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A monitoring study of repetitive surgical oocyte harvest in *Xenopus laevis*

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Abstract

Xenopus laevis oocytes provide a successful electrophysiological model for heterologous de-novo expression of channels and receptors as well as for the incorporation of exogenous membrane preparations rich in receptors/channels for pharmacotoxicological studies. The method of choice for oocyte harvesting is repetitive surgical laparotomy of female adult *Xenopus*. The lack of policies regulating oocyte surgery in France prompted us to design a follow-up study to assess the effect of repetitive laparotomy on *Xenopus*. To this end, we used a batch of 24 female *Xenopus* which were tagged with an electronic chip. The amphibians were housed by colonies of eight in 20-L plastic containers. Groups of six amphibians were operated every two, three, four and six months. The weight of each female amphibian was recorded before and after the operation. The recovery from anesthesia, the incision healing, the *Xenopus* behavior and the skin health were also monitored. The impact of repetitive surgery on the oocytes survival following nano-injection with cDNA, mRNA or Torpedo membranes was systematically assessed. Here, we show that repetitive surgery every two months does affect animal weight. In addition, a four-months-recovery period should be allowed between each laparotomy. However, repetitive surgery does reduce the number of *Xenopus* used for electrophysiological/pharmacotoxicological characterization of receptors and channels.

Suivie des effets de l'extraction chirurgicale répétitive d'ovocytes chez *Xenopus laevis*

Les ovocytes de *Xenopus laevis* constituent un modèle d'électrophysiologie réussi pour l'expression de-novo des canaux et récepteurs, l'incorporation de membranes exogènes riches en récepteurs et canaux et leur caractérisation pharmacotoxicologique. La méthode acceptée pour l'extraction d'ovocytes est la laparotomie répétitive de xénopes adultes. L'absence de régulations concernant l'extraction d'ovocytes de xénopes femelles en France nous a incités à suivre et à évaluer l'effet de laparotomies répétitives sur les xénopes. Dans ce but, nous avons utilisé un lot de 24 xénopes femelles, marquées avec une puce électronique. Les amphibiens ont été placés par colonies de huit dans des bacs en plastique de 20 litres. Des groupes de six xénopes ont été opérés tous les deux, trois, quatre et six mois. Le poids de chaque xénope a été déterminé avant et après l'intervention. Leur récupération de l'anesthésie, leur guérison de l'incision pratiquée, leur comportement ainsi que l'état de leur peau ont également été surveillés. L'impact des laparotomies répétitives sur la survie des ovocytes après nano-injection avec de l'ADN, l'ARN ou des préparations de membranes de torpille a été systématiquement évalué. Nous montrons ici que la chirurgie répétée tous les deux mois affecte le poids de l'animal. De plus, une période de récupération de quatre mois est nécessaire entre chaque laparotomie. Cependant, la chirurgie répétitive réduit effectivement le nombre de xénopes utilisées pour la caractérisation électrophysiologique/pharmacotoxicologique des récepteurs et des canaux.

Keywords : Heterologous expression, voltage-clamp electrophysiology, *Xenopus laevis*.

Introduction

The African Clawed Frog *Xenopus laevis* is an amphibian of the order Anura and the family Pipidae. *X. laevis* is an aerobic amphibian but behaves as an entirely aquatic animal. It needs to breath air but it does not need land-based existence. *X. laevis* is very susceptible to desiccation, and can die from being out of the water for a few hours (Goldin, 1992).

Gurdon *et al.* (1971) originally demonstrated that *Xenopus* oocytes can be used to express exogenous messenger RNA (mRNA) when microinjected into the cytoplasm. The contribution of *Xenopus* oocyte microinjection methods to the rapid progress seen in molecular and cellular biology since their introduction as

an heterologous expression model is remarkable (Soreq and Seidman, 1992; Brown, 2005). The nicotinic acetylcholine receptor (nAChR) was the first functional receptor to be expressed in *Xenopus* oocytes following microinjection of exogenous messenger RNA coding for *Torpedo* nAChRs subunits that allowed the electrophysiological and molecular characterization of the cholinergic receptor (Barnard *et al.*, 1982; Sakmann *et al.*, 1982). Rapidly, *Xenopus* oocytes became one of the most widely used systems for heterologous expression and functional characterization of ion channels, receptors and transport proteins after microinjection of mRNA or cDNA (for reviews, see Lester, 1988; Soreq and Seidman, 1992). *Xenopus* oocytes were also shown to be able to incorporate purified *Torpedo* electroplaque membranes into their plasma membrane (Marsal *et al.*, 1995). Later, human functional receptors issued from surgically resected and from *post-mortem* brain tissues were microtransplanted into the plasma membrane of *Xenopus* oocytes (Miledi *et al.*, 2002; Bernareggi *et al.*, 2007). The advantage of the transplantation of biological membranes into *Xenopus* oocytes is that native receptors/channels can be reconstituted together with their associated lipids and proteins.

Female *Xenopus* produce thousands of oocytes over their lifetime period. The oocytes were morphologically classified into six stages according to their development (Dumont, 1972). Stage V-VI oocytes are used for electrophysiological studies. These oocytes of ~1.2 mm diameter show a clearly defined vegetative pole and an animal pole where the nucleus is located. Repetitive surgery on a single female *Xenopus* is a commonly worldwide accepted practice for oocyte extraction since it reduces the number of laboratory amphibians to be used. However, there is no consensus on the number and frequency of laparotomies that could be performed on one female *Xenopus*. As example, the Guidelines for egg and oocyte harvesting in *Xenopus laevis* limit the number of surgical oocyte harvesting procedures to six (the sixth being a terminal surgery), and fix a recovery time of at least 4 weeks for the female *Xenopus* between each surgery (ARAC, 2005). The Boston University, which is committed to observe Federal Guidelines and AAALAC International Guidelines for humane care and use of animals, recommend 4 repetitive surgeries per female *Xenopus* (the fourth being terminal) and a minimum of one month recovery period between surgical oocyte extractions (Boston University, 2009). In contrast, in Switzerland, only one terminal operation is consented (Bertrand *et al.*, 2008). The lack of guidelines regulating *X. laevis* surgery for oocyte extraction in France prompted us to undertake a monitoring study to assess the effect of repetitive surgery on *X. laevis* tagged with an electronic chip.

Materials and methods

Xenopus laevis culturing

Xenopus laevis tagged with an electronic chip were purchased from the *Xenopus* Biological Resources Center (University of Rennes 1, France; *Figure 1A*). The cylindrical chip FDX B of 12 mm long and 2.12 mm diameter made of transparent bio-glass (Real Trace, Villebon sur Yvette, France) was inserted subcutaneously in the ventral side of the left leg of the amphibians using an injector (Real Trace; *Figure 1B and C*). The *Xenopus* were housed at the animal facilities conditioned at 21°C with a light/dark cycle of 12 h and fed with trout pellets twice a week and with living bloodworms (Aquarelite, France) once a week. The water was dechlorinated by passage through a charcoal activated filter (Cuno, 3M Purification Inc., St. Paul, MN, USA) and was thermo-regulated to a temperature of 20°C. Water quality was controlled twice a week using a potassium iodide starch paper indicator to avoid any chlorine traces that can harm the skin of the amphibians. At the beginning of this study, the *Xenopus* were disposed in groups of 12 amphibians in 20 liters plastic containers (*Figure 1A*). We have observed that the number of female *Xenopus* per tank could affect oocyte quality and therefore, in the third year of this follow-up study, the number of *Xenopus* per tank was reduced to 8.

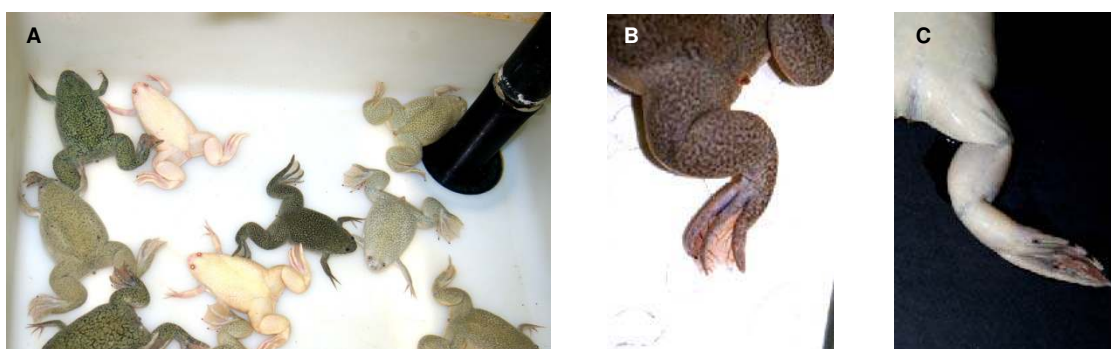


Figure 1. *Xenopus laevis* tagged with an electronic chip were purchased from the *Xenopus* Biological Resource Center (University of Rennes 1, France). (A) Female *Xenopus* were housed in colonies of 12 amphibians at the beginning of this trial. (B) Dorsal view of a "chip"-tagged left leg of a female *Xenopus*. (C) Ventral view of the left leg of a female *Xenopus* tagged with an electronic chip. The FDX B chip of 12 mm long and 2.12 mm diameter is clearly seen. The electronic chips were fully tolerated, without secondary effects, through the whole study (3 years for the first batch of 12 animals).

Figure 1. Les *Xenopus laevis* marquées avec une puce électronique ont été achetées au Centre de Ressources Biologiques de Xénopes (Université de Rennes 1, France). (A) Les *Xenopus* femelles ont été logées par colonies de 12 amphibiens au début de cet essai. (B) Vue dorsale d'une *Xenopus* femelle marquée à la patte gauche avec une "puce". (C) Vue ventrale de la patte gauche d'une *Xenopus* femelle marquée avec une puce électronique. La puce FDX B de 12 mm de long et 2,12 mm de diamètre est clairement visible. Les puces électroniques ont été parfaitement tolérées, sans effets secondaires, au cours de l'ensemble de l'étude (3 ans pour le premier lot de 12 animaux).

Oocyte defollicularization

The ovarian lobes were pulled apart into small pieces using a pair of forceps and placed in a 10-mL OR2 solution (88 mM NaCl, 1 mM MgCl₂, 5 mM HEPES; pH 7.6) containing 1 mg/mL collagenase type I (Sigma). The whole was incubated for 10 min under gentle shaking. The small pieces of partially digested ovarian lobes were washed three times with 50 mL OR2 solution and once with 50 mL Barth's solution (88 mM NaCl, 1 mM KCl, 0.41 mM CaCl₂, 0.82 mM MgSO₄, 2.5 mM NaHCO₃, 0.33 mM CaNO₃, 7.5 mM Hepes; pH 7.6). Finally, the treated ovarian lobes were transferred into a plastic Petri dish (56 mm diameter) containing 10 mL Barth's solution supplied with 2 mg/mL kanamycin (Sigma). The connective tissue and the follicular cell layer of stage V-VI oocytes, were removed under a stereoscopic microscope using a pair of forceps. The defolliculated oocytes were transferred into a 56 mm Petri dish containing 10 mL Barth's solution. The quality of the oocytes was controlled and groups of 50 oocytes were dropped into 35 mm plastic Petri dishes containing 5 mL Barth's solution supplied with 2 mg/mL kanamycin. The oocytes treated in this manner stick to the clean surface of plastic Petri dishes facilitating the microinjection.

Surgical extraction of ovarian lobes from *X. laevis*

The amphibian to be operated was identified by positioning the petSCAN RT 100V5 scanner (Real Trace) 5 - 10 cm over the tagged leg. The amphibian was isolated in a 3-L plastic container for 1 h to reduce handling stress. The *Xenopus* was anaesthetized by immersion for 20 min in a water bath containing 1 g/L ethyl-3-amino benzoate methanesulfonate salt (Sigma, St. Louis, MO, U.S.A.). The laparotomy was performed using autodaved material. An incision of 1 cm through the skin and muscle was performed in the inferior ventral side of the *Xenopus*. Several ovarian lobes were extracted with sterile forceps and immediately immersed in 10 mL OR2 solution. The muscle plane was sutured using undyed braided absorbable suture (Ethicon, Johnson & Johnson, New Brunswick, NJ, U.S.A.), and the epidermal layer was sutured using a blue non-absorbable suture (Ethicon). Afterwards, the *Xenopus* was placed in a water bath taking care not cover the dorsal side of the amphibian to allow respiration through the skin. Once the *Xenopus* was recovered from anesthesia, the recipient was filled with water and the amphibian was observed for 1 day before being replaced into the main tank.

Microinjection of purified membranes, cDNA and mRNA into *Xenopus* oocytes

Torpedo electrocyte membranes, rich in muscle type ($\alpha_{12}\beta\gamma\delta$) nicotinic acetylcholine receptors (nAChRs), were purified as previously described (Krieger, 2007). Aliquots of 20 μ L of *Torpedo* membranes (2.7 mg/mL total proteins) in 5 mM glycine were conserved at -80°C until use. For microtransplantation, a volume of 50 nL of *Torpedo* electrocyte membrane solution was injected into the vegetative pole of *Xenopus* oocytes using a micro-syringe pump controller, Micro4TM (World Precision Instruments, Sarasota, FL, U.S.A.), mounted on a stereoscopic microscope. Glass micropipets for microinjection (3.5 nL, World Precision Instruments) were prepared using the micropipet puller PP-830 (Narishige Scientific Instruments, Tokio, Japan). Following microinjection, *Xenopus* oocytes were incubated at 18°C. Incorporation of *Torpedo* electrocyte membranes into the plasma membrane of the oocyte took place 1 day after microinjection.

To express $\alpha 4\beta 2$ nAChR, 50 nL of an aqueous mixture of pRcCMV cDNA vectors encoding for human $\alpha 4$ and $\beta 2$ nAChR subunits (kindly provided by Dr P. J. Corringer, Pasteur Institute, Paris, France), were injected into the animal pole of *Xenopus* oocytes. A 12 ng amount of each plasmid was injected. Plasmids were purified using an endotoxin-free plasmid maxiprep kit (GenEluteTM, Sigma). The injected oocytes were incubated for 3 days at 18°C *prior* to electrophysiological experiments.

The expression of human $\alpha 7$ nAChR was obtained following injection of 50 nL aqueous mRNA (5 ng) in the vegetative pole of *Xenopus* oocytes. Messenger RNA was prepared using a mMACHINE[®] SP6 kit from Ambion (Austin, TX, U.S.A.) and, as template, pcDNA3.1 cDNA vector encoding human $\alpha 7$ nAChR subunit (kindly provided by Prof. I. Bermudez, Oxford Brookes University, Oxford, U.K.). Translation of mRNA into functional $\alpha 7$ nAChRs took place after 48 h incubation at 18°C.

Experimental design

A group of 12 female *Xenopus* was divided into four groups: Group 1, amphibians operated every two months; Group 2, amphibians operated every three months; Group 3, amphibians operated every four months; and Group 4, amphibians operated every six months. The number of repetitive interventions *prior* to euthanasia was five with the exception of Group 1. The parameters measured for each individual were: (i) the weight *prior* and following chirurgical intervention, in a weekly basis, (ii) the recovery from anesthesia, (iii) the behavior of female *Xenope* following laparotomy, (iv) the incision healing and the skin health of the amphibians and (v), the quality of *Xenopus* oocytes for the expression and incorporation of nAChRs into their membranes.

Results and Discussion

Xenopus eggs and oocytes continue to play a central role in many biological disciplines including developmental studies, structural biology, cell biology, physiology, pharmacology, toxinology, biochemistry to mention some of them (Brown, 2004). For the purpose of electrophysiological characterization of excitable membrane proteins, *Xenopus* oocytes not only efficiently translate exogenous mRNA or cDNA but, in addition, they are able to process post-translational modifications necessary for the correct folding and positioning of a synthesized protein at the plasma membrane of the oocyte (Barnard and Miledi, 1982; Soreq and Seidman, 1992). Finally, the microtransplantation into *Xenopus* oocytes of human brain membranes from frozen tissues containing

functional receptors and channels opens new avenues for pharmacology of receptor-associated brain disorders (Miledi *et al.*, 2002).

Xenopus could be easily stressed. When this happens, they can lose their skin and regurgitate their food. The protocol we apply for surgical extraction of *Xenopus* oocytes is in accordance to the existing international guidelines (ARAC, 2005; Boston University, 2009; IACUC Guidelines, 2001, 2007) to minimize animal distress (Figure 2). Following laparotomy, the amphibians were placed in a separate room at 20°C for observation. Their initial behavior when they were replaced into their colonies was to slide down the other amphibians and to stay quiet for a prolonged period back the black siphon corner of their tank (see Figure 1A). The members of the colony have never shown signs of aggression towards the operated amphibians. There is no need for us to take out the skin sutures since they disappear in a week, and there was no *Xenopus* mortality associated with the laparotomy.

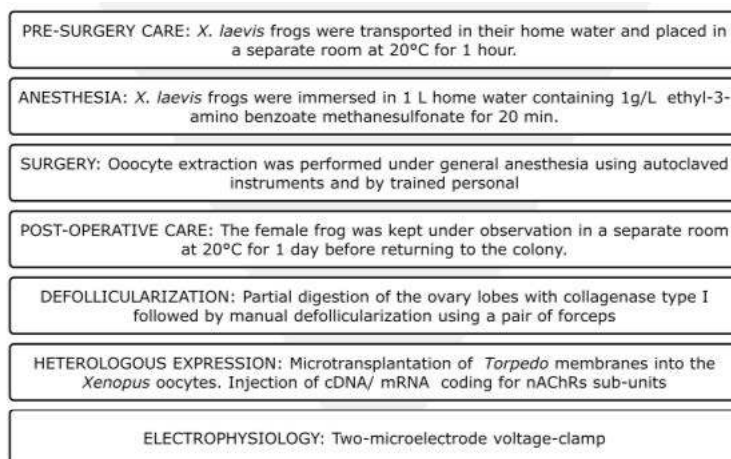


Figure 2. Summarized protocol for surgical extraction of *Xenopus* oocytes for electrophysiological/pharmaco-toxinological studies.

Figure 2. Résumé du protocole pour l'extraction chirurgicale d'ovocytes de xénopes pour les études électrophysiologiques/pharmaco-toxinologiques.

To assess the effect of multiple surgeries of oocyte extraction on female *Xenopus*, we have surveyed the weight of the amphibians in a weekly-basis, before and after laparotomy. The survey of female *Xenopus* from Group 1 that were operated every 2 months showed that their weight was stable although they had a tendency to decrease with negative slopes varying from -0.025 to -0.006 (Figure 3A). Then, the female *Xenopus* of Group 1 have lost 5.6 ± 2.6 g ($n = 3$) in a period of 200 days. In addition, the scar under the incision zone was hypertrophied, which rendered oocyte extraction difficult. These observations led us to stop performing the surgery every two months for the female *Xenopus* of Group 1.

Figure 3B shows the evolution of the weight of Group 2, *i.e.* female *Xenopus* that were operated every 3 months for five times. The weight of these females was stable although a tendency to increase with positive slopes varying from 0.005 to 0.018 was observed. *Xenopus* of Group 2 have gained 4.1 ± 2.3 g ($n = 3$) in the whole period of the study.

Figure 3C illustrates the evolution of the weight of female *Xenopus* of Group 3 that were operated every 4 months. There was a clear increase tendency of their weight, with a positive slope ranging from 0.005 to 0.25. Group 4, *i.e.* amphibians that were operated every 6 months for oocyte extraction, showed a weight profile similar to that of Group 3 (data not shown).

These results indicate that laparotomy performed every two months affects the weight of the amphibians (~ 10% after three operations). In several cases, the scars under the skin of female *Xenopus* of Group 1 were hypertrophied and difficulties for suturing the muscle and epidermal layers were experienced. It was the reason why this group was operated only three times. However, repetitive laparotomies did not seriously affect the weight of the amphibians whenever an adequate recovery time is allowed to female *Xenopus* subject to surgery for oocyte harvest. A recovery time of three months for female *Xenopus* subject to laparotomy for oocytes harvest is thus here proposed.

Figure 3D illustrates a batch of twelve amphibians operated every ~3 months. It is worth noting that the operated female *Xenopus* lost in average 4.53 ± 1.14 g after each laparotomy ($n = 12$; 45 laparotomies). Yet, the amphibians recovered back to their average weight in two weeks. Repetitive surgery for oocyte extraction is an accepted procedure that promotes the reduction of the total number of amphibians used over long term.

The quality of the oocytes also reflects the amphibian health. Therefore, we have also surveyed the quality of the oocytes for heterologous expression of nAChRs and electrophysiological studies. Repetitive laparotomies did not affect the size of stage V – VI oocytes. Oocyte survival after nano-injection was also not affected by repetitive laparotomies. Finally, repetitive laparotomies neither affect heterologous expression of receptor proteins nor *Torpedo* membrane incorporation into the plasma membrane of *Xenopus* oocytes for electrophysiological/pharmaco-toxinological studies.

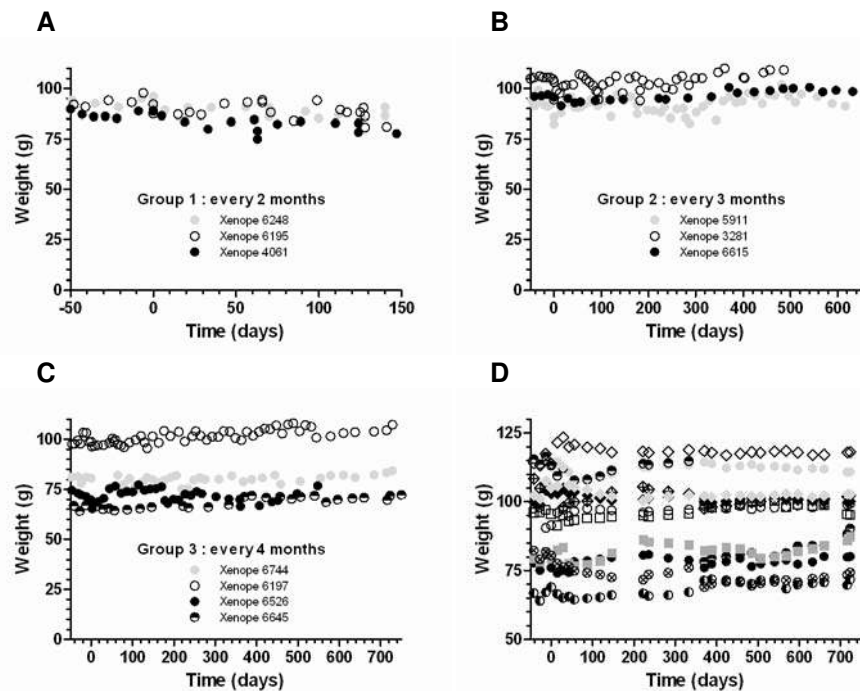


Figure 3. Evolution of the weight of female *Xenopus* subject to repetitive laparotomy. A. Group 1: female *Xenopus* operated every 2 months. B. Group 2: female *Xenopus* operated every 3 months. C. Group 3: female *Xenopus* operated every 4 months. D. Batch of female *Xenopus* of year 2009 which were operated every 3 months: 6094 (●), 6456 (○), 5338 (▶), 4685 (▲), 6588 (◆), 6645 (△), 6836 (▷), 1998 (□), 5334 (■), 6953 (✦), 4273 (▼) and 6156 (✧). The “zero” in the abscise axe indicate the first operation practiced to the female *Xenopus*.

Figure 3. Evolution du poids de xénopes femelles sujettes aux laparotomies répétitives. (A) Groupe 1: xénopes femelles opérées tous les 2 mois. (B) Groupe 2: xénopes femelles opérées tous les 3 mois. (C) Groupe 3: xénopes femelles opérées tous les 4 mois. (D) Lot de xénopes femelles de l'année 2009 qui ont été opérées tous les 3 mois: 6094 (●), 6456 (○), 5338 (▶), 4685 (▲), 6588 (◆), 6645 (△), 6836 (▷), 1998 (□), 5334 (■), 6953 (✦), 4273 (▼) and 6156 (✧). Le “zéro” dans l'axe des abscisses indique la première opération pratiquée à la xénope femelle.

Conclusion

Repetitive surgery for oocyte extraction is a usual practice worldwide accepted which may be justified considering the *REDUCTION* in the total number of animals used over a long term period, without affecting the quality of the obtained results. The aim of this paper was to assess the effects of multiple surgeries on female *Xenopus* by using chip-tagged amphibians to individualize each animal. An electronic chip is a *REFINEMENT* alternative to minimize pain, as well as to distress and enhance *Xenopus* well-being, as it is promoted by the 3 R's in animal research (*REDUCTION*, *REFINEMENT*, and *REPLACEMENT*; Russell and Burch, 1959).

Electronic chips are low-cost devices representing ~1% of the cost of a living female *Xenopus*, and its use should be encouraged to individualize the amphibians for surveying the weight of the animal, which is a measurable indicator of *Xenopus* health.

In conclusion, a minimum of three months recovery between each laparotomy should be allowed to female *Xenopus* subject to repetitive laparotomies for oocyte extraction.

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