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RESEARCH ARTICLE

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A minimally invasive procedure for blood extraction from *Xenopus laevis* allows follow up studies without euthanasia

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ABSTRACT

Blood extraction is extremely important for the development of scientific research; however, the existing methods for amphibian´s blood sampling are invasive, mainly leading to the euthanasia of the animal. Therefore, less intrusive methods that allow the obtention of multiple samples from the same individual, are needed as an alternative to the common methods available. Hence, the aim of this study was to propose a minimally invasive method for obtaining blood from the hind leg of *Xenopus laevis*, that allows continuous sampling without compromising the wellbeing of the organisms. With this method, it was possible to extract blood and plasma from adults and juveniles, and the amount of sample was enough to perform biochemical and molecular assays to assess the viability of the blood. The results also revealed that this method is a convenient alternative to obtain blood without affecting the welfare of the experimental organisms, avoiding the cull of the animals, and the samples are viable for their use in follow up studies.

KEYWORDS
Xenopus laevis; blood extraction; vena dorsalis pedis

Introduction

Studies on amphibians have contributed to the progress of life sciences research for more than a hundred years; particularly species such as *Xenopus laevis* and *Xenopus tropicalis* (Kashiwagi et al., 2010; Green, 2010), have become widely used models to study subjects like cellular, molecular and developmental biology, ecotoxicology, genetics, among others (De Robertis & Gurdon, 2021; Korte et al., 2011; Olson, Hulstrand, & Houston, 2012).

One of the biological materials commonly used in sciences is blood as it is useful to obtain genetic material (Sive, Grainger, & Harland, 2012), to evaluate and compare blood biochemistry, hematology (Wilson et al., 2011) or endocrinology (Luna, Coady, McFadden, Markham, & Bartels, 2013). However, the available methods for blood extraction in amphibians are mostly invasive, such as cardiac puncture (cardiocentesis) for which one to 3 ml of blood are obtained per individual (Chang, Hu, Lake, Bouley, & Johns, 2015; Forzán, Heatley, Russell, & Horney, 2017; American Veterinary Medical Association, 2020). Another technique, leg amputation, yields 2 to 7 ml (Dixon et al., 2021). Although the amount of blood is sufficient for lab analyses, both techniques do not allow subsequent monitoring of organisms (Wright, 2001).

Another commonly used technique for blood collection and the identification of individuals is toe clipping that gives from 1 to 3 ml of blood. In this technique, one of the individual´s digits of the foot is cut (Holsbeek, Mergeay, Volckaert, & De Meester, 2010), but their negative effects have been

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broadly documented, and include impacts on survival, mobility and dexterity of the individuals sampled (McCarthy & Parris, 2004; Parris et al., 2010).

Although through these methods, a somewhat large volume of blood can be obtained in a short time, they are highly invasive or even lethal, preventing the development of studies in which the animal´s response needs to be evaluated over time, requiring new organisms for each sampling event (Russel & Burch, 1959). Alternatively, blood extraction via puncture of the middle region of the abdomen medial vein (Mendoza, García-Ramirez, & Cárdenas-Henao, 2012) allows the obtention of 1 to 3 ml; although this technique is less invasive, it may cause cardiac lacerations if not performed correctly. Additionally, blood collection from the lingual venous plexus can be used for most frogs weighing over 25 g, here the mouth of the animal should be carefully opened with a rigid but smooth instrument, drawing the tongue forward to expose the lingual plexus; nevertheless, this method cannot be used in species that lack a proper tongue such as pipids, like *Xenopus spp* frogs, and it can cause dislocation or even rupture of the jaw if not done carefully (Forzán, Heatley, Russell, & Horney, 2017).

It is undeniable the need and importance of using non-human animals in scientific research to understand basic biological processes, to develop new products and to increase the knowledge and quality of life of a large number of species worldwide. For this reason, bioethics is essential in scientific studies in order to cause less suffering and/or use less animals under the principles of the 3Rs (Replacement, Reduction and Refinement) (Russell and Burch 1959). The 3Rs principles have become synonymous of improved animal welfare in research labs around the world (Hubrecht & Carter, 2019a); in Mexico, bioethics is not as developed as in other countries, and the national regulations that govern these practices have not been updated, considering only mammalian animals, such as is the case for the NOM-062-ZOO1999. However, recently the Mexican Government published a statement (CONBIOÉTICA, 2023) recognizing the lack of information and regulations related to the use of animals in research and teaching, which could be the beginning of a change to establish adequate rules in this context. In our case, the lack of local regulations led us to follow international ethical principles for the study of amphibians, such as the ASPA (Animal Scientific Procedures Amphibia 1986) from the United Kingdom (updated in 2021), and the regulations of the AVMW (American Veterinary Medical Association), in which the use of animals, including amphibians is highly regulated.

Therefore, this work proposes the development of a minimally invasive method for obtaining blood samples from the hind feet of *Xenopus laevis* that allows monitoring the same individual over time.

Materials and methods

All Animal procedures were conducted in accordance with the ASPA (Animal Scientific Procedures Amphibia 1986, UK) and the AVMW (American Veterinary Medical Association) regulations.

Animals and housing

Twelve wild type and albino adults and 24 juveniles of *Xenopus laevis* were acquired from a certified supplier (Aquanimals). Organisms were divided into groups of 3 and kept under captivity conditions in 40 L glass aquaria, each container was filled with 15 L of conditioned water [(NaCl, methylene blue (LOMAS) and water conditioner (ABE Aquarium), following the supplier's instructions)] and maintained in a 12:12-h light: dark cycle, with a temperature range of 18°- 22°C. All groups were fed 6 times a week *ad libitum* for 30 minutes during 16 weeks.

Blood and serum collection

Blood samples were taken every month for four months. The organisms were anesthetized in a solution of water with clove essence in a 1:5000 dilution (Vázquez, Castro, Hernández, Castro,

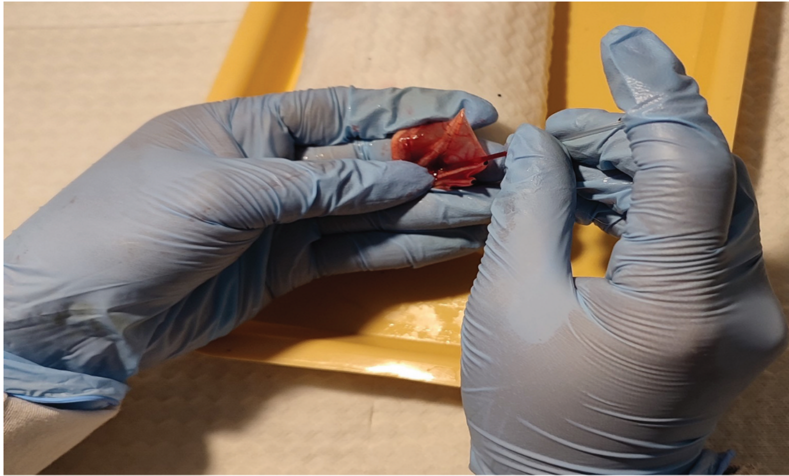

Figure 1. Puncture of the vascular membrane of the hind leg of *Xenopus laevis*, and blood collection from the *dorsalis pedis* vein by capillarity.

& De Lara, 2013) until the loss of consciousness and reflexes was confirmed. The individuals were then placed on an aseptic dissection tray covered with a towel moistened with conditioned water. The right hind foot of each individual was carefully held, and a blood sample was taken from the *dorsalis pedis* vein (Millard & Stephenson, 1940). Access to the vein was through the interdigital membrane (Figure 1). Using a sharp glass capillary tube, a small incision was made, and blood was collected by capillarity with a new 0.05 mm non-heparinized tube (Paul Marienfeld), immediately after collection blood was transferred to 0.5 ml tubes and refrigerated for up to one hour. No signs of distress were observed during blood withdrawal and all individuals fully recovered within 30–40 min. For juveniles, blood was also collected following the same procedure; however, the amount sampled was less than 40 μl. For juveniles, blood samples were kept frozen at −20°C to later extract gDNA.

At the end of the procedure, blood samples of adults were centrifuged at 2000 rpm for one minute, to separate the serum which was transferred to 0.5 ml microcentrifuge tubes and stored at −20°C for posterior ELISA analyses.

Determination of sex steroids

Plasma estradiol concentrations were determined using a commercially available enzyme-linked immunosorbent assay (ELISA Estradiol DRG). Assays were performed according to the manufacturer's protocols. Serum from adult females and males of *X. laevis* was used under two different dilutions: 1:10, 1:100 and whole. As a positive control woman´s serum was used whereas for the negative control, man´s serum was used.

Sexing of the organisms by PCR

gDNA was isolated from whole blood of *X. laevis* juveniles using the Quick-DNA™ Miniprep Plus Kit (Zymo Research, Cat. No. D4068), according to the manufacturer´s instructions.

A multiplex PCR was performed with the primers DMRT1(Fwd: 5'- AACAGGAGCCC AATTCTGAG-3", Rev: 5"- AACTGCTTGACCTCTAATGC-3," 203pb) and DM-W (Fwd: 5"-CCACACCCAGCTCATGTAAAG-3", Rev: 5"- GGGCAGAGTCACATATACTG-3,' 259pb) and the internal control gene Histone 4 [H4, Fwd: 5"-CGGGATAACATTCAGGGTATCACT-3', Rev: 5'-ATCCATGGCGGTAACTGTCTTCCT-3"], 189bp) (Session et al., 2016; Yoshimoto et al., 2008)

under the following conditions: 20 ng experimental gDNA, 1 Taq unit (Taq DNA Polymerase, Invitrogen, Cat. No. 18038), 1X Taq DNA Polymerase PCR Buffer (Invitrogen, Cat No. 18038), 1X Taq DNA Polymerase PCR Buffer (Invitrogen, Cat. No. 18067–017), 3 mM MgCl2 (Invitrogen), 0.2 μM DMRT1 forward primer, 0.2 μM DMRT1 reverse primer, forward primer DM-W 0.2 μM, DM-W 0.2 μM reverse primer, dNTP 0.2 μM, H2O 20 μL. The thermal cycles consisted of 95°C for 5 minutes, 1 minute at 95°C, 35 cycles at 59°C for 1 minute, 1 minute at 72°C, and a final extension at 72°C for 10 minutes. PCR products were separated on a 1.5% agarose gel stained with SyBr green (Jena) and compared with a 1000 base pair (scale size marker).

Results

The blood withdrawal method developed here, allowed us to obtain samples between 35 to 600μ*l*. The amount of blood obtained depended on the weight and size of individuals. For juveniles (45gr) an average of 35μ*l* was taken, whereas for adults, particularly females (90 gr) up to 600μ*l* were collected. This amount of blood was sufficient to get up to 190μ*l*of plasma.

The amount of blood got with this technique fell within international regulations, where it is established that less than 10% of the circulating volume of blood must be withdrawn (ASPA, 1986). Additionally, this method did not have negative consequences for survival or mobility of the organisms, like other methods such as cardiac puncture or toe clipping and allowed continuous sampling of the same individuals over four months.

Furthermore, *Xenopus laevis* plasma samples were stored at −20°C for more than six months during which they were still viable to evaluate hormone concentrations. Moreover, molecular sexing was also performed by identifying DMRT1 and DMW genes using genomic DNA. Hence, blood extracted from the *dorsalis pedis* vein in juveniles can also be used in molecular applications, since organisms of known sex, morphologically identified as male or female by the presence of the cloacal extension, the snout-vent-length (SVL) and the presence of nuptial pads, were confirmed by PCR analyses (Figure 2).

Plasma was obtained from blood samples of 6 females and 6 adult males, and it was collected from the same individuals every month for four months. After storage, plasma was analyzed by ELISA assays to determine estradiol concentrations in undiluted and in 1:10 and 1:100 diluted samples (Estradiol ELISA, 2020). The ELISA Estradiol DRG kits showed cross-reactivity with *Xenopus laevis* hormone. We found a higher concentration of estradiol in females (Figure 3a) than in males, as expected, but only when undiluted plasma was used. The diluted samples had low concentrations, outside the detectable range, so we recommend to use whole plasma when

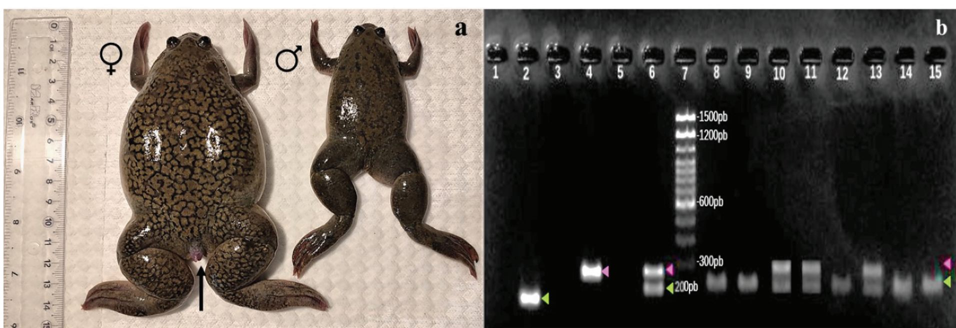

Figure 2. Sexing of juvenile *Xenopus laevis* by PCR from blood samples obtained from the *dorsalis pedis* vein. a) morphological sexual dimorphism: the arrow signals a female showing the cloacal extension, females are also characterized by having a longer SVL than males. a) 1.5% agarose gel for DMRT and DM-W genes. Samples with two bands indicate a female. Lanes 1, 3 and 5 are blank, lane 2 shows DMRT1 (green arrow, 203bp), lane 4 DMW (pink arrow, 259), lane 6 is the multiplex lane, and lane 7 corresponds to the molecular weight ladder (1000bp).

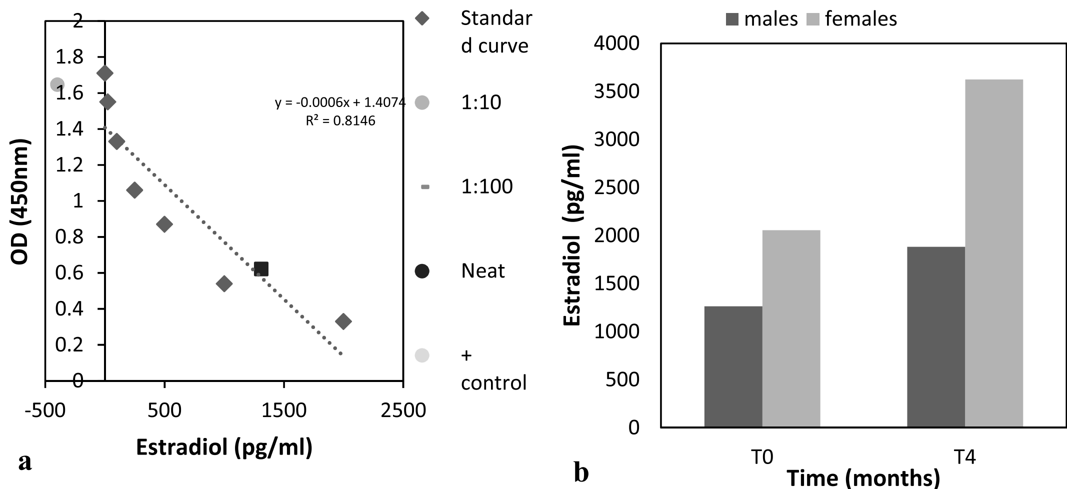

Figure 3. Estradiol concentrations from blood samples extracted from the *dorsalis pedis* vein of males and females of *Xenopus laevis*. a) standard curve testing the cross reactivity of plasma. It is observed that whole plasma concentrations (squares) fall within the standard curve concentrations (rhombuses), whereas diluted samples (circles and lines), and the male negative control (triangles) are out of range because the concentrations were too low. b) estradiol concentrations of the same individuals after four months, T0 (initial time) and T4 (fourth month).

measuring estradiol with this technique. We were able to measure estradiol in the same individual during four months, showing variations in the plasma estradiol concentrations along time (Figure 3b).

Discussion

Due to the importance of blood collection in experimental assays with animals, several standardized methods have been developed for model organisms such as the zebrafish (Zang, Shimada, Nishimura, Tanaka, & Nishimura, 2015), those methodologies allow the monitoring of individuals along time, without compromising the survival of the individuals. However, until now, that is not the case for amphibians such as the African Clawed frog *Xenopus laevis*; therefore, the blood collection technique presented here offers several advantages over other methods used in this or similar species, as it constitutes a viable option for sampling the same individuals (juveniles or adults) more than once during an experimental period. This clearly improves the ethical work with the species, by reducing the number of animals used in experimentation, minimally impacting their welfare and not compromising their life. Hence, with this method it was possible to follow up on the same individual with no effects once they had recovered from the anesthetic. We applied the anesthetic technique developed by Vázquez, Castro, Hernández, Castro, and De Lara (2013) that uses clove oil extract to numb the organisms. Although the time needed to anesthetize each frog was somehow long, it depended on their size, ranging from 25–35 minutes for juveniles and 30–45 minutes for adult females. However, it was also possible to withdraw blood without anesthetic, by holding firmly but carefully the frogs hindlimbs and covering their face with a wet towel to diminish stress. Both techniques were efficient, as they allowed to obtain enough blood, although collection time increased without anesthetic (40 min). It is important to note that all frogs recovered well within 30 min of sampling, and no signs of distress or disease were observed in the following weeks.

Additionally, in the area of the puncture, there were no signs of infection and the wound recovered quickly (maximum 2 days). Although the technique is simple and easy to implement, there are basic practices that can ensure high success and optimal survival rates, such as maintaining

a working area sanitized, having wet towels available, and in order to save time in large-scale experiments, a double-team approach is recommended.

Unlike blood extraction by digital amputation (toe clipping), for which infections have been recorded (McCarthy & Parris, 2004), with this technique the lesion takes two to three months to completely regenerate; additionally, none of the 36 frogs used in this study died or showed signs of infection after the procedure because the vascular membrane closes almost immediately.

As shown in Figure 1, though the hindlimbs of Xenopus runs the vena *dorsalis pedis* which is easily accessed by extending the interdigital membrane, and blood sampling from it requires only the use of a capillary tube. One aseptic glass tube is used to puncture the vascular tissue and blood is readily collected by capillarity. With this method blood continuously flows into a capillary by just pressing gently the vein.

This novel extraction technique allowed us to get enough DNA and to obtain clear bands in the electrophoresis gel, confirming the sex of the individuals, previously established by morphological characteristics, supporting the fact that the biological material obtained has good quality for genetic studies.

Moreover, one aim of this study was to determine whether this extraction technique would allow repeated blood sampling from the same individual, without the negative consequences that other methodologies pose, and collect enough blood for further studies. We collected blood once a month during four months, and measured estradiol concentrations by ELISA assays using purified plasma. Our results demonstrate that the proposed method is adequate and reliable to take samples on a regular basis from the same individual and yields enough blood and plasma for subsequent lab analyses that can be used in different studies like toxicokinetics, pharmacokinetics, endocrinology and hematology.

The estradiol ELISA kits used in this study are designed with mammalian antibodies, and for their use in human hormone quantification; therefore, it was important to validate whether they could detect *Xenopus laevis* hormones, because there is no evidence of their use in amphibians. We demonstrateted that the kits present cross-reactivity, making it possible to determine the concentrations of estradiol. Concentrations of about 1400 pg/ml were obtained in undiluted Xenopus plasma; for women, concentrations of up to 540 pg/ml have been detected at the time of ovulation (the highest peak of the cycle), indicating that the concentrations of the estrogen are high in this organism.

Conclusion

It is possible to collect blood through the *dorsalis pedis* vein of *Xenopus laevis*, becoming a minimally invasive method that allows continuous monitoring of the same individuals, and the use of blood in other lab techniques.

Commercial kits (DRG) of estradiol have cross reactivity with *Xenopus laevis*. The blood withdrawal method developed here proved to be economical, clean, friendly, minimally invasive, and effective for several applications, without presenting repercussions to the health, mobility and survival of the organisms.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

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Data availability statement

No data set associated with this manuscript.

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