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

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

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ABSTRACT:

African lungfish (*Protopterus aethiopicus*) supports many communities in Uganda, and has aquaculture potential in the East African region. Fish farmers access seed from natural environments, which is not sustainable, environmentally and economically. This study uncovers the genetic diversity of *P. aethiopicus* collected from Lakes Wamala, Kyoga, Nawampasa, Bisina, Edward and George with a total of putative 1,437 SNPs generated to inform its future breeding programs. Fertilized lungfish eggs will hatch at a range of 24-32C (optimally at 27C) when bred in captivity. Hatchability in captivity averaged 21.7 ± 7.2 % (SD) and mean fecundity of wild-caught broods was 1922.41 ±1227.6. A combination of salt (0.5g/L) and temperature enhance its hatchability. Larvae will start feeding artificial diets at 18-20 Day-After Hatch. Larval growth and survival is enhanced when fed on combination of decapsulated *Artemia sp.* or live *Moina and* microdiet (35-57% Crude Protein) improves growth and survival rates. This study generates information that guides the domestication of African lungfish in the region, and will improve nutrition and livelihoods of vulnerable communities.

INTRODUCTION

The African lungfish (*P. aethiopicus*) is a high value species in great demand within the lakes region of Uganda. 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, how best to 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.

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.

METHODS AND MATERIALS

Experiment 1: Assess the seasonal African lungfish reproductive cycle 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. 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.

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 was collected, frozen at -20^{o} C, and shipped to North Carolina State University for steroid assays. The steroid levels were 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) were 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* was 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) was employed to test differences in various reproductive parameters over time. The study was conducted for 12 months.

Gonad characteristics.
Somatic index (GSI), condition factor, length at maturity, fecundity and sex ratios were measured. Samples from Lakes Wamala and Bisina were collected monthly for a year, weighed and dissected to expose gonads for staging and gender identification. Standard histological methods (Bancroft and Gamble 2002) were conducted to aid confirmation of sex of gonads (especially tiny gonads) and stages. Digital photographs of the gonad histology were taken.

Gonads were staged following Brown-Peterson et al. (2011) and Wallace & Selman (1981) classification and the mean diameter of the top clutch of oocytes was 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 was determined by analysis of variance (ANOVA). Fecundity was determined by gravimetric method (Kipling and Frost 1969). Significant deviation from the hypothetical 1:1 ratio of monthly and class size sex ratios were determined using a chi-square test and binomial probability.

Sexual maturity was 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.

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.

Sample collection, morphometric, and gender identification.
African lungfish from all size classes were collected from Lakes Wamala and Bisina system and measured for total length and weight. Approximately 40-60 fish were collected using locally available harvesting gears every month. Fish were anesthetized with tricaine methane sulfonate (MS-222) buffered with 0.2 ml $NaHCO_3$, pH = 7.

Morphometric parameters were 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) was characterized using digital images to determine the differences among wild populations of the two lakes. Data were subjected to statistical analysis as described by Mir et al. (2013) to evaluate morphological differences. Fish samples were dissected to identify individual sex, which was correlated to phenotypic observations based on existing scientific knowledge.

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 was used to aspirate gonadal tissue. Fish were anesthetized and oocyte staging of biopsied females was conducted as described above. Additionally, digital photographs of the oocyte stages were compiled with images collected in **1.2 Gonad characteristics** (above) to form a guide for African lungfish ovarian development.

SNP panel for gender identification.
Molecular genetic markers were identified to ascertain and explain sex differentiation and determination of African lungfish since this information is apparently unknown. Validation was 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 were 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 was extracted using a Trizol protocol and stored at -80°C. Libraries for RNA-seq were 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 was identified using Trinity/v2.0.6 software. The total number of SNPs detected was selected based on the SNPs expected heterozygosity and the Polymorphic information content (PIC) as a tool for genetic diversity.

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

Following the Peukert et al. 2013 approach, genomic polymerase chain reaction (PCR)-amplification was 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 were 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 was re-sequenced. Positions of 3'- and 5'-UTR, introns and exons was determined using GeneSeqer.

Using Ansmann et al. (2012) methods, 20 males and 20 females whose sex is phenotypically determined was verified using the SRY/ DMRT1 genes. Fragments of SRY/ DMRT1 genes was amplified using PCR with 20–25 ng DNA, 0.15 M of each primer. The PCR profile was 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 was separated on agarose gel to determine sex based on length differences

Experiment 2: Identify breeding technologies for producing African lungfish seed.
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 was developed. Simple and low-cost breeding technologies were 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 were subjected to simple artificial reproduction techniques to evaluate the working fecundity, egg survival and hatchability.
Larval rearing was 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 was 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).

Identify efficient artificial breeding technologies for African lungfish:
Modifying protocols used by Vijaykumar et al. (1998), mature broods were conditioned for 3-4 weeks in concrete tanks at NaFIRRI facilities, and were 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 included the administration of: GnRHA, HCG, and catfish pituitary extracts.

Gonadotropin releasing hormone analogue (GnRHa) implants (added)
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 was 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).

Human chorionic gonadotropin (HCG) and LHRHa
An application rate of 2.0 mL per 5 kg fish HCG (330 IU/kg) and LHRHa (300*u*g/Kg) to ripe male and female broods was used, following Hodson and Sullivan (1993) protocols.

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 examined fecundity, hatchability and survival of post-hatchlings. Water quality parameters were monitored weekly to understand environmental factors affecting captive breeding. Best approaches were selected based on statistical analysis of factors that produce better quantity, viability, and quality of lungfish spawns.

Compare breeding methods of African lungfish in captivity:

Manipulated environment breeding.

Selected mature broodfish (males and females) were 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 were manipulated to simulate a flood pulse to promote natural ovulation, spawning, and fertilization. Fecundity, hatchability and survival of post-hatchlings was evaluated.

Evaluating hormone induced fish.

Two approaches were used. First, the fish was 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 were used to analyze the egg and larval mortalities and differences in means was evaluated by analysis of variance (ANOVA) or non-parametric analog tests. Water quality parameters—pH, alkalinity, temperature—were monitored weekly to understand environmental factors affecting artificial breeding. The two approaches were 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 was 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 was evaluated for their performance on survival and growth of the larvae. Larvae from induced spawning experiments was 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) was given to the larvae in 4 replicates. The larvae after hatch was raised until the juvenile stage. Sampling was done at intervals to record the weights and lengths of the fish. The analysis of variance (ANOVA) was 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 test was 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 were randomly allocated into six tanks and a commercial feed of varying crude protein content (35% and 40%) was evaluated to establish its performance on the growth of *P. aethiopicus*. The same experimental arrangement was evaluated in ponds at the research station and with some farmers (on farm).

Fish were 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 were determined by an ANOVA. The experiment was conducted for 120 days. All the fish were harvested, counted and weighed individually. The increase in length and weight was calculated from: final - initial length or weight. Survival rate was calculated on the basis of the number of fish harvested from the formula:

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

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

Specific growth rate (SGR) was 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 is multiplied by 100 to express it in percentage per day

Statistical analyses use 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.

RESULTS

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

Seasonal reproductive cycle in Lakes Bisina and Wamala, Uganda Lakes.

Preliminary results from 962 feral lungfish samples indicate that this fish was continuously breeding in Lake Wamala during the seven sampling months.

Hormonal profiles and seasonality

A total of 767 blood samples were drawn from wild-caught live lungfish harvested from Lakes Bisina and Wamala, and stored under -80^{o}C during May, June, July, August, September, October and November 2017. A total of 300 well-preserved blood samples were shipped to North Carolina State University Laboratories for steroid profiles.

Gonad somatic index GSI and seasonality

The GSI was not significantly different among months both in females ($F_{6\text{-}330} = 1.7$, $p = 0.099$) and males ($F_{6\text{-}115} = 0.0972$, $p=0.448$) from Lake Wamala. The highest peak for females was from May –

July and from males was August and September. Results from Bisina show that significant differences occurred among the female monthly GSI ($F_{6\text{-}295} = 2.775$, $p = 0.013$). The highest peaks were recorded in the month of August, September and November. However there were no significant differences observed among the monthly Male GSI ($F_{6\text{-}209} = 1.497$, $p = 0.18$) the highest mean GSI for males was observed in July and November.

Gender identification

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 (Figure 1). 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 can be used to aspirate gonadal tissue. Fish were anesthetized and oocyte staging of biopsied females were conducted as described above. Additionally, digital photographs of the oocyte stages were compiled with images collected in 1.2 Gonad characteristics (above) to form a guide for African lungfish ovarian development**.**

The plastic catheter can only be inserted into the urogenital pore as far as the oviduct is straight. Maturing oocytes in the anterior part of the ovary pass through the anterior loop and then descend posteriorly toward the urogenital pore through the coiled oviduct. The African lungfish has an asynchronous ovary and will ovulate a small clutch (200-500) oocytes at a time that will travel down the oviduct for spawning. Females are iteroparous and may spawn multiple times during the breeding season.

The ovary biopsy technique has been used to sample small amounts of tissue from the gonads of several different species of fishes, including the striped bass (*Morone saxatilis*) and white bass (*M. chrysops*) (Sullivan et al. 1997). The gonads in these species are connected to the urogenital pore by a straight duct that extends from the posterior end of the organ to the exterior of the fish (urogenital pore), thereby allowing direct entry of the plastic cannula into the gonad. In the case of the African lungfish, the plastic cannula can be inserted several cm, however no gonadal tissues may be aspirated (Figure 1; see also Figure 2).

General gross anatomy of the African lungfish abdominal cavity

An overall outline of the fish is presented in Figure 3. Upon dissection, it appears that the lungfish has an unusual paired ovary compared to that of teleosts in that the oviduct originates from the anterior portion of the organ and then transverses posterior to the urogenital pore (Figures 4 and 5). The oviduct appears to be coiled and therefore the cannula cannot be fully inserted for gonadal tissue sampling from the ovarian lamellae (Figure 2). It may be that spawning lungfish with ovulated eggs in the oviduct could be sampled in this manner, however this has not been tested as none of the stage 3 females had ovulated eggs contained within the duct at the time of capture.

Batch Fecundity

A total of 81 females have been collected from Lake Wamala, preserved and later counted. The preliminary results indicate that the relationship between length and fecundity is weakly associate ($R^2 = 0.161$; Figure 3). The Number of eggs per g was 0.8±0.4, which was low. For the 26 females

collected from Lake Bisina the relationship between fecundity and length was moderately associated (R^2 = 0.635). The number of eggs per gram was 0.9±0.5.

SNP panel for gender identification

Comparative genomics revealed 1285 orthologous genes that are common to the species from six lakes. Orthologous genes are unique to each lake (Bisina, 186; Edward, 138; George, 304; Kyoga, 205; Nawampasa, 250; and Wamala, 91), respectively. We also report a set of SNP markers developed by sequencing the study.

A total of 5961 SNPs sites were identified from 71191331 Illumina MiSeq reads. Of these, a total of 277 markers were selected for use in low cost typing using PCR-RFLP and can be used for monitoring the genetic diversity during conservation and management program of the wild stocks of lungfish. Besides, the genomic resources generated in this research can propel further studies of this native species.

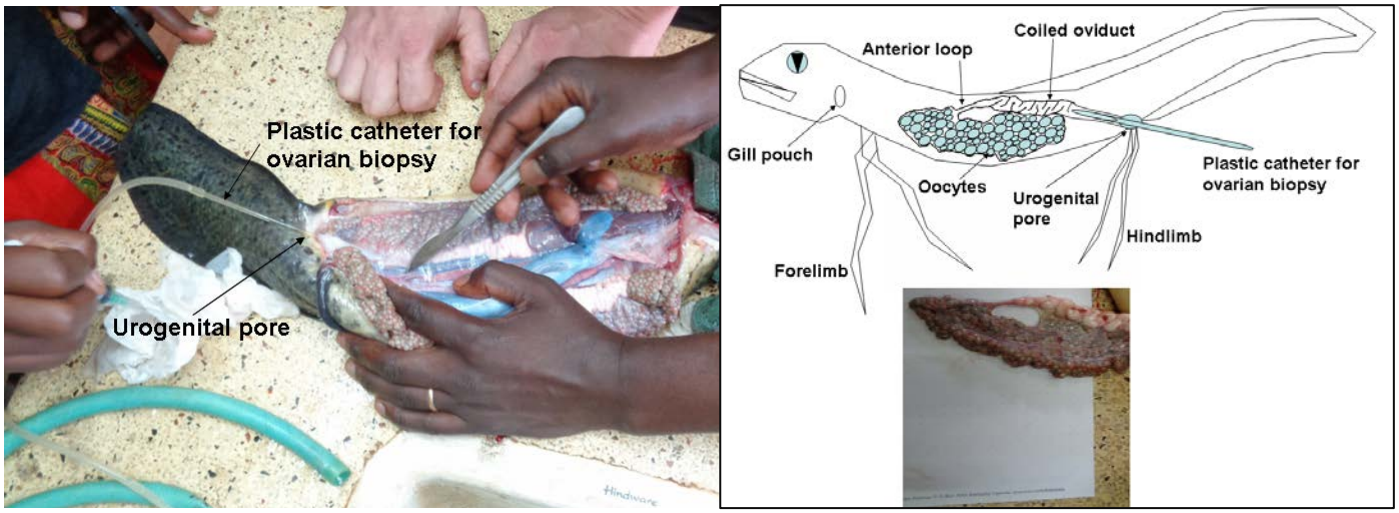

Figure 1. Photo (above) and drawing (below) of the African lungfish ovary anatomy. A plastic catheter has been inserted several centimeters into the oviduct of the African lungfish (*Protopterus aethiopicus*) and the tip of the scalpel indicates where the duct begins coiling. Oocytes pass down the oviduct from anterior to posterior. Due to the anterior loop leading from the ovary proper, it is not possible to sample oocytes directly from the ovary using the biopsy tool unless the oocytes have been ovulated and are present in the posterior oviduct near the urogenital pore.

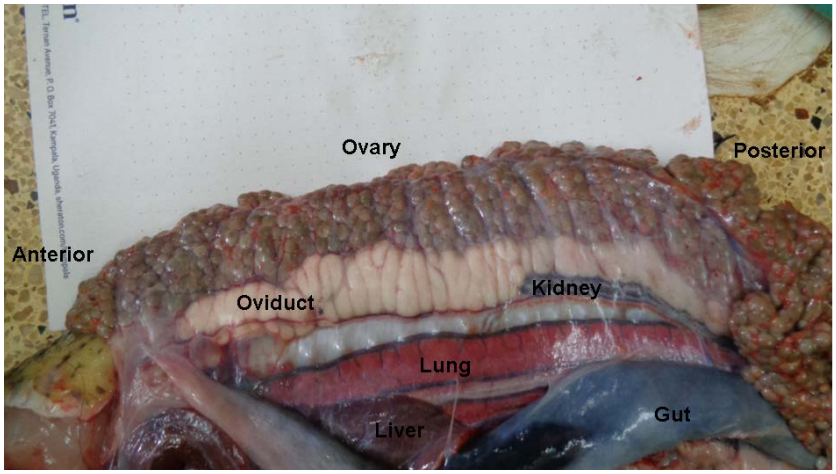

Figure 1 African lungfish (*Protopterus aethiopicus*) gross abdominal dissection showing the olive colored oocytes within the ovary, the white, coiled oviduct and associated kidney, the lung, the liver, and the gut (alimentary canal).

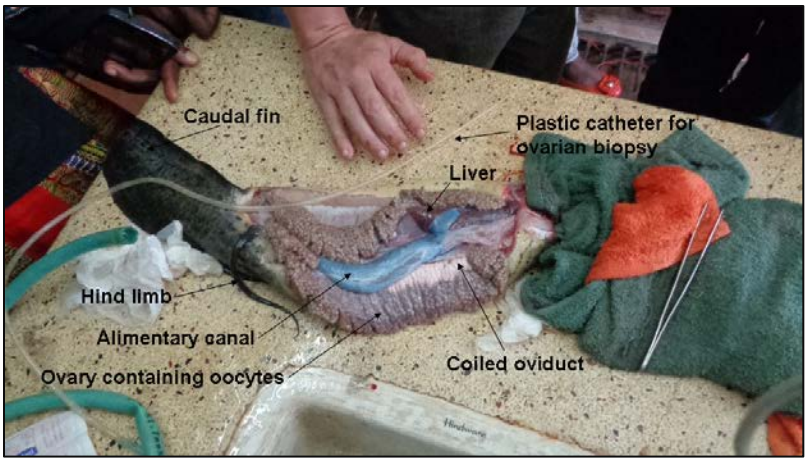

Figure 3 African lungfish (*Protopterus aethiopicus*). African lungfish (*Protopterus aethiopicus*) ovary showing the olive colored oocytes, with the largest clutch being about 3.5 mm in diameter. A short anterior loop connects the ovarian sac containing the oocytes to a coiled oviduct that extends posterior to the urogenital pore. Scale bar = 10 mm

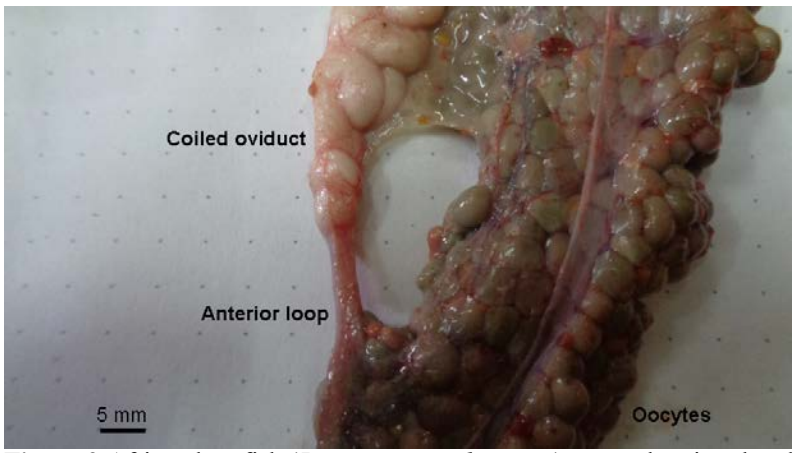

Figure 2 African lungfish (*Protopterus aethiopicus*) ovary showing the olive colored oocytes, with the largest clutch being about 3.5-4.5 mm in diameter. A short anterior loop connects the ovarian sac containing the oocytes to a coiled oviduct that extends posterior to the urogenital pore. Scale bar = 5 mm.

Experiment 2: Identify breeding technologies for producing African lungfish seed.

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 was developed. Simple and low-cost breeding technologies were 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 were subjected to simple artificial reproduction techniques to evaluate the working fecundity, egg survival and hatchability.

Evaluating hormone induced fish

HCG and LRHA analogs

Lungfish (200-300g) responded to doses of HCG and LHRH synthetic hormones but LHRHa had more effect for lungfish to spawn (Table 1). However, most induced broods failed to breed naturally under captive conditions in out-door tanks. Stripping and fertilization was possible with a few ripe wild-caught lungfish fish in hatchery conditions.

Table 1. Induce response of Lungfish broods when subjected to HCG and LHRHa doses.

HORMONE USED	MASS (g)	40% VOLUME/mls 0 hrs	60% VOLUME/mls 24-hours	TOTAL VOLUME/mls	RESPONSE
LHRHa	187	0.24	0.36	0.6	None
(300*u*g/Kg)	221	0.24	0.36	0.6	None
	361	0.32	0.48	0.8	Active
	354	0.32	0.48	0.8	Active
	480	0.4	0.6	1.0	Active
	460	0.4	0.6	1.0	Active
HCG	172	0.24	0.36	0.6	None
(400 IU/Kg)	153	0.24	0.36	0.6	None
	184	0.24	0.36	0.6	None
	258	0.24	0.36	0.6	None
	354	0.32	0.48	0.8	Active
	344	0.32	0.48	0.8	Active

Males

Male lungfish (av. 280g; 43.2cm) subjected to HCG produced milt during stripping. Only one male fish was observed to have motile milt (spermatids) when observed histologically through microscope. Spermatogonia in males dominated immature stages. The primary spermatogonia Sg1 (41± 5.3) developed into secondary spermatogia Sg2 (26.2 ± 3). While the secondary spermatogonia were transformed into primary spermatocytes Sc1 (14 ±1.3) which later developed into secondary spermatocytes Sc2 (9.76 ±1.3). The presence of Sc1 and Sc2 in the testes indicated the beginning of the developing phase. The Sc2 develop into the spermatids St (6.7±1.1) which are encysted in a coat like integument.

The size of the spermatogenic cells progressively reduced from the Sg1 to the spermatids and their color was more basophilic with Hematoxylin and Eosine (H&E). The spermatids finally burst open giving rise to the spermatozoa Sp (4.5±0.6) which are characterized by the presence of the head and the tail (Table 2 and Figure 5). The spermatids and the spermatozoa in the testes indicated stage 3 the capable spawning stage.

Table 2. Lungfish macroscopic and microscopic description of male spermatogenic cell stages.

Spermatogeic cell types	Size µm
Primary spermatogonia	41± 5.3
Secondary spermatogonia	26.2 ± 3
Primary spermatocytes	14 ±1.3
Secondary spermatocytes	9.76 ±1.3
Spermatids	6.7±1.1
Spermatozoa	4.5±0.6

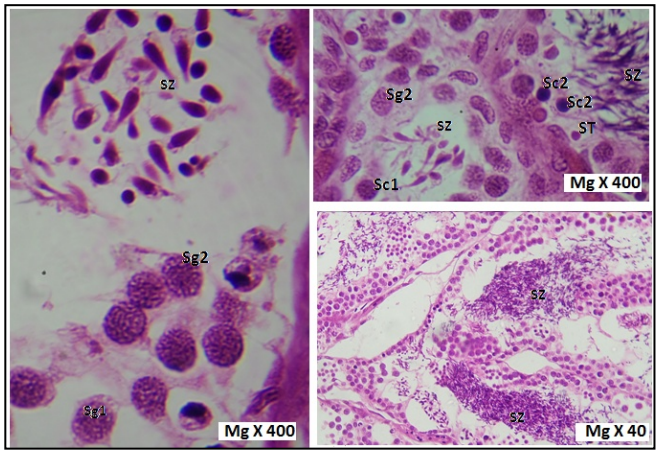

Figure 3. H & E section through lungfish testes showing a) primary spermatogonia (Sg1) b) secondary spermatogonia (Sg2) c) primary spermatocytes (Sc1) d) secondary spermatocytes (Sc2) d) spermatozoan (SZ) and e) spermatids (ST).

Females

One female released a few eggs (61) when stripped while other eggs of suspected ripe females were extracted. Microscopic examination indicated eggs at differential stages (I - IV) of maturity. Most eggs had nucleus not positioned at the animal pole, hence were not ready for ovulation and fertilization.

The ovaries of immature fish are dominated by the oogonia OO and Primary growth oocytes PGO. The oogonia were smallest germ cells with no distinct boundaries found solitary or in clusters of lamellae with a large light nucleus. The oogonia (347.44 ± 50µm) developed into primary growths oocytes (670.6 ±153.05 µm) (Figure 6). The PGO developed into cortical alveolar oocytes CAO which later transformed into the primary vitellogenic oocytes Vtg 1.

The Vtg1 developed into the secondary vitelogenic oocytes (Vtg2). The presence of CAO (1381.7±114.6µm) marked the beginning of the developing phase which also could include the Vtg1 (1400.1±190.9µm) and Vtg2 (1795.8±280.7µm) (Figure 7). The Vtg2 grew into the tertiary vitellogenic oocytes Vtg3 (2472.48 ± 284.8). The Vtg3 developed into mature oocytes MO (3737.3 ± 371.4). The Vtg3 and the MO were differentiated by the location of the nucleus. The Vtg3 had the nucleus in the center and the MO had the nucleus towards or at the periphery (Figures 7). The mean diameter of un-ovulated mature oocytes in females varied from 347.44 ± 50µm to 3737.3 ± 371.4µm (Table 3).

Table 3. Lungfish macroscopic and microscopic description of female cell stages.

Ovarian cells	Size (µm)
Oogonia	347.44 ± 50
Primary growth oocytes	670.6 ± 153.05
Cortical alveolar oocytes	1381.7 ± 114.6
Primary vitellogenic oocytes	1400.1 ± 190.9
Secondary vitellogenic oocytes	1795.8 ± 280.7
Tertiary vitellogenic oocytes	2472.48 ± 284.8
Mature oocytes	3737.3 ± 371.4

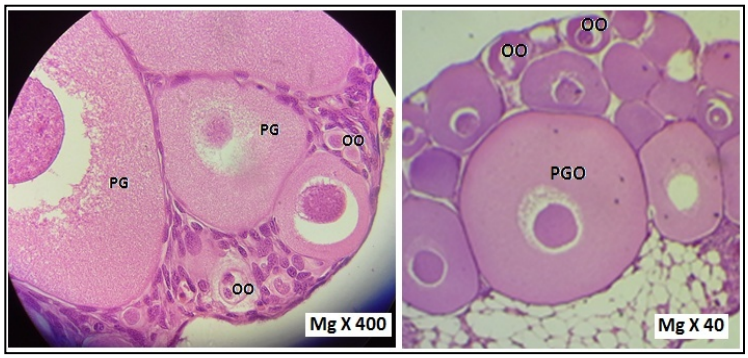

Figure 4. H & E Sections through the ovary of stage I showing oogonium (OO) and primary growth oocytes (PGO).

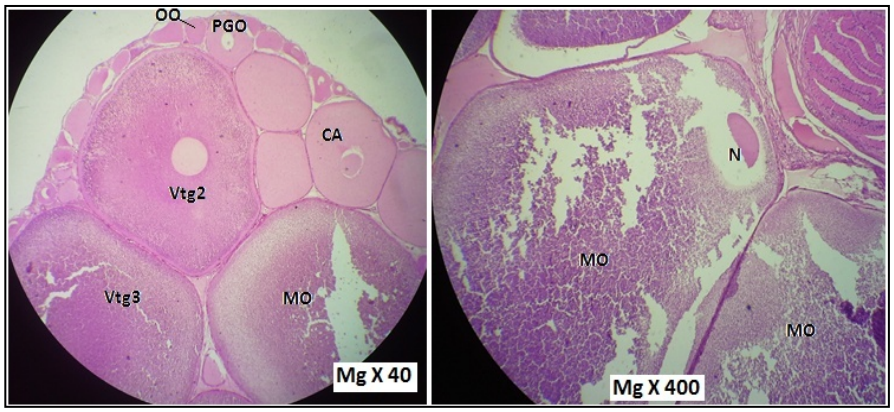

Figure 5. H & E sections through the ovary of stage III showing mature oocytes (MO) with nucleus (N) at animal pole, secondary vitellogenic oocytes (Vtg2), Tertiary vitellogenic oocytes (Vtg3) and cortical alveolar oocytes (CA).

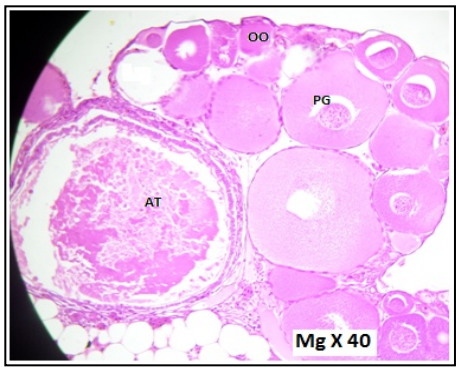

Figure 6. H & E section through the ovary of stage VI showing atretic oocytes (AO).

Hatchability

There was no significant difference ($p > 0.05$) in hatchability of lungfish eggs when exposed to temperatures of 24, 27, 30 and 32^{0}C (Fig. 9 ,10 and 11). Hatchability was faster at 32^{0}C with optimal range of 24 - 32^{0}C ($F_{3, 11}$=1.429; p=0.3.04 but overall rate was relatively low 9 to 14%. The hatchability was generally very low across all temperature treatments with the highest mean of 12.6 at 32^{0}C (Figure 11). Eggs in tanks containing water at 32^{0}C hatched first at 1 Day After Hatch (DAH) followed by eggs at 30^{0}C, 27^{0}C and lastly 24^{0}C. Tanks at 30 and 32^{0}C also developed fungal infections (Figure 12) which happened on the second day of hatching but more prevalent in 30^{0}C.

At hatching, the larvae attained the color of the eggs from which it hatched and they became uniform at the third day after hatch (Figure 13). After washing the eggs in the salt solution, it was seen that the mobility of the newly hatched larvae increased. For the eggs that delayed to hatch, in most cases the hatched larvae were weak and could die within 3 days.

GnRHa implants

There wasn't any response to implants since no lungfish hatchlings were observed in the out-door tanks.

Catfish pituitary extracts (African catfish, Clarius gariepinus).

There was no response to pituitary extracts on wild caught lungfish broods.

Manipulated environment breeding.

Wild caught lungfish broods did not breed in the out-door tanks. No hatchlings were observed.

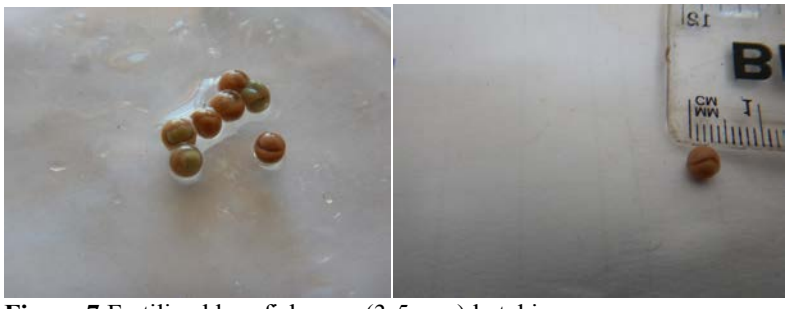

Figure 7 Fertilized lungfish eggs (3-5 mm) hatching.

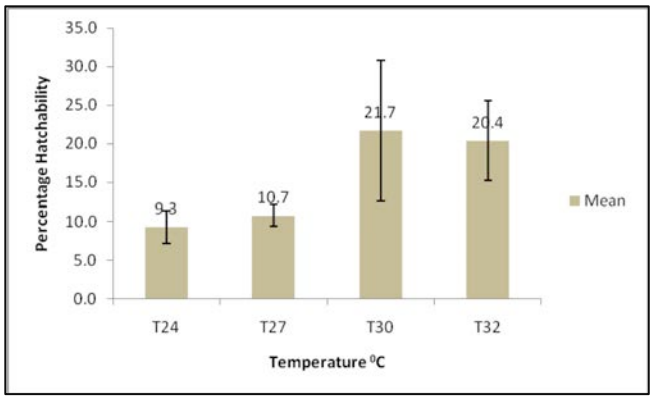

Figure 8. Hatchability rates of lungfish.

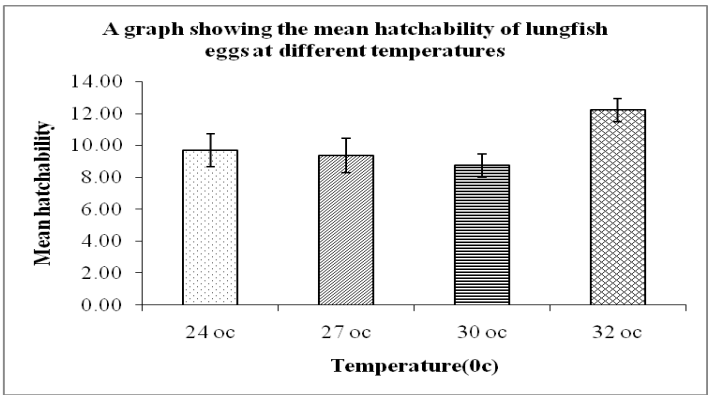

Figure 9. Mean hatchability of lungfish eggs at different temperatures

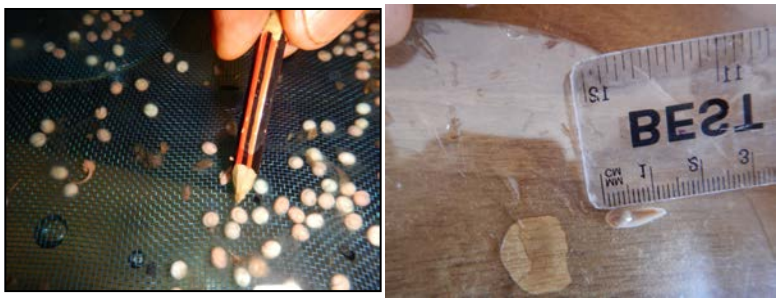

Figure 10. Lungfish eggs hatching to brown and light green larvae (av. 1.2 cm in length). White colored eggs did not hatch

Figure 11 Lungfish hatchlings/larvae in aquarium tank.

Optimal conditions for nursing lungfish hatchlings

Weaning lungfish larvae on decarpsulated Artemia, dry feed (56% CP) and their mixture showed results. However, significant differences between feed treatments ($P < 0.05$) with larvae fed decapsulated *Artemia* providing the best growth, followed by a combination of dry feed and *Artemia*; and the least being dry feed alone (Figure 14). Survival was high in treatments that were given decapsulated *Artemia* followed by the combination diet and lastly the dry feed.

Temperature:

There was no significant difference in larval growth across all water temperatures from 5 to 20-DAH. However, at 25 -DAH (27^0C) larvae grew significantly larger than those in 32^0C (Figure 9). No significant difference ($p > 0.05$). observed in weight gain with larvae raised at 24, 30 and 32^0C (Figure 15, 16, 17 &18). At 3 -DAH larvae at 30 and 32^0C changed color from brown to black while those at 27 and 24^0C turned black when at 4 to 6 DAH. On day four after hatch, blood vessels were visible around the belly.

Larvae in 30 and 32^0C started swimming in schools on 8-DAH and those at 24 to 27^0c, started swimming at 11 DAH. At 5-DAH, all larvae had almost the same weight though that of larvae in 30^0C was slightly higher.

Weaning

Histological sections revealed that development of larval digestive system was a slow transformation with complete development and differentiation of organs (buco-pharynx, oesophagus, intestine, liver and pancreas) complete by 26 DAH. At 5 DAH, the larvae changes color from grey to dark. Artemia was observed in the gut at 18 DAH. Mature columnar cells were observed at Dph 26. At the onset endogenous feeding (5-DAH when yolk reserves were not completely depleted but exhausted at 20 DAH. At this time the gut achieved its complete differentiation from the yolk sac. The mouth is terminal and protrusible, with vomerine teeth and taste buds. The digestive tract is also lined by a stratified squamous epithelium (Figure 16).

Another study was also conducted to determine whether live feed (*Artemia urmiana*), commercially prepared diet and a combination of the two feeds would enhance the growth and survival of lungfish hatchlings. Hence, triplicate of treatments: (1) dry feed (56% CP), (2) decysted artemia and (3) combination of decysted artemia (50% of food) were evaluated. After Six weeks of growth, the final weight (0.22 ± 0.01g) of fry fed on artemia alone was significantly higher ($p=0.000$) than fry fed on (1) and (3); (Figure 18). Survivals were similar throughout all treatments ($p=0.111$) and water quality parameters were within the recommended ranges and did not differ significantly ($p > 0.05$).

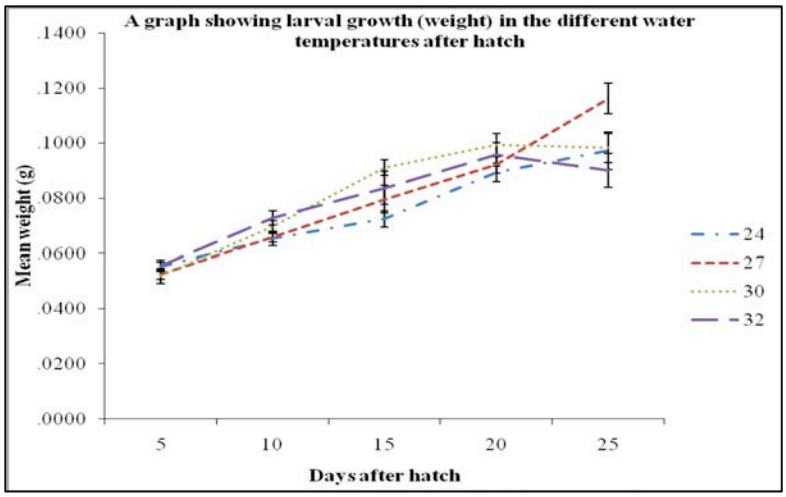

Figure 12. Larval growth at different water temperatures.

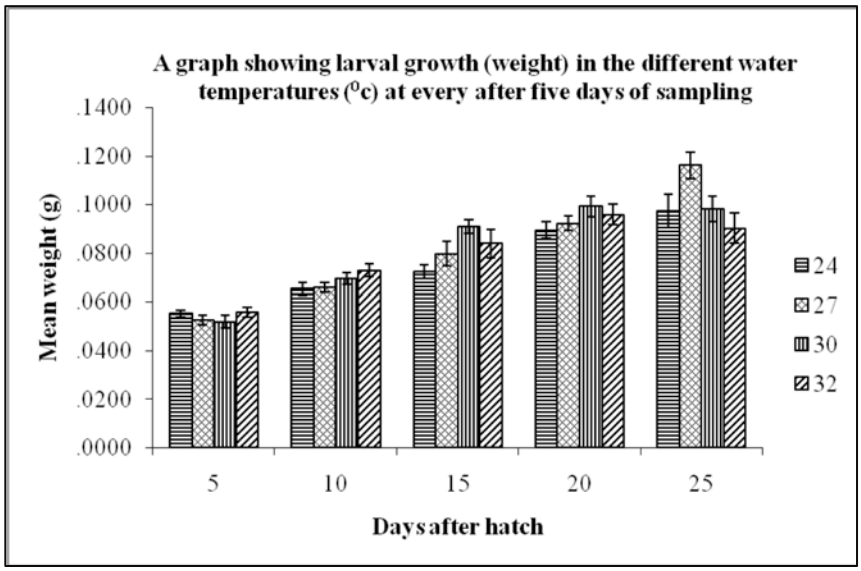

Figure 13. Lungfish larvae weight-gain at different water temperatures.

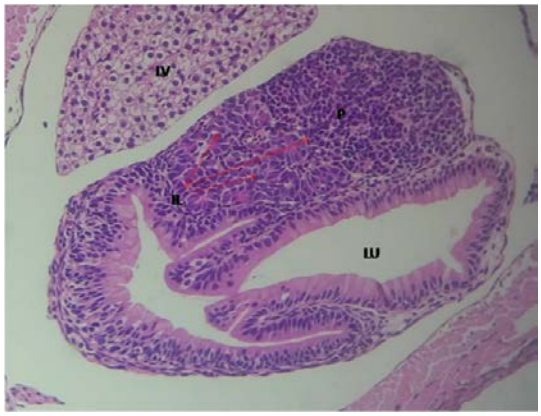

Figure 14. Histological sections of 24-DAH which shows a liver well developed with the pancreas (P) having the Islets of langerhan, the gut lumen was properly developed observed at magnification x100. Complete

exhaustion of the yolk sac, columnar epithelial cells(C), gut lumen (LU), liver (LV), IL- islets of langerhan and enterocytes (E) well observed (H&E staining).

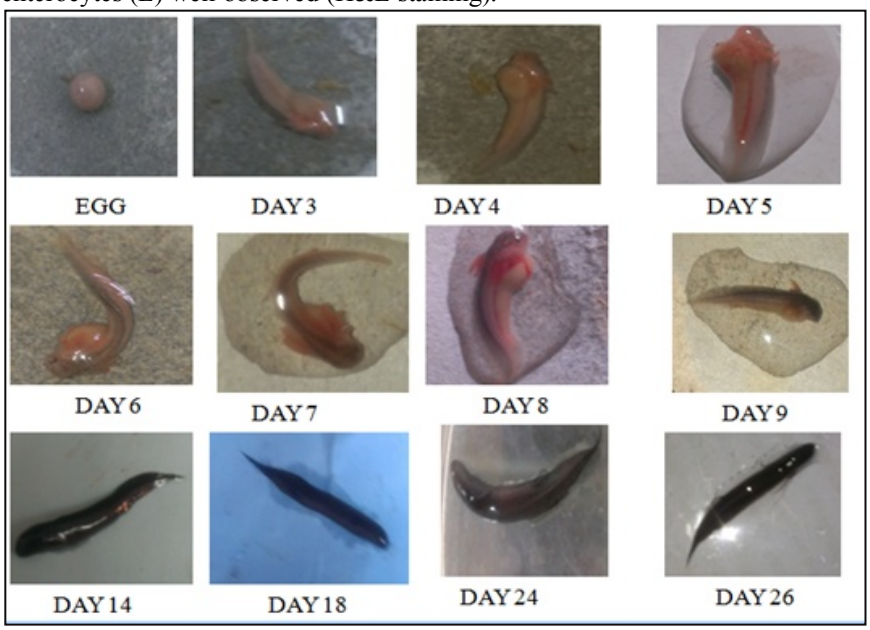

Figure 15. External development of lungfish hatchlings.

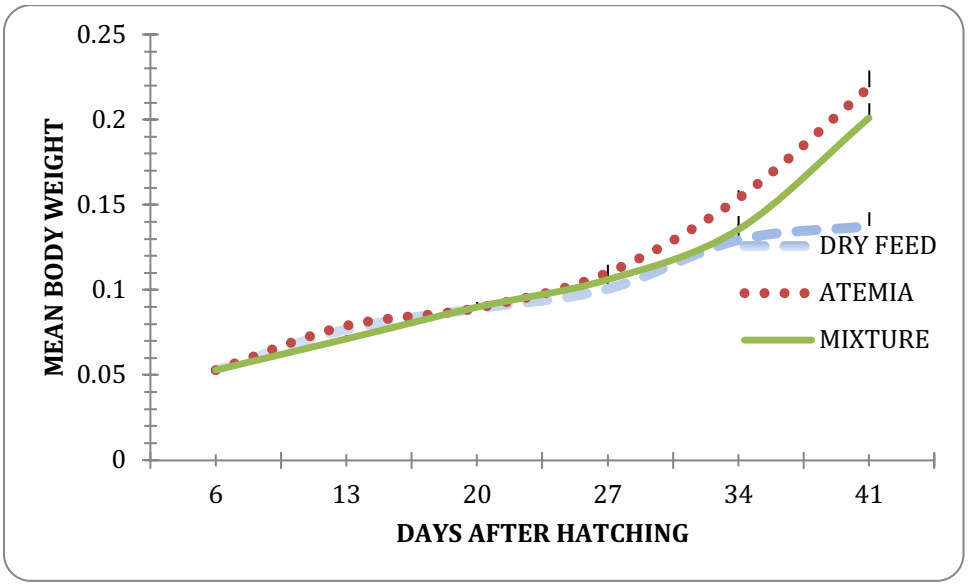

Figure 16. Growth performance of larvae weaned on three diets.

Experiment 4: Growth performance of African lungfish

Lungfish fingerlings (3.48 ± 0.83g) raised in out-door tanks and fed at *ad libitum* marginally grew to 17.13 ± 2.53g in 74 days. Hence, lungfish grew at 2.21% per day, faster than when raising wild-caught lungfish fingerlings observed during Walakira et al. 2014. Survival in this experiment was 92%, slightly higher than when growing wild-caught fingerlings. However, the size achieved in this trial is not the targeted table-size fish (>250g) hence the experiment is still on-going or under observation.

DISCUSSION

The ovary biopsy technique has been used to sample small amounts of tissue from the gonads of several different species of fishes, including the striped bass (*Morone saxatilis*) and white bass (*M. chrysops*) (Sullivan et al. 1997). The gonads in these species are connected to the urogenital pore by a straight duct that extends from the posterior end of the organ to the exterior of the fish (urogenital pore), thereby allowing direct entry of the plastic cannula into the gonad. In the case of the African lungfish, the plastic cannula can be inserted several cm, however no gonadal tissues may be aspirated (Figure 1; see also Figure 2). General gross anatomy of the African lungfish abdominal cavity is presented in **Figure 3**. Upon dissection, it appears that the lungfish has an unusual paired ovary compared to that of teleosts in that the oviduct originates from the anterior portion of the organ and then transverses posterior to the urogenital pore (Figures 4 and 5). The oviduct appears to be coiled and therefore the cannula cannot be fully inserted for gonadal tissue sampling from the ovarian lamellae (Figure 2). It may be that spawning lungfish with ovulated eggs in the oviduct could be sampled in this manner, however this has not been tested as none of the stage 3 females had ovulated eggs contained within the duct at the time of capture.

The past literature (Mosille and Mainoya, 1988 and other older literature by Greenwood), although scant, has suggested that the white coiled oviduct was “ovarian fat” and although it appears to consist largely of an unknown lipid, it is a duct with a lumen as also more recently indicated by Mlewa and Green (2004). Furthermore, an anterior loop has been identified here that connects this coiled oviduct to the ovary sac containing preovulatory oocytes (Figures 5 and 6). The ovary of stage 3 female lungfish sampled contains oocytes of different developmental stages, the largest of which constitute the most advanced clutch being 3.4-3.5 mm in diameter, consistent with an asynchronous ovary described in previous literature (Mosille and Mainoya, 1988; Mlewa and Green, 2004).

Since male African lungfish induced with hormone as described in objective 2.2 produce freely flowing semen upon palpitation of the abdomen, we suggest that this feature be used during the reproductive season to sort fish by gender such as those animals that produce semen are confirmed as males and those that fail to do so, but are of reproductive size as indicated by data from objective 1.2, are considered to be females. Fish sorted in this manner may then be kept in separate tanks based on gender for use as broodstock. Furthermore, there is no evidence of hermaphroditism in this species and all individuals sampled appeared to be gonorchistic.

The unknown, white-colored lipid of the coiled oviduct appears to be consistent with either cholesterol or a wax ester and does not appear to contain substantial triacylglycerol or other types of lipids that would typically leave a “clearing field” on white cellulose paper, as a subsample of the oviduct was streaked on paper and allowed to incubate in sunlight for 1 hour, leaving no trace of a clearing field, however it did produce in a smooth waxy surface on the paper.

Furthermore, the oviduct dissolves in organic solvents used for tissue fixation. A subsample of the oviduct was collected and frozen for subsequent analysis by lipidomics if funds are available to do so. The data collected here and also in objectives 1.1, 1.2 and 1.4 provide a comprehensive description of African lungfish internal anatomy and female gonad morphology.

Temperature is a critical factor in egg /embryo hatchability and survival of fish larvae (Haylor and Mollah 1995; Bjornsson, et al. 2001; Okunsebor, 2015). Results from this study hatchability ranged from 24 to 32^{o}C were all appropriate for hatchability of *P. aethiopicus*. It is unclear why hatchability values are low but this rate has been achieved with other fish species like *Clarius gariepinus* (Mwanja et al., 2013). Low hatchability rates in cultured species are attributed to poor quality of eggs and poor hatching conditions such as water quality(thorsen et al 2003). Since the broods were collected soon after the end of dry season it is necessary to use broods during the rainy season.

Lungfish larvae should be given live and dry feed at 18 and 26 DAH, respectively since histological sections showed well developed lumen and mucosa regions. The poor growth of fry fed on dry feed may be due to the inability of the fry to digest the feed nutrients coupled with its big size which reduced the feed intake and the best performance in fry fed live feed might be as a result of increased feed intake and high nutrient digestibility coupled with the proteolytic enzymes in the artemia.

Fry fed only formulated diet were visually smaller, lying on their sides and remained dispersed at the bottom of the aquaria in comparison with those fed live diet which were more active. It was also observed that live food seemed to be more attractable to the Larvae than other diet. Larvae Fed the combination of the diets (live and formulated) had an intermediate behavior, swimming either near the bottom or the walls. The above observations were in line with reports made by Paulo *et al.,* 2004, Adebayo and Akin 2013. The best growth performance in fry fed live feed might be as a result of increased feed intake and high nutrient digestibility of artemia that is also influenced by the proteolytic enzymes in artemia. Therefore this study indicates that source of food play an important role in delivering nutrients to fish larvae, which invariably influences their behavioral tendencies and survivability.

Previous study indicated lungfish (138 ± 42.46g) grows well with survival rates of 86%, in aquaculture outdoor tanks when poly-cultured with tilapia. In this trial, lungfish grew faster in out-door tanks with higher survival rates. Therefore, farmers can use this technology to raise lungfish in out-door tanks.

CONCLUSIONS

Artemia is the best feed for fry but given its cost, use of a mixture of feeds can be employed or other live feeds like moina or rotifers should be explored. Additionally various challenges are encountered in the species propagation and culture (hatchability, early fungal infections, among others) and their mitigation is paramount before accepting lungfish as a suitable candidate for aquaculture.

This project has unlocked important information on propagating, growing and conserving the biodiversity of African lungfish in Uganda. Government and regional bodies can now develop policies that will ensure protection of lungfish and communities dependent in this resource.

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