Development of Low-cost Captive Breeding and Hatching Technologies for the African Lungfish (*Protopterus aethiopicus* and *P. amphibius*) to Improve Livelihoods, Nutrition, and Income for Vulnerable Communities in Uganda

Climate Change Adaptation: Indigenous Species Development/Experiment/13IND03AU

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ABSTRACT

Culturing resilient species in the prevalent variable climate conditions will be beneficial to African aquaculture. Air breathing fish like the African lungfish (Protopterus sp.) will be desirable, but fish farmers lack aquaculture technologies to propagate and manage this fish. This report summarizes experimental results on diversity, breeding, and management of lungfish reared in aquaculture systems. Relatively higher survival and maturity rates were achieved when lungfish is kept in captivity. A novel SNP panel that will guide a comprehensive lungfish-breeding program is partially generated. Hermaphroditism in lungfish is first reported in this investigation study. These results will guide the generation low-cost technologies for propagating and producing cultured African lungfish to improve household nutrition, food security, and income. Consequently, natural stocks will be protected through this intervention.

INTRODUCTION

Marbled lungfish (Protopterus aethiopicus) is native to Ugandan waters, but its natural stocks are declining mainly due to overexploitation, environmental degradation, and the large-scale conversion of wetlands to agricultural land. Furthermore, climate change continues to influence regional rainfall patterns and temperature regimes, which directly affects aquaculture production. For example, seasonal water deficits caused by prolonged droughts usually constrain management of aquaculture systems. Rearing fish species that are tolerant to drought and poor water quality conditions would be a significant future for African aquaculture development (Allison et al. 2007, Daw et al. 2009, Wagle et al. 2011, Williams and Rota 2011). Air breathing fishes would be suitable candidates in poor water quality conditions because of their ability to obtain and utilize atmospheric oxygen to meet all or part of their metabolic demands (Myers 1986, Pethiyagoda 1991, Graham 1997, Thomson 2013).

The African lungfish, however, is an air breather that may offer some distinct advantages when water quality for fish growth is poor, like low-dissolved oxygen. Lungfish is valued in Uganda and consumer acceptance seems high and widespread, but appropriate aquaculture technologies are not available for fish farmers engaged its culture. Lungfish farmers currently collect seed from the wild environments and either raise them in earthen ponds or tanks. This is not sustainable ecologically, since its captive breeding technology is not known or documented. The absence of breeding and production technologies limits possibilities to explore its potential to generate income and improve nutrition for small-scale holders.

This study seeks to develop low-cost sustainable breeding and culture techniques for African lungfish in the region. Therefore, the underlying molecular information that supports its diversity, reproduction, and/or propagation is important. However, there is insufficient lungfish genomic data since it has the

largest genome (> 130 Gb) among vertebrates (Metcalfe et al. 2012). Hence, novel approaches like Single Nucleotide Polymorphisms (SNPs) panel will facilitate, i) genetic diversity of lungfish, ii) whole genome sequencing, iii) breeding program, and iv) conservation programs.

OBJECTIVES

- Determine the genetic diversity of the endemic African lungfish (Protopterus aethiopicus) fingerlings sourced from four agro-ecological zones (East, North, South western and Central) of Uganda;
- Domesticate the African lungfish using simple, adoptable, and productive captive breeding techniques that integrates indigenous knowledge;
- Assess the reproductive performance of the African lungfish in captivity; and
- Evaluate the culture performance of African lungfish raised to market size in small-scale fish ponds.

METHOD

Study 1: Determining genetic diversity of African lungfish (Protopterus aethiopicus) sourced from six lakes of Uganda using mtDNA and morphometry.

Morphometry. A total of 254 fish samples were collected from Lakes Bisina, Nawampasa, Edward, George-Kazinga, Wamala, and Kyoga in 2014. Sampling stations included open lake ecosystem, in-river, and lake-river interface.

Whole fish samples (Figure 1) of adult and juvenile fish were obtained from fishing grounds using monofilament gill nets (size = 2 to 8”), and long lines. Using truss dimensions (Strauss and Bookstein 1982) each fish was constructed, using calipers, as described by Flemming et al. (1994, 1995).

From each fish, the total weight, total length, truss, and non-truss parameters (Figure 2 and Table 1) were measured to compute morphological disparity among and between the populations. These were done using a digital weighing scale, graduated meter rule, and Vernier calipers. From each fish, a tissue sample of about 1x1 cm was extracted, mainly from the left posterior claspers and preserved in an absolute ethanol and kept at -32OC at ARDC Kajjansi before transferring to BecA-ILRI Hub laboratory facility in Nairobi, Kenya for genomic analysis.

Principle Component Analysis (PCA) was used to determine morphometric differences from each source using XLSTAT software, version 2012. A total of six truss parameters, mainly were fin (claspers), lengths of the head, snout, gape size and orbital distance, and four nontruss morphometric variables were measured on each specimen. All morphometric measurements were transformed in PCA to adjust for variations in fish size (Reist 1985).

Figure 1. Sources of lungfish samples in Uganda.

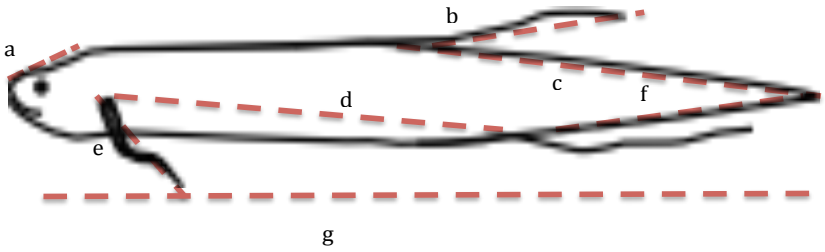

Figure 2. Landmarks used for the morphometric analysis of P. aethiopicus.

Table 1. Morphometric characters used for analysis of African lungfish (P. aethiopicus).

Character	**Description**
Anal fin length (AFL)	Distance between anterior of anal fin to tip of caudal fin.
Dorsal fin length (DFL)	Distance between anterior of dorsal fin to tip of caudal fin.
Gap size (EL)	Maximum height of mouth cavity
Head length (HL)	Distance from tip of snout to posterior margin of opercula
Orbital distance (OBD)	Distance between the eyes
Right and Left pectoral fins (RAC and LAC)	Length of pectoral fin measured from proximal to distal end
Right and Left pelvic fin (RPC and LPC)	Length of pelvic fin measured from proximal to distal end
Snout length (SL)	Mouth size
Total length (TL)	Distance from the tip of the snout to the longest caudal fin ray

D-loop determination. Genomic DNA was extracted from fin clips of 254 fish samples using protocols described by Life technologies (Invitrogen) kit®, but also compared to salt extraction method. The mtDNA control region was amplified using Bioneer kit® conditions and primers developed Protopterus dolloi Boulenger (Zardoya and Meyer 1996).

Amplified products were visualized on electrophoresis through 1.5% agarose gels, amplified fragments were purified using Qiaquick Gel Extraction columns (Qiagen) then mtDNA Sanger sequenced at BecA-ILRI, Nairobi, Kenya. Phylogenetic analysis was applied to further understand the genetic variability of this fish in Ugandan waters. Information generated will enhance strategies to conserve its biodiversity and support the aquaculture program in the region.

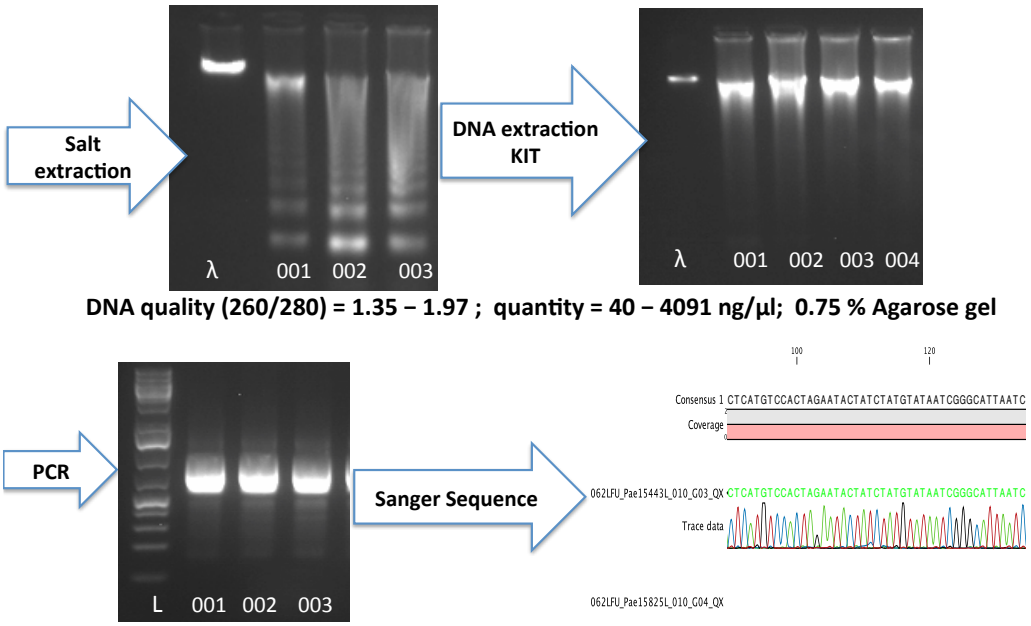

Figure 3. Workflow of DNA extraction and quality assessment of genomic material.

D-loop data generated was analyzed using CLC Main Workbench Version 7.5 (QIAGEN Aarhus, A/S www.clcbio.com) 2014. The phylogenetic tree was constructed using MEGA 6.06 software.

Experiment 1.1: Genetic diversity of African lungfish in Uganda— Relatedness based on single nucleotide polymorphisms (SNPs) and microsatellite markers. This panel comprises a small set of markers that will guide in the selection of broodstock to be recruited into the breeding program. Secondly, changes in the genetic profile of this fish can be determined as the species is developed. In this study, fin clips from 200 lungfish samples (50 per site) were collected from four AEZs (i.e,. Lake- Edward, Kyoga, Bisina, Nawampasa, Wamala, and George) were used and flash frozen in liquid nitrogen and stored at -80°C. Total RNA was isolated from each sample using TRIzolTM (Invitrogen, Carlsbad, CA) protocol. Equal masses of total RNA from the samples of each group were pooled and used for RNA-Seq sequencing.

cDNA libraries were prepared and sequenced through Illumina Genome Analyzer (single-end, 300 bp read length) at the International Livestock Research Institute-molecular laboratory (Nairobi, Kenya) as described by Severin et al. (2010). Variant detection pipeline (Miller et al. 2008) was used to map sequence reads to a reference transcriptome of a double-haploid Latimeria chalumnae fish (Amemiya et al. 2013). SNP detection was done using the Next Generation Sequencing (NGS) pipeline described in Figure 4.

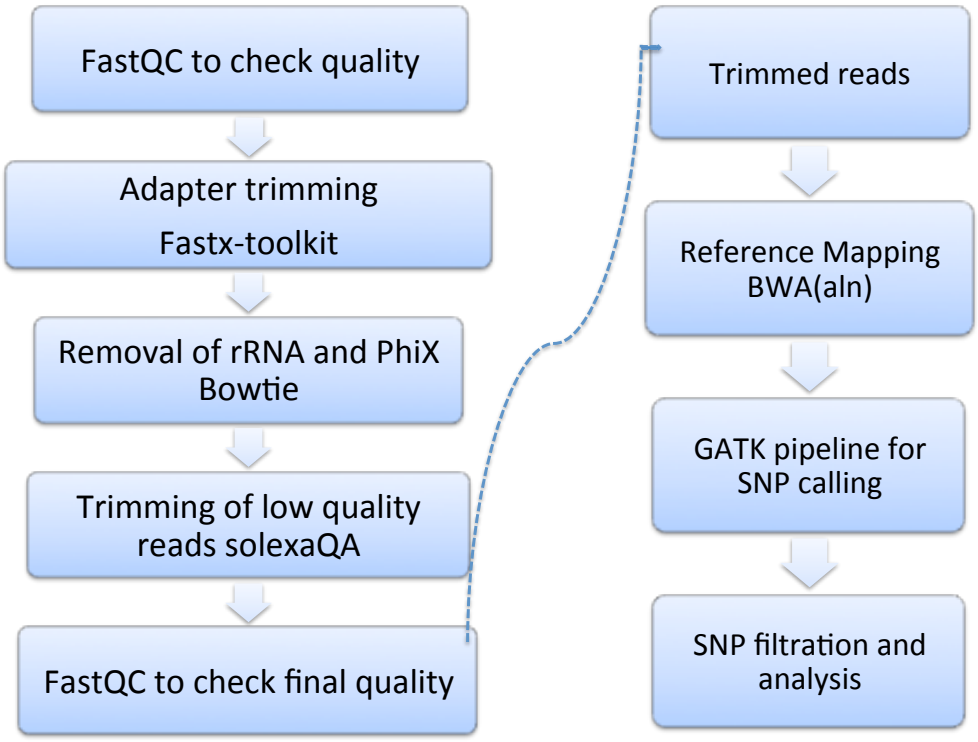

Figure 4. Schematic of transcript assembly and SNP detection pipeline.

Experiment 1.2: Reproductive biology of African lungfish in captivity. To ensure an environmentally sustainable supply of African lungfish seed to fish farmers, artificial breeding technologies have to be well developed. Low-cost breeding technologies will be ideal for rural communities that are dependent on this fish. Lungfish collected from wild populations were visually checked for maturity, and subsequently subjected to artificial reproduction techniques; natural and artificial breeding.

Study 2: Domesticating the African lungfish using simple captive breeding techniques that integrate indigenous knowledge.

Experiment 2.1: Artificial breeding of African lungfish in captivity. Following Vijay Kumar et al. (1998) protocol, mature broods were caught from wild lake environments (Lakes Edward, Bisina, and Kyoga) during rain seasons and conditioned in concrete tanks at NaFIRRI for two weeks. Fish were injected with two selected hormones; human chorionic gonadotropin (HCG) and luteinizing hormone-releasing hormone analogue (LHRHa) to induce spawning. Response to hormone treatment was observed, measured, and recorded. Water quality parameters were monitored to understand environmental factors affecting artificial breeding.

Results on reproduction of lungfish held in captivity at NaFIRRI-Kajjansi show males reach maturity at 300 g (37–57cm) compared to wild conditions where first maturity occurs between 65–85 cm, according to Mosille and Mainoya (1988) and Greenwood (1958).

Lungfish that weighed more than 300 g responded positively to doses of HCG and LRHa synthetic hormones, but failed to breed under captive conditions. Anatomy of mature fish samples revealed that hermaphroditism exists in lungfish populations. Hermaphroditism in lungfish presents a challenge in propagating this fish, but comprehensive research is tracked to understand this phenomenon.

Experimental fish gradually accepted the sinking fish feed pellets. However, lungfish slightly increased in average body weight having highest growth attained using feeds with high protein content. With this diet, lungfish grew with a specific growth rate (SGR) of (0.50 ± 0.06%/d), and feed conversions ratios ranging from 1.61 ± 0.26 to 2.07 ± 0.11 were obtained. The highest survival rate under this experiment condition was 57.50 ± 2.85%, which is higher than previously documented by Mlewa et al. (2009). Cannibalism predisposed most experimental fish to aquatic pathogens; primarily water molds and bacteria causing mortalities.

Common diseases encountered included: bacteria (Aeromonas sp. and Flavabacterium columnaris), fungus (Fusarium spp., Aspergilus sp. and Saprolegnia sp.), and parasite (Dactylogurus sp., Trichodina sp., Tetrahymena sp., Heterorchis sp., and cestodes). Nevertheless, regeneration of injured appendages (fins) was observed, as previously described by Tamura et al. 2010. However, lungfish grows better in aquaculture outdoor tanks when poly-cultured with tilapia.

Cultured lungfish juveniles reached market size of average weight of 138 ± 42.46g in six months when polycultured with mixed-sex tilapia. Size at harvest ranged 50.2–512.9g, indicating a genetic variation within this species, and therefore, a comprehensive selecting program for this fish species is important. Survival rates improved to 86%, compared to indoor experiment, and generally fish appeared healthy.

Experimental 2.2: Natural breeding of African lungfish in captivity. Selected mature brood-fish collected were kept in two concrete tanks and water surface covered with water hyacinth (Eichornia crassipes) to simulate a natural breeding habitat. Water levels were manipulated (0.2 to 0.4m) every week to stimulate natural ovulation, spawning, and fertilization. Water quality parameters [Temperature and Dissolved oxygen (DO)] were monitored weekly to understand environmental factors affecting breeding in these tanks.

Study 3: Evaluating the performance of African lungfish reared in ponds under different management practices.

Indoor experiment was conducted in twelve Crest fiberglass tanks (60 L): testing three formulated diets replicated four times. Each replicate was stocked with 30 lungfish fingerlings of mean weight 9.74 ± 0.12 g and mean length of 13.74 ± 0.33 cm. Systems were aerated with 50% of water exchanged three times per week. Sampling was done weekly.

Outdoor trial was conducted in concrete tank (2 x 7 x 0.5) m3, stocking 100 juveniles (40.84± 19.24g) together with 50 mixed-sex tilapia (Nile tilapia and Tilapia zilli) fingerlings. Fish were fed ad libitum with formulated fish feeds (30% Crude protein).

Morphometry. Findings indicate lungfish from six lakes generally exhibit intrinsic and extrinsic homogeneity. However, samples from Lake Nawampasa showed variations in gape size, snout length, orbital distance, dorsal fin length, and anal fin length.

D-loop (mtDNA) diversity. The phylogenetic tree showing 70 mtDNA haplotypes, 13 reference groups, and one outgroup is shown in Figure 5. The lungfish haplotypes cluster into three distinct groups (henceforth referred to as A, B, and C clades) that differ at about 9.8% sequence divergence. The distinction between lineages was supported at a bootstrap value of 100%. Tajima's D values were

negative for all populations, indicating a low frequency polymorphism compared to expected value, hence, an expanding population.

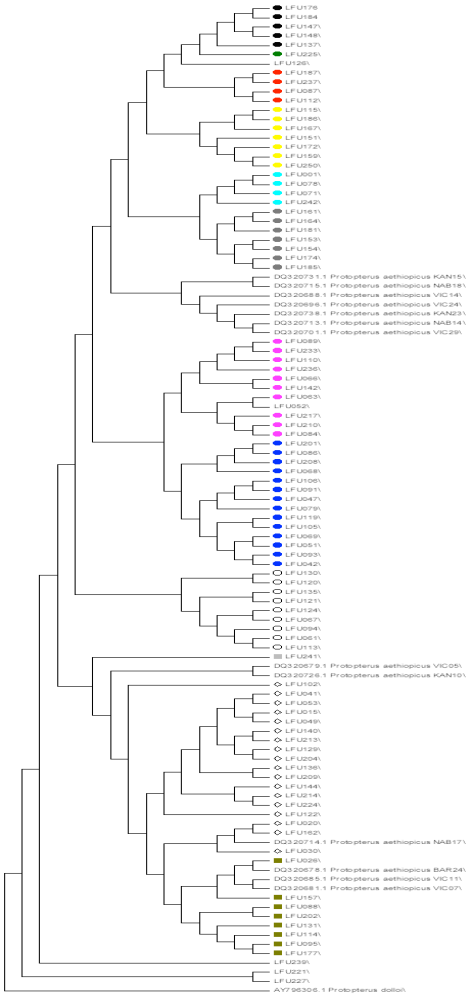

Figure 5. Phylogenetic tree generated showing clades of lungfish collected from six lakes of Uganda. (Different colors indicate haplotypes of P. aethiopicus).

Conversely, Fu's FS tests showed negative values for all populations, indicating an excess of rare haplotypes/alleles expected from a recent population expansion, largely contributed from Lakes Bisina and Edward. However, the nucleotide (Pi) diversity values averaged 0.010 ± 0.001, indicating less diversification between the six populations.

Geographical structure of African lungfish results from the AMOVA (Table 2) show that overall genetic variation within populations (79.01%) is much larger than the variation between populations (20.91%).

Table 2. Comparison of geographical structure of populations by assessments with Analysis of Molecular Variance (AMOVA).

Sources of Variation	d.f.	Sum of Squares	Variance Components	Percent of Variation
Among populations	5	182.9	1.0 V_a	20.9
Within populations	197	751.6	3.8 V_b	79.9
Total	202	934.5	4.824	
Fixation Index	F_{ST} = 0.209			

Single nucleotide polymorphism (SNP) panel for genetic diversity of lungfish from six lakes in Uganda. A total of 80,358,264 Single-end short reads (301-bp) through RNA-Seq using Mi-Seq pipeline were generated (Table 3). After filter using NGS QC Toolkit, a total of 75,647,110 high quality reads with 285 bp were generated from transcriptome. A total of 64,666,158 high quality reads with 285 bp were obtained after mapping to the reference Latimeria cholumnae.

Table 3. Statistics of raw reads with high quality and mapped reads ratio of transcriptome.

	Reads
Total number of reads: raw	80,358,264
Total number of reads: clean	75,647,110
Mapped to reference	64,666,158
Mapped read ratio	85.5%
Average GC content after trimming	43.4%

A total of 12,085 putative SNPs with high quality were identified in the transcriptome. Using the four sets of data together (Wamala, Kyoga Nawampasa, and Bisina), a total of 121 SNPs with high quality were predicted (Figure 6).

The estimated SNP frequency was 0.21% (one per 400 bp). Within the identified SNPs, more transition substitution (41.7%) was found than transversion substitution (68.3%) (Table 4 and 5). In terms of transition substitution, the amount of A/G transitions was similar to that of C/T transition. In terms of transversion substitution, the frequency of four types (G/C, G/T, A/C, and A/T) was equal. The estimated ratio for transition to transversion was 0.7.

Table 4. Statistics of transition and transversion type in the total SNPs.

Type	Transition		Transvertion		
	GA	CT	GC	GT	AC
Number	2433	2614	1374	1613	1912
Percent	20.1	21.6	11.4	13.3	15.8
Total	5047		7038		

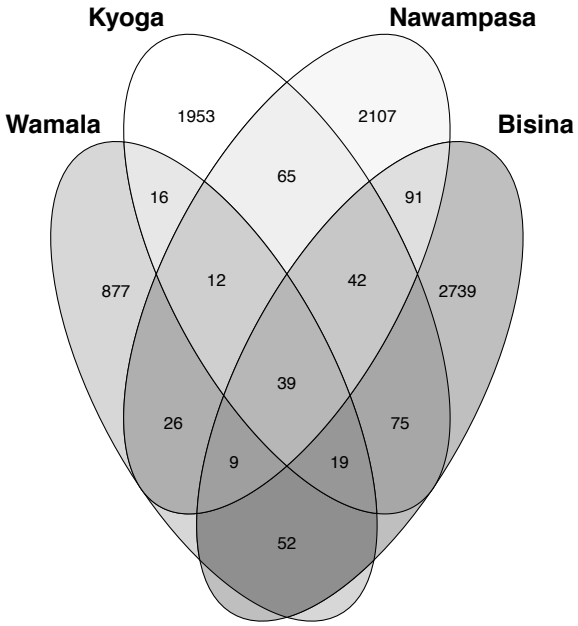

Figure 6. Venn diagram of SNPs using reads of the four datasets. Lakes Wamala + Kyoga + Nawampasa + Bisina represented SNPs discovered using the reads of the four transcriptomes together.

Table 5. Summary of SNPs per lake.

Lake	Number of SNPs
Wamala	831
Edward	1,430
Kyoga	1,811
Nawampasa	2,009
George	2,070
Bisina	2,602

As the read depth in SNPs position was closely related to the prediction accuracy of SNPs, the statistics of read depth for each SNP was calculated. It showed the estimated average read depth was 33. SNPs with read depth between 10 and 50 account for the majority (98%), while SNPs with read depth range from >200 account for nearly 1%. The average read depth was 33.

Table 6. Statistics of read depth represented the number of SNPs with the corresponding read depth.

SNP Read Depth	Number of SNPs
>10-50	11,984
60-100	117
110-150	43
160-200	5
>200	33

CONCLUSION

This study assessed indigenous practices and understanding about lungfish as a potential culture species in Uganda. Fish farmers inadvertently farm lungfish that enter their ponds during flood periods and have realized they can survive and grow alongside other fish (i.e., tilapia). However, optimal feed composition and lungfish grow-out strategies remain to be established. At present, growers rely on wild-caught lungfish fingerlings for the limited culture currently taking place.

Research must clarify the reproductive cycle of lungfish to facilitate farm-based spawning and produce uniform seed stock batches of genetically advantaged fish. A clear foundation to establish a lungfish industry, as well as the biology and manipulation of reproduction processes, is not fully understood.

An experimental program is needed to establish production parameters, since little is known about the growth cycle and nutritional needs of farm-reared lungfish. For example, optimal water temperatures, salinity tolerance, and other basic parameters of the species are not known.

Farmers have developed indigenous means of handling and managing lungfish in natural water bodies and farm ponds. These techniques are beginning to be discovered and codified. Promoting wider levels of lungfish production will require articulation of model production strategies and management systems that account for the burrowing and mobility of lungfish. Clearly, cage culture would overcome some known difficulties, but this work has not yet been accomplished.

Lungfish is a delicacy among groups in northern and eastern Uganda, and in some parts of the western region. Thus, present and potential consumer demand for the species is fairly well established. Field work has assessed potential paths of production and training for lungfish as a cultured species and managed water resource.

Lungfish may be raised on artificial diets since all fish farms feed commercial pellets to catfish or tilapia stock. Efforts to domesticate African lungfish are fundamental to advance a commercial industry capable of providing a valuable food item to people in need of affordable protein.

QUANTIFIED ECONOMIC BENEFITS

- Basic guidance on management of lungfish expressed in a farmer-oriented leaflet is being developed;
- Basic nutrition profile of lungfish grow out was expressed in a technical report for extension;
- Basic fingerling supply and grow out information expressed in a journal article; and
- Inform the merits of continuing research into developing low-cost, artificial breeding technologies for these species.

ACKNOWLEDGMENTS

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