DEVELOPMENT OF CAPTIVE BREEDING, LARVAL REARING TECHNOLOGIES AND MANAGEMENT PRACTICES FOR AFRICAN LUNGFISH (*PROTOPTERUS AETHIPICUS*)

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Climate Change Adaptation: Indigenous Species Development/Experiment/16IND03AU

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Objectives

1. Assess the seasonal reproductive cycles of the African lungfish in two Uganda lakes.
2. Identify breeding technologies for producing African lungfish seed.
3. Determine optimal larvae weaning feed and period in P. aethiopicus.
4. Evaluate the culture performance of African lungfish raised from juvenile to market size in ponds and tanks.

Significance

The general objective of this study is to generate information on aspects of reproductive biology, larval rearing and growth of *Protopterus aethiopicus* for sustainable production in captivity while improving nutrition and household incomes for local populations in Uganda and the neighboring regions. The studies and experiment continue the program of captive reproductive process determination and genetic description accomplished in the previous cycle of research.

The African lungfish (*P. aethiopicus)* is one high value species in great demand within the lakes region. The species natural distribution occurs in the basins of the Congo and Nile river systems in Central and Eastern Africa that include Victoria, Tanganyika, Albert, Edward, George and Kyoga lakes (Greenwood, 1966). The *P. aethiopicus* was a substantial component of the lake fisheries in 1920s (Smith, 1931), but because it dwells largely in swamps vulnerable to human activities, it has been overfished. Attempts to domesticate lungfish through induced spawning and evaluation of growth performance in captivity has been met with little success. Some farmers in eastern Uganda have caught young lungfish from the wild and stocked them in small ponds, but the yields have been reported to be low (Walakira 2012; 2014). We do not yet know how to reproduce the species, to best feed the fish, or how to manage its culture and harvest in captivity.

Aquaculture requires year round supply of quality seed (Bromage et al., 2001). However, production of such seed entails development of appropriate technologies for breeding the fish in captivity and ensuring constant supply of the seed to farmers (Aruho 2013). The domestication process requires knowledge of the reproductive biology and life history of the African lungfish, which has not yet been characterized. This includes addressing questions such as spawning season, reproductive hormone profiles, egg quality and maturation, fecundity, size at sexual maturity, clear gender identification, and expected sex ratios of offspring (Bromage et al., 2001). It is equally imperative to establish larval and juvenile rearing and management protocols for successful growth of African lungfish in captivity by farmers.

This study seeks to develop sustainable breeding and appropriate culture techniques for African lungfish species using commercially available fish feeds. If feasible, culturing lungfish has the potential to improve nutrition, food security, and generate income for local farmers. It will also reduce harvest pressure on wild fish stocks in Uganda.

Successful culture techniques may offer some distinct advantages for income generation for small-scale fish farmers as a high value product grown under controlled conditions. Local people in many parts of Uganda and the East African region cherish the fish because it has a desired flesh quality, especially when fried into pieces coated with cassava, although other forms of preparation are also very common. Small ethnic groups from central Buganda region associate the lungfish with cultural beliefs and desire to preserve it.

Quantified Anticipated Benefits

- Basic guidance on production and management of lungfish expressed in a farmer-oriented leaflet.
- Basic nutrition profile of lungfish grow out expressed in a technical report for extension.
- Larval weaning protocol for the fish established and expressed in a manual.
- Basic fingerling supply and grow out information published in a journal article.
- Inform the merit of continuing research into developing low-cost, artificial breeding technologies for these species.

Research Design and Activity Plan

Location

Kajjansi, Uganda.

Methods

Experiment 1: Assess the seasonal reproductive cycle of the African lungfish in two Uganda Lakes.

Lake Wamala in Central Uganda and Lake Bisina in Eastern Uganda each supports established populations of lungfish. Understanding the reproductive seasonality of lungfish is necessary for identification of the right period and the right size (size at sexual maturity) of ripe broodfish to induce them to spawn in captivity. Apparently the hormonal profiles, gonad somatic indices, fecundity, size at sexual maturity, gender identification and sex ratios of the fish are subject to climatic and micro-environmental changes. Little is known about these aspects of the life history of African lungfish and they are critical to successful rearing in captivity.

*1.1 Hormonal profiles and seasonality***.** Monthly blood samples of more than 30 specimens (at least 15 fish from each gender) of mature *P. aethiopicus* per lake, will be collected from either the caudal peduncle or directly from the heart using a heparinised microsyringe and centrifuged at 6000 rpm for 5 minutes. Plasma will be collected, frozen at -20^{o} C, and shipped to North Carolina State University for steroid assays. The steroid levels will be measured using enzyme-linked immunosorbent assay (ELISA) technique following Yeo & Lim (2015).

Antisera for measuring plasma levels of 17-beta-estradiol (E2), 11-ketotestosterone (11-KT), and testosterone (T) will be purchased from Cayman Chemical Company (Levavi-Sivan et al., 2004). These are the important gonadal steroids regulating gametogenesis in male and female fish and are typically used for characterizing the reproductive cycle (see: Berlinsky & Specker, 1991; Jackson & Sullivan, 1995).

Although we recognize that progesterones also are important mediators of gamete maturation, at this time we do not know which progesterone(s) are active in African lungfish. The progesterone maturation-inducing steroid (MIS) of fishes varies between species (Patino & Sullivan 2002) and would require

characterization for meaningful interpretation of plasma levels. Therefore, measuring plasma progesterone is beyond the scope of this present application.

The seasonal variations of gonadal steroid profiles (E2, 11-KT, and T) in *P. aethiopicus* will be determined by ELISA at North Carolina State University and correlated with reproductive parameters such as gonad somatic index (GSI), condition factor (K), diameter of the top clutch of oocytes, and oocyte stage (see: Wallace & Selman, 1981). Analysis of variance (ANOVA) will be employed to test differences in various reproductive parameters over time. The study will be conducted for 12 months.

1.2 Gonad characteristics. Somatic index (GSI), condition factor, length at maturity, fecundity and sex ratios will be measured. Samples from Lakes Wamala and Bisina will be collected monthly for a year, weighed and dissected to expose gonads for staging and gender identification. Standard histological methods (Bancroft and Gamble 2002) will be conducted to aid confirmation of sex of gonads (especially tiny gonads) and stages. Digital photographs of the gonad histology will be taken.
Gonads will be staged following Brown-Peterson et al., (2011) and Wallace & Selman (1981) classification and the mean diameter of the top clutch of oocytes will be measured using light microscopy. Mean differences in gonad somatic index (GSI) (Weight of the gonads/Eviscerated weight x 100), condition factor K (weight/length L^3*100) and the fecundity between and within the lakes will be determined by analysis of variance (ANOVA). Fecundity will be determined by gravimetric method (Kipling and Frost, 1969). Significant deviation from the hypothetical 1:1 ratio of monthly and class size sex ratios will be determined using a chi-square test and binomial probability.
Sexual maturity will be determined by fitting a logistic ogive to the reproductive active fish captured during the spawning season in two-centimeter size classes. The logistic ogive is described by the equation $P_L = (1+\exp (L-L_{50})^{-1}$ where P_L is the percentage of the mature fish at length L and L_{50} is the length at maturity. A nonlinear minimization formula below was used to estimate the parameters.

$$-\ln L = \sum_L y_L \ln \left[\frac{P_l}{1-P_l} \right] + n_L \ln (1-P_L)$$

Where y_L is the observed numbers of mature fish in $n_{L,}$ fish sampled in Length class L.

1.3 Gender identification. Identification of males and females is a key reproductive aspect required in identifying sexes of some of the cultured fish species that cannot be easily identified as females and males. Accurate sex determination of *P. aethiopicus* is imperative to aid develop simple, user-friendly techniques for captive farming. New technological approaches have been developed to simplify identification of males and females for species that are difficult to separate. Biomarkers such as gene expression and plasma vitellogenin are used to identify gender of some fish species, however, these methods rely on proper characterization of the gene expression or egg yolk systems specific to each fish species and they must be validated prior to any meaningful use (Reading et al., 2009; Reading & Sullivan 2011; Schilling et al., 2015).

Since no coding gene (RNA or cDNA) sequence is available for lungfish vitellogenin, and the species is quite divergent from other fishes with validated vitellogenin assays, measuring plasma vitellogenin is beyond the scope of the proposed project. For example, the Perciform fishes express three different forms of vitellogenin and these are quite different (structurally and functionally) from the forms of vitellogenin expressed by salmonids (Reading et al., 2009).

Little to nothing is known about the vitellogenin egg yolk systems of lungfish, and comprehensive characterization would have to precede quantification. However, characterization of the yolk system of these ancient fishes would be an important future goal to eventually establishing such assays for gender identification, especially in less mature animals. Here, we propose to use expressed SNP markers and minimally invasive sampling procedures to identify mature fish of each gender.

1.4 Sample collection, morphometric, and gender identification. African Lungfish all size classes will be collected from Lakes Wamala and Bisina system and measured for total length and weight. Approximately 40-60 fish will be collected using locally available harvesting gears every month. Fish will be anesthetized with tricaine methane sulfonate (MS-222) buffered with 0.2 ml $NaHCO_3$, pH = 7. Morphometric parameters will be measured following the "Truss Network System" (Strauss and Bookstein, 1982) focusing on the geometric morphology of the African lungfish. Each fish will have homologous anatomical landmarks. These selected *n* inter-land mark distances (modification of Cavalcanti et al., 1999) will be characterized using digital images to determine the differences among wild populations of the two lakes. Data will be subjected to statistical analysis as described by Mir et al., (2013) to evaluate morphological differences. Fish samples will be dissected to identify individual sex, which will be correlated to phenotypic observations based on existing scientific knowledge.

1.5 Ovarian biopsy method for gender identification. A simple technique has been devised for identification of gender in other cultured fishes including the striped bass (*Morone saxatilis*) and white bass (*M. chrysops*) (Sullivan et al., 1997). In these species a short, flexible plastic cannula is inserted into the urogenital pore and a small amount of gonadal tissue is aspirated into the tube and removed for analysis under a microscope. If this tissue contains oocytes, then a positive identification of female gender can be made. If semen or no oocytes are aspirated, then identification as male is confirmed or suggested, respectively.

Ovulated African lungfish oocytes are 3.4-3.5 mm in diameter, therefore a 12 cm long cannula of about 3 to 4 mm internal diameter will be used to aspirate gonadal tissue. Fish will be anesthetized and oocyte staging of biopsied females will be conducted as described above. Additionally, digital photographs of the oocyte stages will be compiled with images collected in **1.2 Gonad characteristics** (above) to form a guide for African lungfish ovarian development.

1.6 SNP panel for gender identification. Molecular genetic markers will be identified to ascertain and explain sex differentiation and determination of African lungfish since this information is apparently unknown. Validation will be measured taken to understand their effects on fish sex determination and differentiation following the Baroiller et al., (2009) method. This information will facilitate sexing the African lungfish; a basic procedure in captive breeding programs. Through a non-lethal sampling approach, fin clips from identified fish will be used to extract genomic DNA and RNA, and stored at 4 °C in 100 % ethanol and -20 °C RNA Later solution respectively. Fin clips from 40 lungfish samples (20 per site) collected from Lakes Bisina and Wamala. Expressed SNPs or Quantitative Trait Locus (QTL) be used to guide gender identification in future breeding for African lungfish.

Total RNA will be extracted using a Trizol protocol and stored at -80 °C. Libraries for RNA-seq will be prepared from total RNA using the TruSeq manual. This study will use the Next Generation Sequencing (NGS) technology to develop a novel SNP panel that can be used for gender identification of *P. aethiopicus*. Using the reference genome and *de novo* assembly putative reads and subsequently SNPs will be identified using Trinity/v2.0.6 software. The total number of SNPs detected will be selected based on the SNPs expected heterozygosity and the Polymorphic information content (PIC) as a tool for genetic diversity.

DNA will be prepared using protocols described by Sambrook and Russell (2001). DNA will be extracted using proteinase K and phenol-chloroform and amplified using polymerase chain reaction (PCR) primers. A complete mtDNA sequence of P. *dolloi* will be used to develop primers as described by Zardoya and Meyer (1996).

Following the Peukert et al., 2013 approach, genomic polymerase chain reaction (PCR)-amplification will be performed in 25 µl volume of PCR buffer (0.01 M Tris, 0.05 M KCl, 1.5 mM MgCl2, 0.01% gelatin) and contained 100 ng of genomic DNA, 0.2 mM of dCTP, dGTP, dTTP, dATP, 0.2 µM of each primer and 1 U of Taq polymerase. After 3 min at 94 °C, 45 cycles will be made with 1 min at 94 °C, 1 min at 55°C, 2 min at 72 °C and a final extension step of 10 min at 72 °C. Successfully amplified gene fragments obtained for reference genotypes will be re-sequenced. Positions of 3' - and 5' -UTR, introns and exons will be determined using GeneSeqer.

Using Ansmann et al., (2012) methods, 20 males and 20 females whose sex is phenotypically determined will be verified using the SRY/ DMRT1 genes. Fragments of SRY/ DMRT1 genes will be amplified using PCR with 20–25 ng DNA, 0.15 M of each primer. The PCR profile will be denatured at 95°C for 4°C then cycled 35 times at 94 °C for 45 s, 50 °C for 45 s and 72 °C for 60 s, and a final extension at 72 °C for 10 min. PCR products will be separated on agarose gel to determine sex based on length differences

Experiment 2: Identify breeding technologies for producing African lungfish seed.
2.1 Domesticate the African lungfish using simple captive breeding techniques. To ensure an environmentally sustainable supply of African lungfish seed to fish farmers, artificial breeding and hatching technologies will be developed. Simple and low-cost breeding technologies will be needed in rural communities that are dependent on this fish. Based on the described information from studies 1 and 2, mature brood-stock from the wild populations will be subjected to simple artificial reproduction techniques to evaluate the working fecundity, egg survival and hatchability.

Larval rearing will be conducted to determine the survival and growth aimed at establishing the larval rearing protocols (technologies) of *P. aethiopicus* to be used by the farmers. An effort also will be made to retain some of these offspring in captivity to initiate a breeding program for domestic lungfish. Even a modest number of generations (i.e., 3 to 5) of breeding in captivity can greatly improve growth, tolerance to handling and poor water quality, and success at captive breeding (Teletchea & Fontaine, 2014).

2.2 Identify efficient artificial breeding technologies for African lungfish. Modifying protocols used by Vijaykumar et al., (1998), mature broods will be conditioned for 3-4 weeks in concrete tanks at NaFIRRI facilities, and will be induced to spawn using selected synthetic and natural hormones as described. The hormonal use will take into account the fact that P. aethiopicus is an asynchronous batch spawner; it releases eggs in batches. Hormones to be used will include the administration of:

2.2.1 Gonadotropin releasing hormone analogue (GnRHa) implants. This is a technique that has been used to induce ovulation in cultured fish including southern flounder (*Paralichthys lethostigma*) (Berlinsky et al., 1996), wild-caught summer flounder (*P. dentatus*) (Berlinsky et al., 1997), and striped bass (Hodson & Sullivan, 1993; Sullivan et al., 1997). A GnRHa-loaded implant will be applied at two doses: 10 µg kg^{-1} BW (IMP-10, 6 females and 5 males); or 50 µg kg-1 BW (IMP-50, 6 females and 5 males).

2.2.2 Human chorionic gonadotropin (HCG) or Chorulon. At an application rate of 2.0 mL per 5 kg fish (330 IU/kg hCG to male and female broods, according to Hodson and Sullivan (1993).

2.2.3 Catfish pituitary extracts (African catfish, Clarius gariepinus). These are widely used to induce most cultured species in Uganda at a dosage of 0.014g ml^{-1}. The analysis will examine fecundity, hatchability and survival of post-hatchlings. Water quality parameters will be monitored weekly to understand environmental factors affecting captive breeding. Best approaches will be selected based on statistical analysis of factors that produce better quantity, viability, and quality of lungfish spawns.

2.3 Compare breeding methods of African lungfish in captivity:
2.3.1 Manipulated environment breeding. Selected mature broodfish (males and females) will be stocked in concrete tanks or hapas suspended in earthen ponds, and then covered with macrophytes such as water hyacinth (*Eichornia crassipes*) or other aquatic plants that are usually present in natural breeding habitats. Water levels will be manipulated to simulate a flood pulse to promote natural ovulation, spawning, and fertilization. Fecundity, hatchability and survival of post-hatchlings will be evaluated.
2.3.2 Evaluating hormone induced fish. Two approaches will be used. First, the fish will be allowed to spawn volitionally in ponds or tanks. Second, stocking the mature fish without hormone induction in the prepared ponds or tanks to mimic the natural environment.

Cross tabulation methods will be used to analyze the egg and larval mortalities and differences in means will be evaluated by analysis of variance (ANOVA) or non-parametric analog tests. Water quality parameters—pH, alkalinity, temperature—will be monitored, weekly, to understand environmental factors affecting artificial breeding. The two approaches will be evaluated based on the relative quantity, viability, and quality of lungfish spawn produced.

Experiment 3: Determine optimal larvae weaning feed and period in P. aethiopicus.
In larval development identification of better feed must be synchronized with the development of the weaning protocol. The optimal feed and the period for weaning are critical factors for improving the larval survival and growth for seed production (Mai et al., 2005). Weaning will enable introduction of dry feeds at a particular time and stage when the fish can easily digest and absorb the feeds. The introduction of micro diets to the developing larvae is an important strategy that is cost effective in minimizing expenses of preparation of live feeds or artemia (Gordon et al., 2000).

There is currently no weaning protocol farmers could use to produce the required larval weaning technologies (protocols). Weaning will be tailored towards identifying when it is best to introduce the micro dry diets to improve the survival of larvae as well as juveniles. In this study a local commercial micro diet Ugachick (U) and Sabra & Sons (30, 35 & 45% crude protein) and live feed (de-cysted artemia and moina), and combination will be evaluated for their performance on survival and growth of the larvae. Larvae from induced spawning experiments will be randomly allotted to 20 tanks each of 50 liters. Five larval feeds (including one with a combination of live and dry feed and a dry feed alone) will be given to the larvae in 4 replicates. The larvae after hatch will be raised until the juvenile stage. Sampling will be done at intervals to record the weights and lengths of the fish. The analysis of variance (ANOVA) will be used determine any differences in growth parameters such as weight gain, condition factor, specific growth rates and survival rates between various feed treatments. Duncan's tests will be used to establish significant variations between feeds at 95% confidence levels.

Experiment 4: Evaluate growth performance of African lungfish raised from juvenile to market size in different culture systems.
Fish development occurs in stages and each developmental stage may require feeds with varying nutrient requirements within the same species (De Silva and Anderson, 1995). Juvenile lungfish will be randomly allocated into six tanks and a commercial feed of varying crude protein content (35% and 40%) will be evaluated to establish its performance on the growth of *P. aethiopicus*. The same experimental arrangement will be evaluated in ponds at the research station and with some farmers (on farm).
Fish will be sampled monthly to collect their weights and the lengths until an acceptable market size. The differences in mean weight gain, feed conversion ratios, specific growth rates and survival rates will be determined by analysis of variances (ANOVA). The experiment will be conducted for 120 days. All the fish will be harvested, counted and weighed individually. The increase in length and weight will be calculated from: final - initial length or weight. Survival rate will be calculated on the basis of the number of fish harvested from the formula:

$$\text{Survival rate} = \frac{\text{No of fish harvested}}{\text{No of fish stocked}} \text{ X } 100\%$$

- Gross production will be determined from the expression;
- Gross production = (Average final weight of fish X Total no of harvested fish)
- Net production will be determined from the expression;
- Net production = (Average final weight increase of fish X Total no of harvested fish) kg

Specific growth rate (SGR) will be calculated with the formula:

$$SGR = \frac{[\ln(Wt) - \ln(Wi)] * 100}{t}$$

- InWt = natural log of the weight of the fingerlings at harvest
- InWi = natural log of the weight of the 21 days old stocked larvae.
  t = the nursing period, in days
- SGR will be multiplied by 100 to express it in percentage per day

Statistical analyses of the data will be done by one-way ANOVA to determine differences between the means of growth, survival and production of different treatments taking at 1 and 5 percent significance levels using the computer program.

Trainings and Deliverables

Item	Mechanism (e.g. podcast, reports, factsheets).
Captive breeding results	Basic nutrition profile of lungfish grow out expressed in a technical report for extension
Captive reproductive results	Journal article
Captive growth results	Basic guidance on management of lungfish expressed in a farmer-oriented leaflet

Schedule

Task	9/16	10/16	1/17	3/17	6/17	12/17	2/18
Collect fingerlings from 4 zones	x	x	x				
Develop captive breeding	x	x	x	x	x	x	x
Assess reproductive performance	x	x	x	x	x	x	x
Assess captive performance (On-farm and station)		x	x	x	x	x	x

APPENDIX: Hormone protocol

Synthetic gonadotropin releasing hormone analogs or GnRHa are effective on ovulation and spermiation and last longer in the fish's system.

Approach

Determining the hormone dose;

1. Weight of fish: Brood fish will be weighed under water to stress conditions.
2. Recommended dose: using the formulae below,

$$\text{Hormone Concentration} = \frac{\text{Recommended Dose x Fish Weight}}{\text{Desired Injection Volume}}$$

3. Total weight of dose;

$$\text{Total Weight of Hormone} = \text{Recommended Dose x Total Weight of Fish}$$

4. Ripe broods of lungfish (*Protopterus aethiopicus)* will be selected from the culture systems (tanks/ponds). To induce ovulation, intra-muscular injection of synthetic GnRHa will be done using the dose described by Rottmann et al., (1991). The recommended dose for GnRHa will not exceed 300 *u*g/Kg
5. Broods will be injected twice using calculated volume;

Volume of Injection = Recommended Dosage x Fish Weight Hormone Concentration

- First injected dose will be 40% volume
- Second/resolving dose will be 60% after 18 hours from the time of administering the first dose.