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Overview

Factors Affecting Oogenesis in the South African Clawed Frog (*Xenopus laevis*)

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***Xenopus laevis*, commonly known as the South African Clawed frog, is a hardy adaptable species that is relatively easy to maintain as a laboratory animal. Gametogenesis in wild *Xenopus laevis* is continuous and under ideal conditions, reproduction can occur year round. This unique aspect of amphibian reproduction offers an advantage over mammalian model systems: the eggs and oocytes collected from laboratory maintained *Xenopus laevis* provide an abundant and readily obtainable supply of material for cellular and biological research. However, many investigators report that laboratory *Xenopus laevis* go through periods of unexplained inefficient or complete failure of oocyte production or the production of poor quality oocytes. This results in experimental delays, inability to reproduce data, and ultimately the use of more animals. There is a lack of evidenced based information regarding the housing conditions that are necessary to optimize the health and fecundity of this species in captivity, but studies of wild *Xenopus laevis* have shown that temperature, age of the female, and nutrition are of key importance. The objective of this report is to review oogenesis with a special emphasis on these factors as they pertain to laboratory *Xenopus laevis* maintained for the purpose of providing a steady supply of eggs and oocytes. Harvesting methods and other experimental techniques that affect the quality of eggs and oocytes are also discussed.**

Xenopus laevis, commonly called the South African Clawed frog due to the presence of small black curved claws on the inner three toes of the hind feet, has been used extensively as a non-mammalian model for basic research in vertebrate embryology, and in cellular biology, physiology, and biochemistry. An aquatic anuran, *Xenopus laevis* is especially suitable for the laboratory because of its short generation time, its longevity in captivity, and its ability to adapt to a range of laboratory conditions. Gametogenesis can occur year round in this species. Oocytes and eggs harvested from laboratory *Xenopus laevis* provide a continuous and primary source of material for cellular research.

Oocytes from *Xenopus laevis* are large (~ 1mm) fully grown egg precursor cells, which accumulate in large numbers in the ovary. They are very active in gene transcription and in protein synthesis and their cell-free cytoplasmic extracts can be used for biochemical analysis of cellular processes (1-3). For example, the cytoplasmic cell-free extracts from *Xenopus laevis* oocytes and or eggs are widely used as an in vitro system to study the mitotic cell cycle (4-6), molecular signaling pathways (7-14), nuclear assembly and nuclear transport (15), and the transcription, translation, and expression of a variety of mRNAs (16-20). In addition, the oocytes and eggs from laboratory *Xenopus laevis* yield sufficient quantities of proteins to study numerous other cellular processes such as cytoskeletal structure and function, phosphorylation, receptor synthesis, protein secretion, compartmentalization, expression of recombinant proteins, and the "loss of function approach" using antibody depletion techniques (21-24). *Xenopus laevis* oocytes are also large enough for microinjection of RNA or cloned DNA, and for analysis of ion channels via electrophysiological recording and expression cloning (20, 25, 26). Given the range of applications, it is not surprising that the *Xenopus laevis* oocyte system has been referred to as a "living test tube" (26, 27). The use of this model system in basic research has resulted in significant advances in the field of cellular biology, physiology, and cellular biochemistry and in understanding the cellular mechanisms of a variety of human diseases, especially cancer.

The number of published studies making use of *Xenopus spp.* has tripled in the last 10 years (28). Of the non-mammalian animal models supported by NIH, budget figures from 1998 rank *Xenopus spp.*, as number three at ~ $90 million dollars, after yeast (~$150 million) and *Drosophilia* (~$110 million) (http://www.nih.gov/science/models/xenopus/). Colonies of *Xenopus laevis*, ranging from as few as 20 animals, up to several thousand frogs or more, are currently maintained at laboratory animal facilities across the United States and around the world in order to provide researchers with a steady supply of oocytes and eggs. Although the exact number of adult female *Xenopus laevis* used annually in laboratory research for this purpose is unavailable, a conservative estimate would approach several hundred thousand frogs or more.

Despite the increasing demand for laboratory *Xenopus laevis* (28), there is a lack of evidenced based information regarding the water quality, environmental conditions, or the nutritional requirements that are necessary to optimize the health and fecundity (e.g. egg and oocyte production) of this species in captivity. In contrast, years of investigation have established universally accepted protocols for laboratory rodents that optimize conditions

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for health and reproduction. For *Xenopus laevis*, laboratory protocols describing long-term housing of adult females for the purpose of egg and oocyte production are generally based on the biology of wild amphibians, institutional experience, time-honored techniques, and recommendations from amphibian hobbyists, zoos, and aquariums. Several groups (29-32) have described the care of laboratory maintained *Xenopus laevis,* but they do not provide experimental data to support their recommendations. Hilken and colleagues (33) examined the affect of water temperature and level, population density, feeding, cover, tank color, and other variables on the growth of postmetamorphic *Xenopus laevis* but found a large and unexplained variability across the treatments. The small numbers of animals evaluated and the short study times limited usefulness of the study. Factors affecting egg and oocyte production were not specifically addressed. The National Academy of Sciences offers sound guidelines regarding the husbandry and care of laboratory *Xenopus laevis*, but the document specifically states that the recommendations should be considered tentative as few established and tested guidelines are available (34).

Many investigators report that entire colonies of laboratory *Xenopus laevis* undergo unexplained prolonged periods (weeks to months) of inefficient oocyte or egg production or the production of poor quality material (28, 35, 36). This phenomenon causes experimental delays, inability to reproduce data, and ultimately the use of more animals. Once disease or parasitic infection is ruled out, housing and husbandry practices are usually investigated as a potential cause. Laboratory animal veterinarians are frequently asked to correct the situation, but there is frustratingly little information in the laboratory animal and veterinary literature that addresses this topic specifically. The objective of this report is to summarize what is known about reproduction in female *Xenopus laevis* both in the wild and in captivity, with special attention to the factors that affect oogenesis and egg production. Harvesting methods and experimental techniques that affect oogenesis and egg quality are also briefly discussed.

Factors affecting oogenesis *Xenopus laevis*

The most detailed data describing factors that affect the reproductive cycle and oogenesis in *Xenopus laevis* comes from amphibian biology textbooks and from field reports on feral frog populations in California (37-39), where conditions are optimal for year round reproduction. Of all factors that affect oogenesis in wild anurans (population density, cover, water depth, food source, etc.), temperature, the age of the female, and nutrition appear to be the most critical to ovulation and the production of large egg clutches (37-43).

Temperature. *Xenopus laevis* can reproduce year-round in tropical and Mediterranean climates and typically prefer warm (21°-24°C), quiet, brackish water; healthy populations can also be found around the world at temperatures ranging from 13°C to 58°C (38). Although gametogenesis in female *Xenopus laevis* can occur year round, in temperate climates with annual seasons, the reproductive cycle is interrupted (40). Gonadal development and oogenesis are under the control of the adenohyphophysis (pars distalis of the pituitary gland) and are strongly correlated with seasonal environmental changes, particularly temperature. Lower ambient temperatures have a negative effect on the secretion of gonadotropins by the pituitary gland, the sensitivity of germinal epithelium to gonadotropic hormones, and on vitellogenesis (the synthesis of yolk necessary for incorporation into growing oocytes) (40, 41). In the fall and winter months, *Xenopus laevis* typically enter a period of torpor, lay relatively few eggs, and reduce food intake in preparation for winter hibernation (43, 44). Emergence of *Xenopus laevis* from winter hibernation, subsequent oocyte growth, ovulation, spawning, and breeding tend to coincide with warming temperatures and the end of the rainy season. In addition, daylight hours usually lengthen as the ambient temperature increases, but there is little specific information on the effect of photoperiod on *Xenopus laevis.* Other laboratory amphibian species housed under constant lighting do not produce healthy eggs (45). Bellerby (46) suggests that photoperiod does not play a significant role in stimulating oogenesis in *Xenopus laevis*, since they normally live year round in darkened water and reproduce over a range of diel cycles.

Most laboratory-maintained *Xenopus laevis* are kept year-round under constant light cycles (12h light/12h dark) and in water between 19° to 23°C (28). Under these conditions, they are quite capable of continuous gametogenesis and of producing a steady supply of oocytes. Nevertheless, many investigators report failures in oocyte or egg production and poor quality eggs and oocytes, usually beginning in the late summer months and fall (30, 35, 36). For the most part, the cause is unexplained, but may be due in part to the naturally occurring and innately regulated period of topor as described in wild frogs. Barring disease, parasitic infections, or other sources of stress in the colony, egg quality and production usually improves again in late fall and winter.

There is evidence that vitellogenesis and oocyte growth are regulated by estrogen (47-49), which is thought to increase in cold-adapted *Rana pipens* as they divert energy and nutrients to oocyte production during hibernation in preparation for the spring breeding season (50). *Xenopus laevis,* like *Rana pipens* and other ectotherms, may actually benefit from a period of cold exposure or hibernation to promote the production of estrogen and metabolic conditions necessary for oogenesis and ovulation (48-53). This might account for the improvement in egg quality and egg quantity that investigators report after they keep their colony housed at 16°C and gradually (over 48 h) reintroduce the frogs to warmer temperatures, prior to collecting eggs or oocytes. Alternatively, *Xenopus laevis* are stressed by prolonged exposure to temperatures below 14°C and above 26°C (30). Adults may survive, but the quality and quantity of oocyte and eggs produced deteriorates (30). Water temperatures approaching 30°C are not ideal for gametogenesis and can in fact be lethal to *Xenopus spp.* adults and embryos and other amphibian species (38, 54-57). In the wild, as temperatures approach this range, adult *Xenopus laevis* will aestivate (burrow into the mud) to prevent overheating and dehydration, and resume the reproductive cycle when conditions become more hospitable.

Cold acclimation of laboratory *Xenopus laevis* for the purpose of improving the quantity and quality of oocytes is not without risk to the animals' health. Immunosuppression, characterized by low levels of complement, lymphopenia and diminished ability of lymphocytes to proliferate is well documented in cold-adapted *Rana pipens* (58-60). It is a normal physiologic response during cold exposure and hibernation (58-60). Cold adaptation has also been reported in laboratory *Xenopus laevis* (61). A marked increase in the hematocrit and hemoglobin levels, and a neutro-

philia, lymphopenia, and a marked eosinopenia are consistent with the changes reported in the hematological profiles seen in cold-adapted amphibians (58-61). Increases in hemoglobin and RBC count in cold-adapted amphibians are thought to be an evolutionary, compensatory mechanism to increase the oxygen carrying capacity of the blood in response to declining temperatures (59). Eosinopenia may indicate a decreased need for histamine-releasing cytotoxic cells during periods of low temperature (58, 60). The increase in neutrophils observed in cold-adapted amphibians is attributed to the need for phagocytic protection and rapid defense against bacterial and fungal pathogens (58, 60).

Aeromonas hydrophilia, *Flavobacterium spp.*, *Mycobacterium spp*. and *Saprolegnia spp.* are opportunistic pathogens of laboratory *Xenopus laevis* that could cause disease in cold-adapted immunosuppressed frogs. *Flavobacterium meningosepticum* in particular, are bacteria that prefer to grow in cold water temperatures and have caused disease outbreaks associated with an increase in population density and colder water temperatures (62). In addition, the stress of handling and multiple experimental manipulations will further increase the susceptibility of cold-adapted laboratory *Xenopus laevis* to infection.

While *Xenopus laevis* are remarkably tolerant to gradual increases or decreases in temperature, they are, like most amphibians, susceptible to thermal shock (42, 56, 57, 61-63). Mortalities occur if they are abruptly exposed to more than a 2 to 5°C variation in temperature (56, 57). In laboratory settings this may occur by either failure to control temperature fluctuations during water changes or by abrupt reintroduction of cold-acclimated frogs into warmer water (61).

Age of the female. Some of the best estimates of age and maturity have been based on certain populations of wild *Xenopus laevis* in California and determined by the snout to vent length (SVL) (37, 39). Depending on temperature and the availability of food, wild female *Xenopus laevis are* sexually mature as early as 6 months post-metamorphosis when they achieve a SVL of approximately 65 mm (30, 31, 39, 40). At this age, they may produce as many 1000 secondary follicles containing mature oocytes (39, 40, 64). By 2 to 3 years of age, both wild and laboratory-reared *Xenopus laevis* (SVL 80-104 mm) have reached their peak reproduction and can lay up to 3 or 4 clutches/year (30, 31, 37, 39, 40, 64-66). Each clutch can contain up 10 to 20 thousand eggs or more (30, 37, 40, 64). Very old wild frogs (4.5-15 years old, SVL ~119 mm) are capable of multiple spawnings and producing hundreds of thousands of good quality eggs that hatch to larva (37). In contrast, the fecundity of aged and very large (greater than 140 mm) commercially reared *Xenopus laevis* does not appear to be as good (66).

Wild *Xenopus laevis* are estimated to grow only about 1.2 mm/month at 8-15°C (39). Given the current annual per diem rates at most laboratory animal facilities and the lack of established protocols that optimize the growth and fecundity of *Xenopus laevis* in laboratory settings, it may be more cost-effective and efficient for investigators to purchase older, albeit more expensive, adult female frogs to begin with, rather than attempt to rear young frogs until they reach the optimal age and size for consistent egg production.

Nutrition. *Xenopus laevis* are carnivorous opportunistic feeders whose food appears to include just about everything available in the aquatic environment (38, 67-71). In field studies, the stomach contents of *Xenopus laevis* have yielded the remains of fish, birds, and other amphibians, but small insects, slugs, worms, and other aquatic invertebrates predominate. *Xenopus laevis* are predators and prefer live prey, but will scavenge the carcasses of dead animals. They also readily adapt to eating pelleted food or raw meat in a laboratory setting (28). Adult *Xenopus laevis* will consume their sloughed skin, eggs and offspring, which normally make a significant contribution to the nutrition of the parental population (37, 71, 72).

The best diet for laboratory adult *Xenopus spp.* is a topic of some controversy and the feeding practices at different laboratory animal facilities vary (28). Diets fed to laboratory *Xenopus spp.* range from chopped liver and other meats, frozen invertebrates packaged and sold as fish food at pet stores, various cultured worms, small crickets, and commercially prepared pelleted foods (67, 73). None have been critically evaluated. One author (73) cautions against using pelleted food intended for omnivorous fish and turtles. Such food may not meet the protein, fat, and fat-soluble vitamin needs of a strict carnivore such as *Xenopus laevis*.

Feeding large colonies of laboratory *Xenopus* is also somewhat dictated by convenience and ease of cleaning the residues from the water and tank drainage systems. How much and when to feed, particularly to large colonies housed in tanks that hold several hundred frogs, is largely determined by trial and error, the body condition and health of the frogs based on visual inspection, and whether or not there is leftover material in the tank after the feeding frenzy. The standard metabolic rates of anurans (number of kcal required/day by body weight and body temperature) are available (73) and can offer a rough guide for feeding laboratory housed *Xenopus spp.,* but the dietary requirements that optimize oogenesis are unknown. It is known that ovary production in *Xenopus laevis* is profoundly affected by food supply: during food shortages the ovaries regress (32, 46, 49). *Xenopus laevis* are remarkably tolerant of starvation and can significantly reduce energy consumption for many months during hibernation and under adverse conditions such as drought and limited food supplies. However, under such conditions, gonads represent additional energy reserve (32, 38, 46, 49).

It remains to be determined how to adequately feed laboratory housed *Xenopus laevis* so that nutritional needs are met and oogenesis continues year round. Certainly extreme variations in housing conditions and feeding can be avoided in laboratory settings, but frogs that receive inadequate nutrition due to their subordinate position in the colony, or stress related to disease or other factors are not likely to produce large egg clutches or good quality eggs.

"Bad eggs" as a result of harvesting methods and experimental techniques

The various laboratory protocols used to collect oocytes and eggs from *Xenopus laevis* are beyond the scope of this report but can be reviewed in several references (30, 41, 74, 75). All of the methods will to some degree, affect the quantity and quality of material obtained. The biochemical and physiological criteria that define "good quality" versus "bad quality eggs" has not been standardized for a particular experimental application. This can make it difficult to suggest improvements in colony management or harvesting techniques, when egg production is adequate and the eggs appear to be healthy morphologically.

Oogenesis in *Xenopus laevis* is asynchronous, that is all stages

of eggs are present in the ovary at any given time (75). Under Dumont's classification system (51), there are 6 stages of oocytes, stage 1 being the smallest (least mature) and stage VI the largest full-grown oocytes. Typically, investigators require healthy looking mature stage VI oocytes, characterized by a diameter > 1200 μm, a smooth surface, and clearly defined line of separation between the pigmented vegetal hemisphere and the light colored animal hemisphere to complete biochemical experiments involving protein synthesis, enzyme systems, and microinjection (75). Sexually mature *Xenopus laevis* may produce thousands of oocytes, of which 30% in a clutch will be in stage VI (75). Usually only a small number (200-300) of stage VI oocytes are needed for each experiment (30, 75) and oocytes can be repeatedly collected from the same frog by surgically removing one or more small pieces of ovary, followed by several months recovery period between surgeries. If *Xenopus laevis* eggs are required, they are usually collected from the female after administration of human chorionic gonadotropin (HCG), given to stimulate ovulation and egg laying. After the injection, frogs are isolated and allowed to lay the eggs, or the eggs are collected by squeezing or "milking" them from the abdomen. Frogs should then be rested several months before further stimulation for egg laying.

Over-harvesting of either oocytes or eggs without an adequate rest period in between may result in a decrease in the overall quantity or quality of eggs and oocytes collected from any one frog. As recommended by Keem et al, (76) and Wu and Gerhart (30), most laboratories can repeatedly collect an adequate quantity of oocytes or eggs from the same frog, after allowing the animal to rest 2-3 months between the collections. The long-term effects of repeated administration of hormones is unknown, but some investigators report maintaining the same animals for several years using this regime. Alternatively, Mikamo (53) reports that maintaining laboratory *Xenopus laevis* for long periods without ovulation results in an increase in the percentage of degenerating follicles in the ovary and eggs that develop poorly.

Poor quality oocytes or eggs are typically "white-banded," wrinkled, and atretic with an irregular shape due to collapse of yolk away from the surrounding membranes, with a brown irregular pigmentation of the animal hemisphere, and increasing pigmentation of the vegetal hemisphere (36, 75, 77). Eggs and oocytes showing these characteristics are degenerating and are not useful for biochemical experiments or microinjection. Clearly, more information is needed that includes the physiological, biochemical, and genotypical traits of eggs and oocytes that are considered desirable (or not) for experiments. Morphological descriptions of health stage VI oocytes are available (36, 75, 77), but investigators at our institution have reported that on some occasions, oocytes with an acceptable physical appearance did not yield usable data.

Colonization of the egg with bacteria or ubiquitous watermolds such as *Saprolegnia spp*. is one important factor related to harvesting and experimental technique that contributes to degeneration (77). Colonization of eggs by fungal and bacterial opportunistic pathogens is a normal mechanism of decomposition and will occur in eggs that are laid and allowed to sit for long periods in fecal contaminated water. In addition, females will eat freshly laid eggs if given the opportunity, so eggs should be removed from the water as soon as possible. Contamination of oocytes with bacteria can also occur as a result of unclean surgical technique (36).

Some harvest protocols recommend treatment of the pieces of surgically harvested ovarian tissue with collagenase to release all of the oocytes (75). Use of collagenase makes the vitelline envelope of the oocyte fragile and more difficult to inject (75). Also, use of collagenase-treated oocytes too soon after the enzyme treatment is associated with a depression of endogenous protein synthesis (75). These are all technical problems related to harvesting methods and experimental techniques that are correctable, but frequently result in the animals' being blamed for "bad eggs."

Summary

Based on the biology of the amphibian reproductive cycle, there do appear to be certain factors, namely temperature, age of the female, and nutrition, that are especially important to consider for laboratory *Xenopus laevis* that are intended to produce a steady supply of oocytes and eggs. Amphibian reproductive biology supports the practice of housing laboratory *Xenopus laevis* in either cold or warm water for the purpose of optimizing the quantity and quality of eggs and oocytes produced. With regards to age, 2 to 3 year old *Xenopus laevis* have usually matured enough to continuously produce large clutches of good quality oocytes and eggs. Lastly, because inadequate nutrition is detrimental to oogenesis in wild *Xenopus laevis*, laboratory frogs expected to continuously produce eggs and oocytes must be provided with the proper source and amount of food. These facts can provide a basis for decision-making regarding the housing conditions that optimize fecundity in laboratory *Xenopus laevis* maintained over the long term for the purpose of providing a regular supply of material for biological research. The experimental effects of genetic background and strain variation in laboratory *Xenopus laevis* needs to be investigated.

The demand for *Xenopus spp.* is predicted to increase over the next decade, perhaps approaching the numbers of rodents. There is currently a large initiative to develop *Xenopus spp.* EST's, full-length cDNA libraries, microarrays, and genomic maps, information that will significantly enhance the current utility of both *Xenopus laevis* and the complementary species *Xenopus tropicalis* in biomedical research (http://www.nih.gov/science/models/xenopus/). Both NICHD and the Trans-NIH Non-Mammalian Models Committee have recommended the establishment of stock centers to maintain 200-300 (and eventually up to 1000 lines) of both *Xenopus laevis* and *Xenopus tropicalis* transgenic and gene trap lines (http://www.nih.gov/science/models/xenopus/). Maintaining such large populations of this species in a laboratory environment will be a historical precedent. It will require the knowledge and experience of research scientists, amphibian biologists, and laboratory animal veterinarians alike. Studies that investigate the housing conditions that optimize the health and reproduction of *Xenopus laevis* in laboratory animal facilities are needed to ensure the future success and growth of such populations.

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