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Efficacy of Hormones Produced in the Primary Culture of Pituitary Cells on Induction of Spawning, Survival Rates and Growth Performance of African Catfish (*Clarias gariepinus*, Burchell 1822) Larvae Produced

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Abstract

Pituitary cells have tendencies to undergo continuous mitosis. Pituitary cells were cultured in RPMI 1640 medium for 12 hours under 30°C at 5% CO_2. Female broodstocks were induced for hypophysation with freshly cultured pituitary cells (FPC) and Ovaprim (GNRH analogue). The spawned eggs were fertilized with sperm cells in vitro. The hatched larvae from their inductions were reared for eight weeks and analysed for survival rates and growth performance. The induced spawning bio-marker for gonadotrophin specific function revealed that 97.5 g/kg of mature oocytes were spawned from FPC and 157 g/kg from Ovaprim induced catfishes respectively. The specific growth rates and survival percentages showed no significant differences in both groups (P>0.05). Gonadotrophins from the primary culture of pituitary cells were potent enough to spawn mature metaphase II oocytes that produced healthy larvae with non-significant growth performance.

Keywords:
Pituitary cells, Primary culture, Continuous mitosis, Metaphase II oocytes, Induced spawning

Introduction

Pituitary gland is the main source of the major hormones responsible for reproduction in animals. The pituitary cells have been reported to undergo continuous mitotic process (Yeung *et al.,* 2006). The pituitary gland can be cultured and proliferated in vitro in order to use their secretions for induction of spawning in catfish (Lugo *et al.*, 2008, Chen *et al.,* 2010). Cells growth, division and multiplication can be achieved by addition of fetal bovine serum and culture medium to the pituitary cells in a culture plate at 30°C and 5% CO_2 (Gupta *et al.,* 2005; Gupta and Saxena, 1999; Huhtaniemi, 2000; Lubzens *et al.,* 2010). Gonadotrophins are the hormones responsible for the ovulation and spawning of eggs in fish (Sahoo, 2006). The two pituitary gonadotrophins luteinizing hormone (LH) and follicle stimulating hormone (FSH) are glycoprotein hormones. They are composed of the common α- subunit and hormone specific β-subunit which is coupled by non-covalent interactions (Ando *et al.,* 2006). The main physiological functions of the FSH is to stimulate follicular maturation and granulosa cells oestrogen production in the ovary and sertoli cell functions in the testis thereby contributing to spermatogenesis (Christians and Williams, 2002). LH stimulates theca cells androgen production in the testis, producing a substrate for granulosa cell oestrogen production, triggers ovulation, spawning and maintaining the progesterone production of the corpus luteum in the ovary (Huhtaniemi, 2000; Lubzens *et al.,* 2010). *In vitro* culture has proved to be the most valuable method to study the functions and mechanism of operations of many cells. A particular group of cells can be cultured in large quantities to study their

cellular activities and differentiations. A growing number of cell types have been identified and proliferated in the *in vitro* systems to replace their natural progenitors. Pituitary gland is made up of a number of cell types that are essential for physiological processes such as growth, development, homeostasis metabolism and reproduction. Normal cells can divide a limited number of times before they undergo senescence and death. This property can make them grow continuously and develop in *in vitro* model to study their mechanisms of secretions and regulations of hormones.

This study evaluated the efficacy of the hormones produced in the primary culture of pituitary cells to induce spawning in African Catfish broodstocks and their effects on the survival rates and growth performance of larvae produced.

Materials and Methods

Isolation of pituitary gland

The brain case of the head of catfish was removed using the sharp pointed edge of a surgical knife and carefully lifted up to expose the pituitary gland. The pituitary gland is very tiny and has a high tendency of getting mixed up with the two lobes of the brain. It was gently removed with the aid of a scalpel or forceps, and placed in a petri dish containing physiological normal saline solution.

Homogenisation and trypsinization of the pituitary tissues

Ten donor catfish weighing between 500g and 700g were used for this study. The isolated pituitary glands were weighed with an electronic balance and later rinsed in 2 ml chilled normal saline before being homogenized with vortex mixer. Then, 0.5 ml of trypsin (SIGMA) was added to the homogenized pituitary tissues to aid dissociation of pituitary cells. The solution was warmed in the shaking water bath of 30^0C for one hour (Lugo *et al.,* 2008; Oyeleye *et al.,* 2016). The trypsinised cells were observed under binocular microscope (Mofic, China) to determine the initial cell counts using hemocytometer. Cell per ml = the average count per square (from 10 squares of hemocytometer) × dilution factor × 10^4.

Total cells = cells per ml × the original volume of the fluid from which cell sample was removed.

Culture media preparation

225µl of the culture medium(RPMI) containing 10 % (20µl /250 µl) of Fetal Bovine Serum was measured into the culture microplate. 20µl of the trypinised pituitary cells was gently added to the medium and mixed thoroughly with micropipette.

Culture in CO_2 incubator

The cells were cultured for 12 hours under 30^oC at 5% CO_2 in CO_2 incubator (Labline, USA). The proliferation rate and population doubling time of the cells were determined after 12 hours of culture. Cell viability was evaluated using 0.4% trypan blue stain. The cells that were unstained were viable cells while the stained cells by trypan blue were referred to as dead cells. This experiment was repeated three times to authenticate the data collected.

Proliferation rate: The proliferation rate is the rate of growth per unit amount (weight or numbers) of biomass. The proliferation rate, $r = 3.32 \times (\log N_F - \log N_I)/T_2-T_1$ (Griffiths, 1972; Gupta *et al.,* 2005). Where, r = Proliferation rate, N_F= number of Final cell count, N_I =Number of Initial cell count, T_2= Time at harvest, T_1=Initial time.

Population doubling time (PD): This is the number of times the inoculums has replicated within 24 hours. (PD) = 24/(r), where r is proliferation rate.

Induced spawning bio-marker

Induction of spawning was carried out under optimal laboratory conditions required for catfish oocyte ovulation and maturation. Two inducers were used; freshly cultured pituitary cells and gonadotrophin releasing hormone analogue (Ovaprim; Syndel, Canada) which served as control. An aliquot of 1 ml each of freshly cultured pituitary gland cells as well as 0.4 ml/kg of Ovaprim (Fagbenro *et al.,* 1998; Olaleye, 2005) were injected intramuscularly into quadruplicate female catfish per inducer. The mature and ovulated eggs were later fertilized in vitro with milt from the male catfish. Incubation was done in hatching tanks (1.0 m × 1.0 m × 0.5 m) of 50 L capacity(Akankali *et al.,* 2011).

Nutrition and post-hypophysation management

Larvae produced were fed with *Artermia naupli* shell free feed (54% crude protein) (INVE® Aquaculture Nutrition, Ogden, Utah, USA) *ad libitum*. The fry were fed 5% body weight (45% crude protein) with size 0.5 -2mm) feeds. They were fed four times a day at 7.00 h, 11.00 h, 15.00 h and 19:00 h. The water physical parameters observed fell within the optimum range: water temperature 26.00-29.00°C, pH 7.8-8.5, dissolved oxygen: 6.9– 7.7mg/ll, total NH_3; 0.1-0.2mg/l, Nitrite: 0.07-0.1 mgHam Nitrate: 1-3mg/l).

Determination of survival percentages for the two experiment units

The percentage survival (Equation 3) was calculated following Omitogun *et al.,* (2012) and Olaniyi and Omitogun (2012) methods.

i. % Fertility (c)= Number of fertilized eggs /Number of eggs set * 100..... Equation (1)

ii. % Hatchability = N_H* 100 /N_F..... Equation (2)

iii. % Survival (X_z) = n * 100 / [i * (c/100)] = n * 10^4/ (ixc)..... Equation (3)

Where, c = The absolute percentage of fertile eggs (% of morula at 5h of incubation).
i = The initial number of the egg set
n = The number of fry which survived up to a given development stage,
N_H = Number of larvae hatched, N_F = number of eggs fertilized.

Evaluation of the growth performance

The growth performance of fry produced was evaluated after eight weeks of rearing. The following parameters were measured and determined: The initial weight at one week, final weight at eighth week, initial length at one week, final length at eighth week, average daily weight gain, average daily length gain and specific growth rate. The initial length and final length were measured by plastic metre rule while the initial weight was determined by putting the whole 10 hatchlings in a 50 ml beaker (Pyrex, England) filled with water up to the middle for each experimental unit. The weight was measured by electronic sensitive weighing balance (Toledo, England). Ten fry were taken from each experimental unit to determine their growth performance as stated below:

Average daily weight gain = $\frac{\text{Final weight} - \text{Initial weight}}{\text{Number of trial days}}$ .....Equation (4)

Average daily length gain = $\frac{\text{Final length- Initial length}}{\text{Number of trial days}}$ .....Equation (5)

Specific growth rate (% day^{-1}) = $\frac{\text{Log of Final weight- Log of Initial weight*}}{\text{Number of trial days}}$ 100.....Equation (6)

Data analysis

Data were analysed using one-way ANOVA (SAS, 2002) `and the level of significant difference among means was determined at $P< 0.05$ using Duncan Multiple Range Test.

Results and Discussion

Morphometric characteristics of catfish pituitary gland

The fresh pituitary gland of African catfish is a multifunctional group of complex tissues that have negligible weight (Figure 1). Ten pituitary glands of 500-700g catfish weighed 0.1g and an average weight of 0.01g. The diameter of each of the gland ranged between 0.2 and 1.5 mm (Table 1). The initial cell counts of the trypsinized pituitary cells were found to be 4.2 x 10^6 cells per ml. The initial cell count depends on the age of the fish that is being used. The trypsinized cells appeared round and oval in shape.

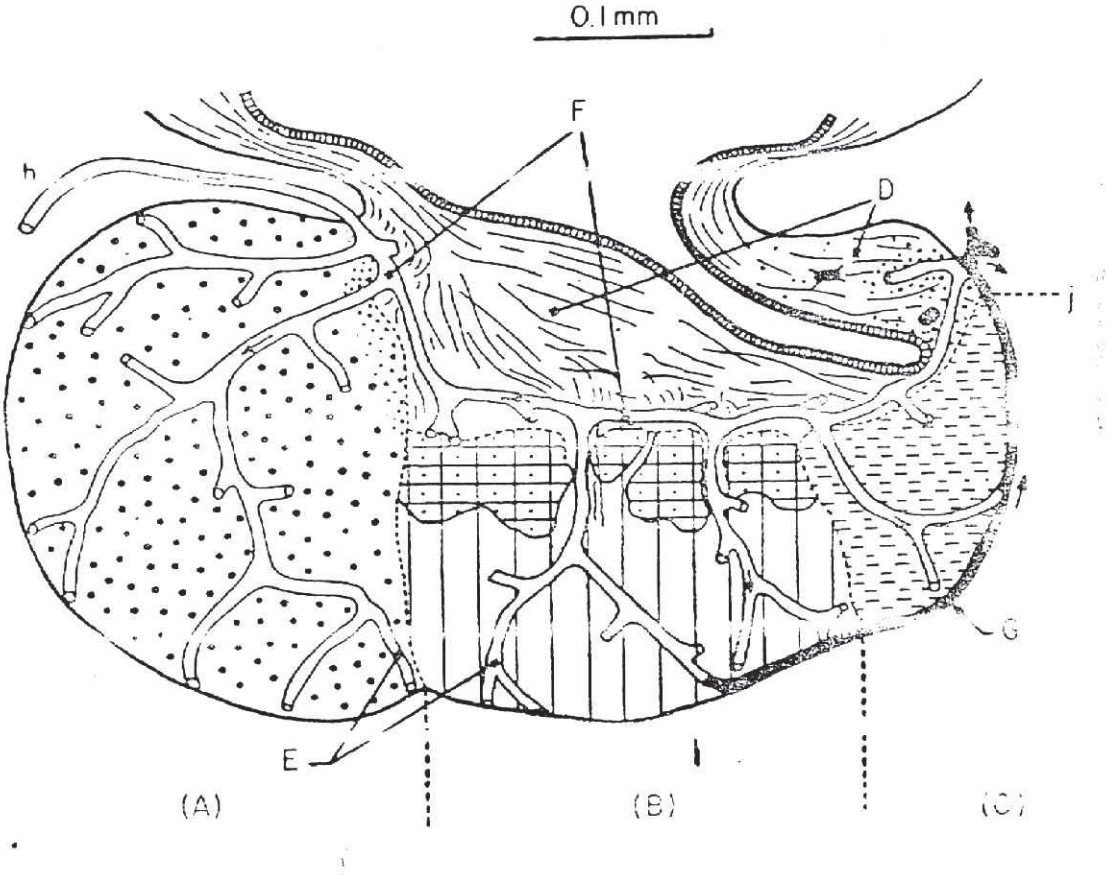

Figure 1: Fresh pituitary gland of African catfish

Table 1: Catfish pituitary gland morphometric characteristics

Weight of Donor fish(g)	Diameter of Pituitary gland (mm)	Number of Donor fish	Average Weight of 10 Pituitary gland(g)	Initial Pituitary cell count /ml
500-700	0.2-1.5	10	0.01	4.2 x 10^6

Growth yield of catfish pituitary cells in culture

The growth yield of pituitary cells was presented in Table 2 and showed that the initial seeding density of about 41.4 x 10^6/ml was seeded into culture medium. The increase in the cell count occurred from the 3rd hour of culture to the 12th hour of culture. The final count after 12 hour of culture showed that RPMI had 31.51±17.5 x10^7cells/ml which was a significant increase over the initial seeding density. The pituitary cells in RPMI culture medium had a proliferation rate of 0.71 cells/h and a population doubling time of 34.05h.

Table 2: The growth yield of catfish pituitary cells in culture

Medium	Initial count (cell/ml)	12h (cell/ml)	Proliferation rate(cell/h)	Population doubling time (h)	N
RPMI (x10^6)	41.4±1.11^a	315.1 ±17.5^b	0.71±0.029	34.05±1.48	10

Means with the same letter were not significantly different at P< 0.05.

Induction of spawning

The GnRH analogue induced female catfish spawned 157g/kg of mature eggs while FPC spawned 97.5g/kg. The water quality parameters fell within the optimal levels throughout the period of incubation and rearing of larvae (Boyd, 1990). There were significant differences in fertility percentages (P<0.05). The hatchability percentages showed no significant differences in both groups(P<0.05).

Table 3: Induction of spawning with freshly cultured pituitary cell solution

Inducer	Weight of egg spawned (g/kg)	Fertilization (%)	Hatchability (%)
FPC	97.5±12.76^b	75.65±3.43^b	73.95±3.23^a
Ovaprim	157±13.66^a	87.34±1.32^a	76.54±1.21^a
n	4	4	4

Means in the same column with the same letter were not significantly different P<0.05.
Keyword: FPC: Freshly Cultured Pituitary Cells; Ovaprim: GnRH analogue

Survival rates of catfish larvae reared for 8 weeks

There was no significant difference in survival rates between fry from FPC and GnRH analogue at all the periods of rearing (Table 4).

Table 4: Survival rates of catfish larvae reared for 8 weeks

Inducer	**A week survival (%)**	**4 week survival (%)**	**8 week survival (%)**
Ovaprim	76.88^a	60.03^a	56.50^a
FPC	71.67^a	58.33^a	50.33^a

Means in the same column with the same letter were not significantly different P<0.05.
Keyword: FPC: Freshly Cultured Pituitary Cells; Ovaprim: GnRH analogue.

Evaluation of the growth performance of catfish larvae produced from induction with freshly cultured pituitary cells and Ovaprim (GnRH analogue)

Table 5 presents the growth performance of the catfish larvae reared to fingerlings' stages within the period of eight weeks. The initial weight of the catfish larvae reared from the 1st week ranged from 0.72g to 0.70g. The final weight of 10.53g was found in the FPC and 11.73g in GnRH. There was no significant difference between the fry from FPC and GnRH in final weight after eight weeks of rearing (P>0.05). The final lengths differed significantly in both groups (P<0.05). The GnRH group and FPC differed significantly in average daily length gain. Average daily weight gain in both groups differed not significantly(P>0.05). The specific growth rates showed no significant difference in both groups(P<0.05).

Table 5: Growth performance of catfish larvae produced from induction with freshly cultured pituitary cells and ovaprim (GnRH analogue)

Treatment	IBW(g)	FBW(g)	IL(cm)	FL(cm)	ADWG	ADLG	SGR	N
FPC	0.72^{a}	10.53 ± 3.05^{a}	1.01 ± 0.12^{b}	7.21 ± 0.42^{b}	0.29 ± 0.001^{a}	0.11 ± 0.04^{b}	5.85 ± 0.42^{a}	10
Ovaprim	0.70^{a}	11.73 ± 3.55^{a}	1.42 ± 0.42^{a}	10.75 ± 0.64^{a}	0.33 ± 0.001^{a}	0.15 ± 0.01^{a}	5.98 ± 0.53^{a}	10

Conclusion

Gonadotrophins from the primary culture of pituitary cells were potent enough to spawn mature metaphase II oocytes that produced healthy larvae with non-significant growth performance. Gonadotrophins released from pituitary gland are responsible for ripening and maturation of oocytes in fish. The protocol adopted in this study can be used to develop *in vitro* model to study synthesis, actions, mechanisms and regulations of hormones in fishes especially African catfish which has a lot of potentials which have not been fully explored.

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