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Effect of Sublethal Concentrations of *Lepidagathis alopecuroides* (Vahl) on Sperm Quality, Fertility and Hatchability in Gravid *Clarias gariepinus* (Burchell, 1822) Broodstock

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ABSTRACT

This study aimed at investigating the effect of sub-lethal concentrations of *Lepidagathis alopecuroides* on the qualitative and quantitative parameters of the milt of the freshwater catfish *Clarias gariepinus*. Fifteen gravid Clarias *gariepinus* broodstock were exposed to sub-lethal concentrations of *Lepidagathis alopecuroides* for 21 days and the milt obtained for quality assessment visually with binocular light microscope and by fertility tests. The predominant color of the milt was creamy white with a mean pH of 6.80±0.26. There was a significant ($p<0.01$) linear relationship between sperm motility and the concentration of *L. alopecuroides* characterized by the equation $Y= -58.45x+83.64$, $R^2 = 0.938$ and a significant ($p<0.01$) negative correlation coefficient ($r = -0.973$). Similar trends were observed between sperm concentration ($Y = -10.24x+14.25$, $R^2 = 0.976$, $r = -0.988$; $p<0.01$) and sperm output ($Y = -39.15x+51.82$, $R^2 = 0.968$; $r = -0.983$; $p<0.01$). A highly significant ($p<0.01$) negative correlation ($r = -0.969$) was also observed between percent hatchability and concentration of the extract. Bodyweight weight ($p>0.05$), Testis weight ($p<0.05$) decreased with increased concentration of *L. alopecuroides*. However, the mean gonadosomatic index (0.79±0.02), the milt volume (3.45±0.64) were not affected. Sperm concentration and sperm output per gram of testicular weight were significantly ($p<0.01$) reduced by increasing concentrations of *L. alopecuroides* extracts. Since these two indicators of spermatogenic efficiency were reduced, it was concluded that *L. alopecuroides* is not only piscicidal but also exhibits anti-fertility effects in *Clarias gariepinus* with a potential to reduce fertilizing ability, hatchability and deplete the population of Clarias gariepinus in the wild.

Key words: Anti-fertility, sperm count, plant leaf extract, motility, output

INTRODUCTION

Plants and their products have been used as natural alternatives for treatment and management of various diseases and as pesticides (Maikai *et al*., 2008) molluscicides (Azare *et al*., 2007) and piscicides (Singh and Singh, 2002). Several commonly used plants have been reported to adversely affect male reproductive functions in wildlife and humans. The effects observed with such plants have been attributed to their ability to impact adversely on spermatogenesis and steroidogenesis (D'Cruz *et al*., 2010). Other plant extracts reported to alter the morphology of the sperm or to diminish its motility include *Abrus precatorious* (Adedapo *et al*., 2007) *Cola nitida rubra* (Adisa *et al*., 2010), *Croton zambesicus* (Ofusori *et al*., 2010), *Kigelia africana* (Adeparusi *et al*., 2010), pomegranate juice (Turk *et al*., 2008).

Plants from different families have been applied for catching fish all over the world. The toxic parts of the plants employed as fish poisons include the roots, seeds, fruits, barks or leaves. These botanical extracts used as piscicides in fisheries management are considered advantageous when viewed against the backdrop of using persistent chemicals that are not eco- friendly. Frequent applications of high concentrations of these ichthyocides in ponds and flowing rivers may have adverse effects not only on fish species but also on other aquatic fauna. *Lepidagathis alopecuroides* (Vahl) is a tropical shrub belonging to the family Acanthaceae and commonly found in the coastal countries of West Africa. In Rivers State the leaves are used for treatment of sores and crude extract administered in the local gin as a cure for stomach disorders. The leaves are used to immobilize or kill fish for human consumption in many communities of Cross River and Rivers State of Nigeria. In Rivers State, it is used for quick kill of hardy fishes like mudskippers (Obomanu *et al*., 2007) and the Clariids. The phytochemistry of the plant revealed that it contains flavonoids, saponins, alkaloids and rotenones (Obomanu *et al*., 2006). *Clarias gariepinus* is not only the most predominant fish species raised in aquaculture in Nigeria, but has also served as an experimental model of aquatic vertebrate for over two decades (Cavaco *et al*., 2001).

Despite the apparent toxicity of *Lepidagathis alopecuroides* extract (Gabriel and Okey, 2009; Gabriel *et al*., 2010), there is a paucity of information on the effects of sub-lethal concentrations of its extract on spermatogenesis and other aspects of the reproductive physiology of teleosts in general and specifically, on the Nigerian fresh water Catfish *Clarias gariepinus*. The catfish predominates the aquaculture activities in Nigeria and successful culture of this fish species requires a good understanding of its reproductive physiology, sperm production, motility, viability and morphology. This is because knowledge of the mechanism of action of frequently used ichthyocides with respect to long term exposure to sub-lethal concentrations on fertility, fecundity and fertilizing ability is crucial to fish population dynamics in the natural aquatic environment. There is no previous investigation, to our knowledge, on the impact of *Lepidagathis alopecuroides* on the reproductive system of any fish. This study was thus conceived to critically study the effect of sub-lethal concentrations of *L. alopecuroides,* used indiscriminately and frequently in the wild, on total sperm quality, fertility and hatchability in *Clarias gariepinus*.

MATERIALS AND METHODS

Experimental location: This investigation was carried out in the Postgraduate Laboratory of the Department of Applied and Environmental Biology, Rivers state University of Science and Technology, Port Harcourt (Coordinates: 4°48'14"N 6°59'12"E) from May 2010 to July 2010.

Experimental fish management: Fifteen gravid male *Clarias gariepinus* (1845-1706 g, mean total length 65±2.50 cm) and 14 months old purchased from Tonebo Farms Ltd., in Rumuibekwe, Port Harcourt and transported in aerated aquaria to the Postgraduate Laboratory were acclimated individually in 40 L circular aquaria (water temperature 25°C, pH 6.8) and fed standard breeders pelleted diet once a day for 7 days, while the aquaria water was renewed daily. The experiments were conducted according to the institutional animal care protocols at the Rivers State University of Science and Technology Nigeria and followed approved guidelines for the ethical treatment of laboratory animals. Feed was withheld 24 h prior to the commencement of the experiment.

Experimental setup: Stock solution of 4 g L^{-1} (4000 ppm) was made from air-dried leaves of *Lepidagathis alopecuroides* ground into fine powder with Moulinex blender and soaked in distilled

water for 2 h before use. From this, four sub-lethal concentrations previously established (Gabriel *et al*., 2010) were prepared at 0.00 ppm as control, 0.50, 0.75, 1.00 and 1.25 ppm in triplicates in 40 L of water.

Experimental fish were introduced individually into the aquaria and covered securely with net to prevent the fish from escaping. Each aquaria was washed daily to remove waste matter and leftover feed and fresh water added to both control and test aquaria.

Milt collection: Qualitative and quantitative parameters of the milt were determined at the end of the experiment from 15 males, 3 from each treatment group were sacrificed, dissected and the testes removed intact, weighed and placed in a dried teacup. Thereafter, the testes were rapidly cut into small pieces using a scissor and finally the milt was pressed out with a teaspoon.

Milt qualitative and quantitative analysis: Milt color and consistency were assessed by direct visual examination and abnormal change in color or deposits were recorded.

Milt volume: The volume of milt per fish was measured using 5 mL hypodermic syringes and recorded to the nearest 0.01 mL.

Sperm concentration: Sperm concentration was determined using Neubaer haemocytometer after dilution with deionised distilled water at 1:2000 v/v ratios. Total number of cells counted mL^{-1} (magnification x400) was expressed in billion ($\times 10^9$).

Sperm output: The total number of sperm cells in the volume of milt obtained (output) was the product of the sperm concentration per mL and the milt volume expressed in billions.

Milt pH: Milt pH was measured immediately after collection by transferring a small drop of it with 20λ Eppendorf micropipette on a piece of pH indicator paper. The pH value was read by comparison with standardized pH values.

Sperm motility: Sperm motility was assessed in fresh activated (milt to which 20 μL of the activating solution (deionised distilled water) had been added. A drop of the milt was examined (×400) under a cover slip with a light microscope. Only progressive straight line movement across the field of vision was noted and expressed as percentage spermatozoa motility.

Live/dead ratio: The proportion of live to dead spermatozoa in each volume of milt was estimated from milt films stained with Nigrosin-eosin vital stain freshly prepared. The percentage of spermatozoa considered as dead (by partial or total staining of the cytoplasm) was calculated from 200 cells per slide under oil immersion. The same slides were used to evaluate the morphology and to determine the incidence of abnormal spermatozoa.

Induction of ovulation, fertilization and incubation: Ovulation was induced in the female with Ovaprim™ administered intramuscularly at o.50 mL kg^{-1} b.wt. Stripping took place 10 h after injection at a mean temperature of 28±2°C. The ovulated eggs oozed out on slight pressure by thumb onto the plastic bowl. About 600 eggs (1 g) were put in each of the five separate plastic containers and a drop of milt added and mixed with plastic spoon. Stripping, fertilization and incubation were accomplished in 3 min.

Fertilization was determined when the eggs reached the 4-8 celled stage of embryonic development. For calculating percentage fertilization of each replicate a sample was taken on petri-dish containing water and the number of fertilized and unfertilized eggs was counted under a microscope.

Percent hatchability was calculated as:

$$\frac{\text{Total No. of hatchlings}}{\text{Total No. of eggs incubated}} \times 100$$

Statistical analysis: The data obtained from this study were subjected to analysis of variance (ANOVA) for the assessment of effect of varying concentration on sperm quality and hatchability, correlation and regression analysis were also carried out between extract concentration and sperm quality indices as well as with hatchability according to Steel *et al.* (1997).

RESULTS

A trend toward reduction in body weight and testes weight was observed with increasing concentrations of *Lepidagathis alopecuroides* extract. Figure 1a shows a non-significant linear relationship of body weight to increase in concentration characterized by the equation Y= -17.578x + 1813.3, a non-significant ($p>0.05$) coefficient of determination (R^2) =0.1578 and a non significant ($p>0.05$) negative correlation coefficient r = -0.397.

Similar trend was observed between testes weight and extract concentration (Fig. 1b). A regression analysis (Y = -2.3768x+14.19, R^2 = 0.3785, r = -0.61) showed significant ($p<0.05$) negative correlation with the concentration. Sperm concentration obtained in the exposed fish were significantly ($p<0.01$) lower than the control and this reduction was concentration dependent as reflected in the linear regression equation Y = -10.24 x +14.25, R^2 = 0.976 (Fig. 1c) and significant ($p<0.01$) negative correlation coefficient (r = -0.988).

Varying concentrations of *Lepidagathis alopecuroides* also reduced significantly ($p<0.01$, R^2 = 0.968, r = -0.983, $p<0.001$) the sperm output (Fig. 1d) and inhibited hatchability of incubated eggs of *Clarias gariepinus*. The concentration dependent reduction was characterized by the regression equation Y = -24.27x+65.18, R^2 = 0.938, r = -0.9685, $p<0.001$) (Fig. 1e).

A linear decrease in the percentage of sperm motility was observed with various concentrations (0.50-1.25 ppm) of the leaf extract, with motility falling to almost absolute zero within 30 sec of exposure to 1.25 ppm (Fig. 1f).

A light microscopic examination of the morphology of the sperm cells revealed an increasing number of dead cells viewed in Nigrosin-eosin vital stain film.

The weight of vital organs (Heart, Spleen and Kidney) remained unaffected while the weight of the liver tended to increase non-significantly ($p>0.05$) with increase in the extract concentration (Table 1).

Despite the non significant effect of the extract on the gonadosomatic index, milt volume, total length of fish, other derivative parameters were highly significantly ($p<0.01$) reduced. Sperm concentration per gram of the testicular weight reduced from 1.01±0.06 $x10^9$ in the control to 0.22±0.08 $x10^9$ in 1.25 ppm of the extract. The sperm output per gram of testicular weight (0.69±0.05 $x10^9$) in 1.25 ppm was significantly ($p<0.01$) lower than (3.88±0.18 $x10^9$) the control (Table 2).

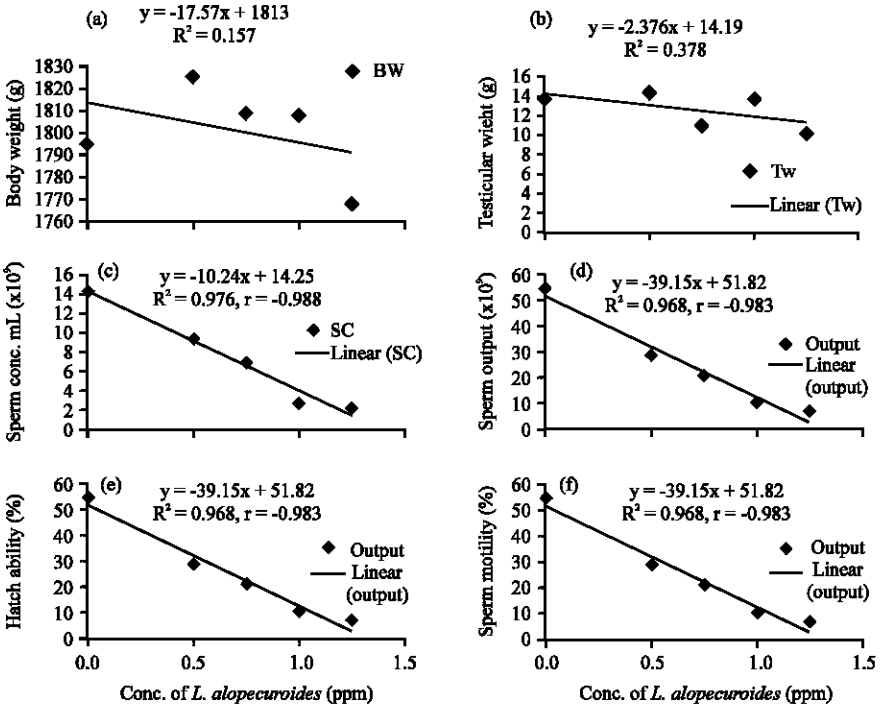

Fig. 1: (a-f) Effect of sub-lethal concentrations of *L. alopecuroides* aqueous extract on sperm quality indices in *Clarias gariepinus* broodstock

Table 1: The organosomatic indices of *Clarias gariepinus* exposed to sub-lethal concentrations of *Lepidagathis alopecuroides*

	Organosomatic indices (%)			
Conc. of *L. alopecuroides* Extract (ppm)	Heart	Liver	Kidney	Spleen
0.00	0.10±0.02	0.81±0.02	0.56±0.03	0.08±0.01
0.50	0.13±0.02	0.77±0.23	0.54±0.02	0.07±0.01
0.75	0.13±0.01	0.88±0.27	0.46±0.03	0.15±0.02
1.00	0.14±0.01	0.97±0.31	0.53±0.02	0.10±0.01
1.25	0.12±0.01	0.95±0.22	0.47±0.02	0.06±0.01

Values are presented as Mean±SEM. SEM: Standard error of mean

Table 2: Effect of sub-lethal concentrations of *Lepidagathis alopecuroides* on milt volume, sperm production efficiency and spermatozoa morphology of *Clarias gariepinus* Broodstock

	Concentration of *Lepidagathis alopecuroides* (ppm)				
Parameters	Control	0.50	0.75	1.00	1.25
Body weight (g)	2415.22±1.01	2054.12±5.85	1808.55±8.22	1808.68±8.65	1768.45±6.21
Total length (cm)	60.65±2.1	61.02±1.10	60.75±1.02	63.29±1.25	63.22±1.01
Gonadosomatic index (%)	0.81±0.04	0.80±0.01	0.79±0.03	0.76±0.03	0.76±0.02
Milt volume (mL)	3.85±0.71	3.10±0.14	3.05±0.71	3.75±0.32	3.20±0.45
Sperm concentration g^{-1} of testis ($x10^9$)	1.01±0.06	0.68±0.01*	0.62±0.04**	0.20±0.05**	0.22±0.08**
Sperm output g^{-1} testis weight ($x10^9$)	3.88±0.18	2.12±0.15*	1.91±0.17**	0.75±0.06**	0.69±0.05**
Live sperm cells (%)	92.64±1.5	60.28±2.32*	43.34±5.42**	22.86±4.87**	10.25±5.14**

Values are Means±SEM. SEM: Standard error of mean. *Value are significant ($p<0.05$). ** Values are significant ($p<0.01$)

DISCUSSION

Sub-lethal concentrations of *Lepidagathis alopecuroides* aqueous extract induced reduction in the bodyweight and testicular weights of *Clarias gariepinus* exposed to it for 21 days. Changes in bodyweight and gonad weight are indicators of toxicity and weight loss especially of the gonads are characteristic of anti-fertility agents. Gonadal weight loss directly impacts on sperm concentration since heavier testes produce and store higher quantities of spermatozoa. This result agrees with earlier reports (Orlu and Egbunike, 2009, 2010; Mishra and Singh, 2009). The gonadosomatic indices of the treated group did not differ significantly from those of the control group. Significant ($p<0.01$) linear decrease in the percentage of sperm motility was observed with various concentrations of the extract, as the motility reduced from 82% in the control to less than 10% in 1.25 ppm in about 25 sec. This result is in agreement with Mathur *et al.* (2010) who reported significant reduction in motility, sperm count and fertility in albino rats treated with *Tecoma stans*. Other works with similar results include Rao (1990), who reported anti-fertility effect of aqueous seed extract of *Abrus precatorius* in rats without alterations in organ or body weight. Also, in support is the linear decrease in the percentage of sperm motility with concentrations of neem leaf extract (Awasthy, 2001) and *Zingiber officinale* (Jorsaraei *et al.*, 2008). Rotenones are the major components of piscicides/ichthyocides in *Lepidagathis alopecuroides* extract and reduction in sperm motility is indicative of reduced energy harvested from aerobic metabolism for spermatozoa motility. Rotenone is a highly specific metabolic poison that affects cellular aerobic respiration, blocking mitochondrial electron transport by inhibiting NADH ubiquinone reductase (Ling, 2002). This renders the extract spermicidal and cytotoxic to sperm cells. A critical observation of the sperm cells in Nigrosin-eosin vital stain film also revealed that the sperm cell membranes were damaged. This result is quite similar to the reaction induced by *Cestrum parqui* reported to be spermicidal at 250 μg mL^{-1} (Souad *et al.*, 2007). However, these findings are only in partial agreement with Adeparusi *et al.* (2010), Adisa *et al.* (2010) and Etuk and Mohammad (2009), all reported lack of significant changes on bodyweight and testis weight, but, increased sperm motility with administration of *Kigelia africana*, *Cola nitida* and *Lophira lanceolata*, respectively.

Sperm concentration and output declined in a concentration dependent manner typified by significant ($p<0.01$) coefficient of determination $R^2 = 0.976$ and 0.968, respectively as well as significant negative correlation coefficients ($r = -0.988$, $p<0.01$) and ($r = -0.983$, $p<0.01$). Over 30-50% reduction of sperm concentration and output were recorded at 0.50 and 0.75 ppm, respectively. From 1.00-1.25 ppm sperm concentration had declined below 20%. Decrease in sperm concentration and output might not be unconnected to the cytostatic and apoptotic effect exhibited by *Lepidagathis alopecuroides* which induced mitotic inhibition, reduction in spermatogenic efficiency and germ cell loss during spermatogenesis (Orlu and Gabriel, 2011). This effect is characteristic of plant materials containing potentially anti-fertility agents. The findings support earlier reports (Ogbuewu *et al.*, 2009) on mild depressive effect of *Azadirachta indica* on spermatogenesis in buck rabbits, inhibition of spermatogenesis by decrease in testis, epididymal and accessory sex organ weights of albino rats by *Dendrophthoe falcate* (Gupta and Kachhawa, 2007) and spermatogenic arrest in albino rats by *Juniperus phoenica* L. (Shkukani *et al.*, 2007).

Gross reduction in hatchability observed in this study is attributed to reduction in motility and viability as well as increase in the percentage of dead sperm cells, reduction in sperm concentration and sperm output. Thus, *L. alopecuroides* reduces the concentration of sperm produced per gram weight of the testicular parenchyma and the total sperm output per gram of testicular weight as a direct effect of early apoptosis of germ cells and general inhibition of spermatogenesis. Based on

these observations it is concluded that *Lepidagathis alopecuroides* is not only toxic but possesses anti-fertility properties at sub-lethal concentrations. Its continued use as an ichthyocide would result in reduction of reproduction potential and depletion of the population of *Clarias gariepinus* in the wild. Further research is needed to evaluate the effect of this biocide on aquatic invertebrates.

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