Harmful Effects on African Clawed Frog (*Xenopus laevis*) Reproduction as Expression of High Water Phosphates Levels

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Abstract: Amphibians are considered as reliable indicators of environmental quality. In Europe, a general decline of amphibians population parallels a worldwide decline, and some of the factors which are thought to be responsible to this decline are habitat loss, introduction of exotic species, disease and decreasing water quality. Recent investigations suggest that these eutrophic conditions may be associated with frog reproduction problems. Water quality criteria settled for the different species of amphibians do not currently exist, but in the present paper, the authors report their observation about water phosphate concentrations in association with hypofertility in African clawed frogs (*Xenopus laevis*). The study considered two different groups of African clawed frog showing hypofertility (22 patients) hosted in captivity. The animals were visited, and the filtration systems functionality and the water quality were checked, i.e., temperature, pH, ammonia, nitrite, nitrate, phosphates, hardness and alkalinity. In the first group, it was decided to perform ultrasound scans, parasitological tests on fecal samples, and microscopical/macrosopical examination, as well as microbiological analysis on collected oocytes. In the second group, only fecal samples were collected in order to perform parasitological exams. No pathological findings were showed by veterinary tests. Water changes were carried out in both facilities and after two months, the fertility in African clawed frogs improved, evidencing the involvement of phosphates values in the onset of the problem and even more in its resolution. More studies are needed to further define this correlation.

Key words: *Xenopus laevis*, water phosphates level, oocytes' quality.

1. Introduction

A good filtration system is necessary to guarantee the maintenance of aquatic organism. In an aquarium, a filter should provide both the physical removal of large organic debris (mechanical filter), as well as denitrification through biological processes (biological filter). Biological filtration, operated by different bacterial classes, is necessary to perform a biochemical transformation of ammonium that would otherwise be toxic to aquatic organisms. This process is known as "cycle of nitrogen" and involves the transformation of total ammonia nitrogen in nitrite or

nitrate [1].

Amphibians use their skin and gills for respiration and osmoregulation [2]; for this reason, the control of waterborne toxins, including ammonia, nitrite, heavy metals, disinfectants, pesticides, chlorine and chloramines, is very important to maintain a good amphibian's environment [3-5]. The toxic effects of high levels of these agents are well described in literature, such as Bealeu [6] showed that amphibians living in an aquarium with high levels of nitrite can develop methemoglobinemia or brown blood disease leading to respiratory compromise, like other aquatic animals. Also, temperature, pH and dissolved oxygen have an important role in maintaining good health. In fact, amphibians are unable to maintain body heat, and

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the ideal temperature maintenance should consider the species, the developmental stage and the season [7]. A good level of dissolved oxygen is necessary for nitrifying bacteria and heterotrophic organisms that are responsible for the initial breakdown of organic waste [8, 9] developed in an aquarium; the necessary amount of dissolved oxygen varies with species, and even if these animals tolerate well low levels, a level of oxygen over 80% is always unsanitary for resident amphibians, as a supersaturated level can result in gas bubble disease [10]. While many species can tolerate wide swings in pH for short period of time, this can be stressful, cause immunodepression and promote the development of secondary diseases [3].

In an aquatic environment, there is another group of chemical substances deriving directly from the food, namely, the phosphates. The biological filter is not able to transform these chemical products which are used by plants or removed by routinely changing water. To our knowledge, there is no report about specific amphibian's toxic effect of high level phosphates. Therefore, this study was focused on the harmful effects on the reproduction of African clawed frog (*Xenopus laevis*) as a result of prolonged exposure to high levels of phosphates in the water.

2. Materials and Methods

The study considered two different groups of *Xenopus laevis* kept in two different facilities, i.e., a national research institute and a private collection.

Fourteen *Xenopus laevis*, all females, were hosted in a national research institute in four different isolated tank, equipped with a biological filtration system and located in a controlled temperature room (20 °C). Six animals were hosted in a tank having a capacity of 80 L, four in a tank with 60 L and the others equally divided in two tanks with 40 L. This distribution was decided according to the different size of the subjects.

The animals were fed three times a week with specific pellet for *Xenopus* (Nasco frog brittle).

These animals were routinely used for research studies authorized by the Ministry of Health in accordance with the Italian laws; the purpose of these studies are intended to check the expression of proteins in oocytes injected with RNA and perform electrophysiology studies.

The animals were anesthetized with tricaine methane sulfonate (MS222); the anesthetic solution was prepared with 1 g of MS222 dissolved in 1 L of deionized water buffered to pH 7 with bicarbonate [11].

A surgical level of anesthesia was confirmed by gently pinching the fleshy part of both rear feet 70 with gloved hands (metals or other can scrape the mucous layer) and ensuring that the frogs were non-responsive to painful stimuli. The frogs were placed on a clean, moist surface and the skin undergoing the surgery was gently swabbed with cotton, at 1-10 dilution of povidoneiodine. An approximately 1 cm incision on the abdomen in paramedian position was made with N° 10 surgical scalpel, scissors were used to dissect through the fascia and muscle to visualize the oocytes. Oocytes strands were then gently externalized and cut.

The incision was closed by suturing both the fascia and skin layer in two layers. Absorbable 5-0 PDS suture was used for the fascia and skin closure.

The extracted oocytes were stored in maintenance solution, i.e., Barth modified solution (NaCl 96 mM, KCl 2 mM, MgCl₂ 1 mM, CaCl₂ 1 mM, Hepes 5 Mm, pH 7.5) at 4 °C until their use [12]. The same animal was operated up to a maximum of seven times.

The second group of African clawed frogs was housed in a private breeder collection, and included eight animals—six females and two males, hosted in four isolated tanks having a capacity of 120 L equipped with a biological filtration system and located in a controlled temperature room (18 °C). The two males were grown in the same tank and the females equally divided in the other tanks if it was not the breeding season, otherwise moving the animals

according to the breeding programme.

The animals were fed four times a week with specific pellet for *Xenopus* (Nasco frog brittle).

After a period of about six months, the animals from both groups started to have reproduction problems.

The animals of both groups were visited, and the filtration systems functionality and the water quality were checked in both cases (temperature, pH, ammonia, nitrite, nitrate, phosphates, hardness and alkalinity).

During *Xenopus* veterinary examination, it was decided to perform an ultrasound examination, parasitological and microbiological analysis on the collected oocytes and to collect fecal samples to check for parasites. Four animals were anesthetized with the same protocol normally used during the surgery, and ultrasound was performed with a portable Esaote MyLab30 ultrasonograph and a 7.5 MHz sectional probe. After the ultrasonography, surgery was performed to collect and analyze the oocytes.

Swabs were taken from the oocytes in the coelomic cavity during surgery to test bacterial and fungal disease. The collecting was made with aluminium wire dry swabs, which was followed by direct seeding of Columbia III agar + 5% sheep blood, mannitol salt agar, MacConkey II agar, Chromagar orientation, Chromagar Candida medium, Sabouraud dextrose agar + CAF 400 µg and Hektoen enteric agar, and seeding in liquid enrichment media (thioglycolate medium and selenite broth) for storage of the sample and the following assays. Analysis was focused on aerobic bacterial flora, micro-aerophilic bacterial flora and fungi. The plates were incubated either in aerobiosis at 37 °C for selective medium or at 35 °C with 5% of CO₂ for blood agar.

In the second group, only fecal samples were collected looking for parasitic diseases, because the frogs experienced a natural breeding programme and the animals did not receive surgery to collect oocytes.

3. Results and Discussion

For the first period, the animals in the research center have had good quality oocytes. The good quality of the *Xenopus laevis* oocytes was expressed considering some morphological and physiological characteristics summarized in Table 1.

After six months, all the frogs showed a bad oocytes' quality. Also in the private collection after the first period, all the animals stopped to reproduce.

Both groups showed no evidence of disease during the veterinary check, and ultrasound analysis showed no pathological findings; fecal samples were negative for parasites in all cases.

Microbiological analysis performed on the *Xenopus laevis*, oocytes of which were collected in the research institute, were negative for bacterial or fungal disease.

The filtration systems functionality was fine and also the water quality was good except for high levels of phosphates [13] in both *Xenopus laevis* groups (Table 2).

Analyzing the results obtained, the authors hypothesized that the cause of low quality of the oocytes in the laboratory group of *Xenopus laevis* and the stop of the reproduction of those hosted in the private collection could be related to high levels of phosphates in water. The authors decided to increase the water changes in both facilities. For about two months management, changes were carried out performing 30% water changes three times a week in all the tanks; and when the level of phosphates was less than 2 mg/L, the quality of *Xenopus*' oocytes was much better. The morphological and physiological characteristics of oocytes improved and they regained the ability to express the protein injected in the experimental stages without dying, confirming their enhanced quality and strength. The change of oocytes quality is well focused in Figs. 1 and 2.

The reproduction of the *Xenopus* began after two months of continuous water changes when phosphate levels was able to maintain below 2 mg/L.

Table 1 Critical points to define the quality of *Xenopus laevis* oocytes.

Critical point	Good quality	Bad quality
Coloration oocyte checked with a stereomicroscope	The animal pole is brown and distinct than the vegetative pole that is white; There are no stains or frustules on the surface of the oocyte	There is no clear colorimetric distinction between the two poles; There are splashes of color with different shades of brown and yellow on the surface of the oocyte
Oocyte size	Approximately 1 mm	Far less than 1 mm
Oocyte' ability to resist elastically to the injection of the protein	The oocyte membrane does not break with the injection and responds elastically; The cytoplasm does not come out	The membrane ruptures and the oocyte cytoplasm comes out
Oocyte' ability to express the injected protein	The injected protein is expressed in the oocyte	The injected protein is not expressed in the oocyte
Oocyte' duration when it is placed in a maintenance solution of 1 mM Ca without enzymes after the collection from the ovary and stored at 4 °C	8-10 days	Up to 2-3 days. In some cases, the oocyte dead immediately after injection for the membrane rupture
Prevalence or not of endogenous currents after injection with protein. (The <i>Xenopus</i> oocyte is a model for experimental electrophysiology studies for its almost total absence of endogenous currents)	A good <i>Xenopus</i> oocyte has very few endogenous currents; After the protein injection, a current starts and prevail over the endogenous current	Endogenous currents occur and prevail over the one started after protein injection

Table 2 Correlation between high phosphates level and *Xenopus laevis* oocyte quality.

Period	Phosphates level (mg/L)	<i>Xenopus</i> oocytes quality
Week 1	1	Good
Week 2	2	Good
Week 3	1	Good
Week 4	5	Good
Week 5	10	Bad
Week 6	20*	Bad
Week 7	20	Bad
Week 8	10	Bad
Week 9	10	Bad
Week 10	5	Bad
Week 11	2	Bad
Week 12	2	Good
Week 13	1	Good
Week 14	2	Good

* means from this week the changes water increased.

The reproductive disorders in amphibians are still not well documented, especially because their multiple causes [3, 14, 15]. In physiological conditions, successful reproduction involves ovulation/spermiation, fertilization, oviposition, embryonic development and metamorphosis [16, 17]. Every disease that involves one of this phases could be the responsible for reproductive problems [18, 19]. In many cases, the

specific causes of amphibians unsuccessful breeding are impossible to determine, primarily for the many causes, of stress they are subjected to.

If we consider the fish reproduction, the problematic is better analyzed owing to the parallel expansion of the aquaculture industry [20]. Fish literature describes the effect of parasites on reproduction [21], the relationship with immunocompetence [22, 23] and the correlation between nutritional deficiencies and low reproductive index [9]. Many reports describe the relationship with different poisonings, like the “new tank syndrome” or “brown blood disease”, and the beginning of a series of problems included the stop of the reproduction [1, 9]. To our knowledge, a correlation between high phosphates levels and low fertility has not been described so far also in fish.

In this study data obtained from the group hosted in the national research institute, it was excluded all the causes related to stress, such as sex ratio, size of the animals, the incompatibility of individuals because of the group characteristics; indeed, the frogs were all females and no changes in the tanks or in the routine management protocols were done.

Fig. 1 *Xenopus laevis* oocytes of poor quality.

It is not possible to distinguish the vegetative pole from the animal pole (BQO = bad quality oocyte). The coloring is spotty and not clearly distinguished. It is also possible to see the presence of immature oocytes (IO = immature oocyte).

Fig. 2 Oocytes after 1 month changing water to decrease the high phosphates levels (A) and *Xenopus laevis* oocytes of good quality (B).

It is possible to distinguish the vegetative pole from the animal pole (GQO = good quality oocyte). The coloring is clear. It is also possible to see the presence of immature oocytes (IO = immature oocyte).

Because the sanitary protocol provided for weekly monitoring of water parameters, it was possible to compare data obtained from water analysis performed in the period preceding the onset of reproductive problems with those obtained from water collected at their resolution; the authors' observations led them to

conclude that phosphate levels are critically involved both in the onset of fertility problems and in their resolution.

From ultrasound analysis, the results of collected fecal samples and the microbiological tests confirmed the authors' theory.

4. Conclusions

The results of this study highlight the importance of using management protocols to compare situations that otherwise could hardly be clarified and resolved; in particular, the proper management of the water in amphibians husbandry is very important.

This is the first report that describes the correlation between high levels of phosphates and reproduction diseases in *Xenopus laevis*. Moreover, this study showed that the phosphates concentrations influences *Xenopus*' reproduction, and the best results in terms of fertility/oocytes quality were obtained at less than 2 mg/L phosphates levels.

More studies are needed to further define this correlation and to analyze possible additional variables in order to fully understand amphibian reproduction. But, this study can be a helpful starting point. The interest for amphibian reproduction in captivity needs to be increased because of the global decline of wild populations, and *Xenopus laevis* could be a very useful model to study due to its high rate of reproduction in captivity.

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