tanks and (F) holding tank of the static water frog facility.

counting. Counts per minute were transformed into nanomoles K^+, and NKCC1 flux was expressed in nmol K^+/oocyte/hour.

Statistical analysis. Differences between the weight and length of frogs distributed into the 2 study groups (Table 1) was analyzed by using unpaired *t* tests (InStat version 3.01, GraphPad, La Jolla, CA). K^+ uptake in individual oocytes was measured by scintillation counting. For every time point, each experimental group was represented by 20 to 25 oocytes from a single frog housed in either the static or recirculating tank. Therefore, we gathered matched pairs of values for every time point and used the nonparametric Wilcoxon matched-pair test to determine significance ($P < 0.01$) of differences between the 2 facilities.

Table 1. 2-tailed *P* values (n = 8 frogs per group) obtained by using unpaired *t* tests

	Weight (g)		Length (cm)	
	Recirculating	Static	Recirculating	Static
	137	146	10.2	11.6
	159	172	11.8	12.8
	144	121	11.0	11.5
	112	123	10.0	11.1
	144	119	11.2	10.8
	134	111	11.2	11.3
	100	110	10.2	10.7
	109	133	10.4	11.2
Mean	128.5	129.4	10.86	11.38
1 SD	19.8	20.8	0.56	0.65
P	0.9327		0.1152	
t	0.086		1.68	
degrees of freedom	14		14	

Differences between the standard deviations were not significant, and each sample passed the normality test.

Results

Veterinary examination of the 16 frogs at the beginning of the study found no physical abnormalities and determined that the animals all were in good health. The frogs were allocated randomly into 2 groups (n = 8), weighed, and measured from snout to vent. There were no statistical differences in weight and length between the 2 groups of frogs (Table 1). The frogs were kept in their individual facilities (recirculating or static) for a period ranging from 19 to 23 mo, depending on when they had their first surgery. Over that extended period of time, no frog maintained in the static facility exhibited clinical signs of disease (bloating, open wounds, skin infections). However, in the recirculating facility, 1 frog died and 2 were euthanized based on the veterinarian's recommendation. Necropsy records on 1 of the frogs euthanized revealed a chronic coelomitis and heavy growth of *Aeromonas hydrophila*. We did not establish a standing order to have any dead animals undergo a necropsy or histopathologic examination, and as such, the staff simply discarded the carcasses. Therefore, the cause of morbidity or mortality is unknown.

We compared several water quality parameters between the static and recirculating facilities. Despite the similarity of the room temperature in both facilities, the tank temperature of the static facility was 2 °C lower than that in the recirculating facility. Because of the addition of salts in the recirculating facility, water alkalinity and hardness differed significantly between the 2 facilities (Table 2). Conductivity was not measured in the static facility because salt was never added to the water supply; however, the conductivity (mean ± 1 SD) in the recirculating facility averaged 963 ± 14 µS (target, 1000 µS). No detectable levels of ammonia nitrogen or nitrate nitrogen were observed in the recirculating facility. Because the water in the static facility aquaria was changed twice weekly, we observed an increase in ammonia nitrogen over the 3- to 4-d cleaning cycle. Despite the presence of ammonia nitrogen in the static facility water, no nitrate nitrogen was ever detected (Table 2). During the 3- to 4-d cleaning cycle, we measured increasing carbonate alkalinity, carbon dioxide, and ammonia nitrate levels in the water in the static tanks (Figure 3 A to C). Although there was no statistical difference in pH between the 2 facilities (Table 2), we observed both decreasing pH and dissolved oxygen levels during the 3- to 4-d cycle (Figure 3 D and E).

Oocytes were isolated every 4 to 6 wk from frogs taken from each facility. The visual quality of the oocyte was scored under the dissecting microscope, and the score did not change over the 5-d period that the oocytes were maintained at 16 °C. We noticed that on average the oocytes from the recirculating facility were lighter in color than were the oocytes from the static facility (score of 2 compared with 3 to 4). In fact, light-brown oocytes were observed 12 times among the 20 observations for the frogs in the recirculating facility but only twice over 20 observations for those in the static system. The survival rate of isolated oocytes did not differ between the static and recirculating facilities: the percentage of oocytes lost on the second day after surgery was 9% compared with 8%, respectively, whereas that on the third day was 4% for both groups. Minimal loss (approximately 1%) was observed over the remaining 2 d. Although there was one instance where frog oocytes isolated from the recirculating facility appeared to be more fragile during injection, there were no significant differences in cotransporter activity between the 2 facilities.

The primary goal of this study was to compare the heterologous expression and physiologic function of a membrane ion transporter between groups of oocytes isolated from frogs housed in a 17 °C aquarium connected to a recirculating water system and frogs housed in a 15 °C aquarium, which underwent water changes twice weekly (static facility). The activity of the mouse cotransporter NKCC1 was assessed through K^+ influx experiments, by using ^{86}Rb as a tracer.[4] To fully assess the ability of the oocytes to functionally express the cotransporter, we also injected 2 kinases (SPAK and WNK4), which stimulate NKCC1 activity. Previous work from our laboratory has shown that WNK4 binds to SPAK and, through phosphorylation of specific residues, activates the kinase. Activated SPAK then binds, phosphorylates, and stimulates NKCC1 activity.[6,7] This previous work also has shown that in *Xenopus laevis* oocytes injected with NKCC1, the vast majority (approximately 90%) of the Rb^+ (K^+) flux is mediated by the cotransporter. Furthermore, we have shown that NKCC1 activity peaks 4 to 5 d after injection, whereas the kinase expression peaks 2 d after injection.[6]

We have summarized the isosmotic K^+ influx measured in oocytes from both the recirculating and static facilities coinjected with NKCC1, SPAK, and WNK4 over the course of our 23-mo study (Figure 4 A). To better visualize long-term trends, each data point in the continuous lines is an average of 3 weekly observations (preceding, current, and following weeks). The first observation is that oocytes isolated from frogs in the static facility produced more robust K^+ influxes than did oocytes isolated from frogs in the recirculating facility. Second, there was a significant decrease in the ability of oocytes isolated from frogs housed in the recirculating facility to functionally produce kinase stimulation of NKCC1 activity during the summer months (April to September). This seasonal decrease was minimal with oocytes isolated from frogs housed in the static facility. Third, although no seasonal decrease was observed under hypertonic conditions, we still observed a more robust K^+ influx in oocytes isolated from frogs in the static facility compared with oocytes isolated from frogs in the recirculating facility (Figure 4 B). As reported in previous studies,[6] the cotransporter flux is maximally activated under these conditions. To further document the difference between the 2 groups of oocytes, we performed nonparametric Wilcoxon matched-pair tests. We found a significant difference in NKCC1 fluxes stimulated by the SPAK

Table 2. Water quality in the static- and recirculating-water facilities

	Recirculating	Static
Room temperature (°C)	18.0 ± 0.7 (n = 89)	18.8 ± 1.8 (n = 70)
Tank temperature (°C)	17.5 ± 0.1 (n = 89)	15.2 ± 0.3 (n = 70)
Alkalinity (ppm)	41.8 ± 10.9 (n = 13)	77.7 ± 10.9 (n = 18)
Hardness (ppm)	286.9 ± 37.6 (n = 13)	107.1 ± 4.8 (n = 18)
Conductivity (μS)	963 ± 14 (n = 89)	not measured
Ammonia nitrogen (ppm)	0 (n = 13)	1.72 ± 1.48 (n = 18)
Nitrate nitrogen (ppm)	0 (n = 13)	0 (n = 18)
pH	6.70 ± 0.12 (n = 13)	6.94 ± 0.16 (n = 18)
Luminosity (lux)	75	220

Temperature was monitored daily over 3 mo for both the facilities. Luminosity was measured once in the vicinity of the aquaria. Conductivity was measured daily in the recirculating water facility. Water-quality tests for alkalinity, hardness, ammonia, nitrate, and pH were measured daily for 3 wk in the static facility and once weekly for 3 mo in the recirculating facility.

and WNK4 kinases (P = 0.0068) or hypertonicity (P = 0.0018) between oocytes isolated from frogs housed in the static and recirculating facilities.

Discussion

We have used the oocyte heterologous expression system for several years to elucidate the regulation of the cation–chloride cotransporters.[5-7,18,22] We house our frogs in a static aquarium facility, changing the water every 3 to 4 d. Our studies have always yielded robust functional cotransporter activity year-round. Interestingly, other laboratories that house their frogs in recirculating water facilities anecdotally report seasonal variability in their experiments. Therefore, the purpose of the current study was to compare heterologous functional expression of the NKCC1 cotransporter in oocytes isolated from frogs housed in either our static water facility or the medical center's recirculating water facility.

Our data demonstrate a statistically significant difference in the functional activity of NKCC1 under both kinase and hyperosmotic stimulation between oocytes obtained from frogs housed in our static facility and those obtained from frogs housed in the recirculating facility. In addition, as a result of the duration of our study (23 mo), we clearly demonstrated that oocytes isolated from frogs housed in the static water facility exhibit minimal seasonal variation when used for heterologous expression of membrane transporters.

We want to make it clear that our data do not equate optimal housing for animal health and welfare with that for a specific type of oocyte production. Indeed, the medical center frog facility uses a state-of-the-art water recirculating housing system that maintains healthy frogs used to produce oocytes for many other types of studies (for example, embryology, developmental biology). It is worth noting that all 3 frogs that died or were euthanized during the 23-mo study were housed in the recirculating water facility. Furthermore, none of the deaths were attributed to the surgeries performed. We should acknowledge that during the same time period of the study, 3 animals in the static facility did exhibit signs of morbidity (that is, lethargy, open sores, and excessive skin shedding), but quarantine in a separate aquarium and regular water changes enabled each frog to recover fully and be included in future experiments. None of the frogs (whether on study or not) housed in the static facility died during the course of the study.

From the beginning of the study, several parameters (that is, tank temperature, water quality) clearly differed between the 2 housing facilities. However, our primary goal was not to identify which parameter produced a better-quality oocyte for heterologous expression studies but to confirm that housing frogs in a static water facility eliminated the seasonal variability observed by other laboratories. Several parameters were similar between the 2 housing facilities. The distribution of the 16 frogs between the recirculating and static facilities was sufficiently random to have no significant difference between the 2 populations (Table 1). Although Nasco (Fort Atkinson, WI) does not explicitly state the lineage of their lab-bred *Xenopus laevis* frogs, they guarantee "healthy, oocyte-positive females which are 2+ years old."[17] Second, the light:dark cycle in both facilities was identical, and the difference in luminosity was only moderate (Table 2). Finally, the frogs in both facilities were fed the same food (Frog Brittle, Nasco), although the frequency of feeding differed.

The first major difference between the 2 housing facilities involved water quality. The static water facility used dechlorinated tap water with no addition of buffering salt, whereas the recirculating water facility adds cichlid salts to buffer the water. Addition of these salts increased water hardness. A study published in 2004[10] examined the effect of salt and water hardness on oocyte quality, as measured through survival and normal development of embryos. The study demonstrated that the hardness of the water was the factor that contributed the most to oocyte quality. Whether the addition of cichlid salts in the recirculating water facility was the principal cause of the observed seasonal variability in oocytes heterologous expression is unknown but, if true, would contradict the results of the previous study.[10]

A second difference is related to the use of the static facility. In the static facility, the frog aquaria were cleaned twice weekly, with a complete water exchange. As a result, we observed increasing alkalinity, ammonia nitrogen, and carbon dioxide levels with simultaneous decreases in pH and dissolved oxygen levels between water exchanges. In contrast, as a result of continuous water exchange, the frogs in the recirculating facility never experienced changes in any of these parameters. It is difficult to assess which of these factors played the most significant role in oocyte quality. Interestingly, the authors of the previously cited study[10] mentioned a history of poor oocyte quality when female frogs were housed in a recirculating system in the absence of salts.[10] It is also worth noting that although *Xenopus laevis* frogs sometimes are found in streams, they more often thrive in ponds and lakes where the water is more stagnant.

A third difference between the 2 facilities was the height of the water: 11 to 12 cm for the aquaria in the static facility compared with 16 to 17 cm for those in the recirculating facility. Although this 4- to 5-cm variation appears negligible, the 11- to 12-cm height allowed the frogs to reach the surface just by standing on

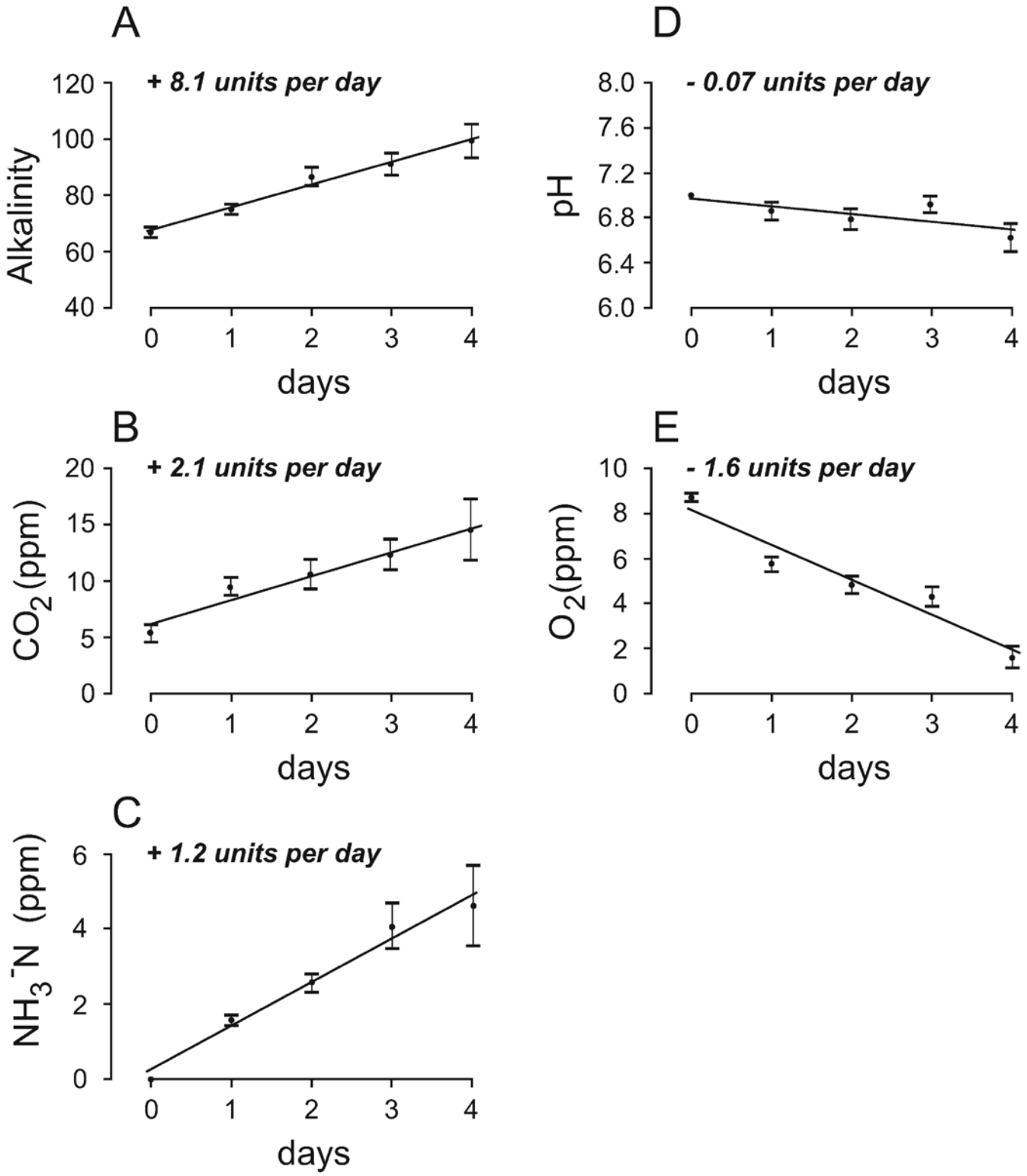

Figure 3. Water-quality tests of static water frog facility. Alkalinity, carbon dioxide, ammonia, pH, and dissolved oxygen were measured at 4 time points between tank cleanings in the static facility. Each point represents mean ± 1 SD (n = 4 to 9 measurements).

the bottom of the tank, a behavior that the frogs in the recirculating facility could not accomplish. A fourth difference between the 2 animal facilities was the cooler tank temperature of the static facility. Although relatively small, the water temperature difference is significant and could account for the seasonal variability that we observed with oocytes isolated from frogs housed in the recirculating water facility. It is noteworthy that the water temperature used in the static facility was 2 °C higher than that reported to be harmful to frogs.[26]

Although any one of these variables may have a significant role in oocyte quality, the more likely possibility is that some combination of these parameters is responsible for the sea-

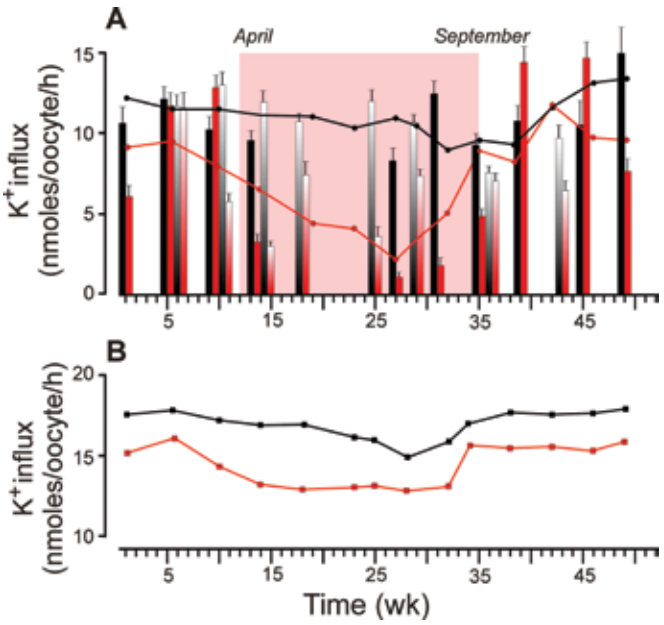

Figure 4. K^+ influx measured in oocytes isolated from frogs in recirculating and static water housing systems. (A) NKCC1-mediated isosmotic K^+ influx in oocytes isolated from frogs from both facilities and coexpressing SPAK and WNK4 cRNAs. Solid-colored bars represent data collected during the first year, and bars with gradient colors represent data collected during the second year of the study. All bars represent mean ± SEM (n = 20 to 25 oocytes). To smooth the data, we averaged the fluxes over 3 successive time points. The black continuous line represents data obtained with oocytes isolated from frogs in the static-water facility, whereas the red continuous line represents data obtained from oocytes isolated from frogs in the recirculating-water facility. The shaded box highlights the period from April through August, when frogs in the recirculating-water facility produced low-quality oocytes. (B) Smoothed data for hyperosmotic-stimulated fluxes in oocytes isolated from frogs in the static-water (black line) and recirculating-water (red line) facilities.

sonal variability in oocyte heterologous expression between the static and recirculating water housing facilities. The differences associated with the static housing facility might isolate the frogs from external environmental cues related to the seasons and prevent physiologic changes associated with natural biological rhythms.

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Note added in proof: Since the conclusion of the study, as refinement, we now buffer our tricaine solution with 3.4 g $NaHCO_3$ per L and use 4-0 monofilament nylon suture for our surgeries.

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