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Effect of Sublethal Concentrations of Aqueous Extract of *Lepidagathis alopecuroides* on Spermatogenesis in the Fresh Water Catfish *Clarias gariepinus*

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ABSTRACT

The effect on spermatogenesis of sublethal concentrations of *Lepidagathis alopecuroides* indiscriminately used for capturing fish was evaluated on fresh water catfish *Clarias gariepinus* using standard Hematoxylin-eosin histological techniques. Matured testes from 15 adult fish exposed to sublethal concentrations of *L. alopecuroides* for 21 days were processed by Hematoxylin-eosin histological technique and the effect of the leaf extract on testicular somatic and germ cells of the seminiferous epithelium assessed by light microscopy. Spermatogenesis occurred in cysts and proliferating Spermatogonia B were surrounded by extensions of Sertoli cells. The last mitotic division of the B spermatogonia produced the Preleptotene primary spermatocytes which differentiated in clones through Leptotene, Zygotene, Pachytene to Diplotene, secondary spermatocytes, round spermatids and spermatozoa before spermiation. This sequence of events demonstrates the presence of recognizable cycle of spermiation in *Clarias gariepinus*. Changes in the germinal epithelium during spermatogenesis showed that sublethal concentrations of this extract induced mitotic inhibition in proliferating spermatogonia populations, inhibited the last mitotic division in B-type spermatogonia and reduced the number of spermatogonia population entering meiotic division. This led to germ cell loss and reduction of primary spermatocytes population, consequently, a decrease in sperm production potential. Exposure of this fish species to sublethal concentrations of *Lepidagathis alopecuroides* even for short periods induced testis structure alterations and functions including inhibition of spermatogonia A renewal after spermiation. It is thus concluded that freshwater catfish *Clarias gariepinus* exposed to sublethal concentrations of *L. alopecuroides* would exhibit reduction in sperm production in the subsequent reproduction cycles.

Key words: *Lepidagathis alopecuroides*, germinal epithelium, catfish, mitotic inhibition, spermatogonial proliferation

INTRODUCTION

Spermatogenesis is the process by which diploid primordial spermatogonia divide mitotically to produce primary spermatocytes which undergo two meiotic divisions and massive cellular differentiation to produce haploid spermatozoa. This process is highly organized, coordinated and

regulated by the presence of Sertoli and Leydig cells in mammals (Sharpe *et al.*, 2003) birds (Orlu and Egbunike, 2009) and teleost (Batlouni *et al.*, 2006; Bizzotto and Godinho, 2007; Schulz *et al.*, 2005; Billard, 1986). However, in contrast to avian and mammalian spermatogenesis which take place in the seminiferous tubules with definite and identifiable cellular associations known as stages of the cycle of the seminiferous epithelium, teleost spermatogenesis generally occur in cysts formed from the association of Sertoli cells with spermatogonia A within the seminiferous tubules in which single germ cell clones can be identified (Schulz *et al.*, 2005; Alvarenga and de Franca, 2009). Recent studies on teleost spermatogenesis include works of Billard (1986) and on Atlantic cod (Almeida *et al.*, 2008), Nile Tilapia (Alvarenga and de Franca, 2009), African catfish (Schulz *et al.*, 2005), *Heterobranchus longifilis* (Oteme *et al.*, 1996), Protogynous fish (Koulish *et al.*, 2002), common snook (Grier and Taylor, 1998), Gilthead seabream Sparus aurata (Chaves-Pozo *et al.*, 2005),Cardinal fish (Fishelson *et al.*, 2006). In teleost, just like in mammals and birds (De Rooij, 2001) the number of mitotic divisions of spermatogonia preceding meiosis is species-specific and may reach peaks of 5-14 prior to differentiation into primary spermatocytes (Ando *et al.*, 2000; Vilela *et al.*, 2003; Fishelson *et al.*, 2006; De Rooij and Russell, 2000). The number of spermatogonial mitoses reflect the Sertoli cell efficiency and the sperm production capacity in any species.

The use of icthyotoxic plants crushed and introduced into slow flowing water to stun and kill fish is a usual practice in coastal communities in Nigeria (Ekanem *et al.*, 2003). Several species of these plant extracts have been reported to be toxic and piscicidal not only to targeted fish but also to other aquatic fauna, inducing biochemical changes (Tiwari and Singh, 2003; Singh and Singh, 2002), hematological and histopathological changes (Fafioye *et al.*, 2004; Olaifa *et al.*, 2004). Among the indigenous botanical toxicants is *Lepidagathis alopecuroides* used by fishermen in Rivers and Cross River States of Nigeria to immobilize and kill mudskippers *Periophthalmus papillio* (Obomanu *et al.*, 2007) as well as tilapias and catfish (Ekanem *et al.*, 2003). The effect of the leaf extracts of *L. alopecuroides* on various aspects of the physiology of Clariid fish has been documented and include, acute toxicity and mortality in Heterobranchus fingerlings (Gabriel and Okey, 2009), inhibitory effect on selected enzymes activity (Gabriel *et al.*, 2009a), changes in hematology and plasma enzymes of *Clarias gariepinus* (Gabriel *et al.*, 2009b). Frequent applications of these piscicides in aquatic environment may produce long term adverse effects on the reproductive potentials of fish and other aquatic fauna.

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 two decades (Cavaco *et al.*, 2001). Despite the indiscriminate use of *L. alopecuroides* and other piscicides in the wild, there is a paucity of information on the sublethal concentrations of this plant extract on spermatogenesis and general reproductive physiology of the catfish. This fish species forms the bulk of fish protein in Nigeria both from the wild and from aquaculture farms and its ability to reproduce in captivity increases its potential for aquaculture. For successful and effective development of economically viable breeding program of this species, a sound knowledge of its reproductive physiology and spermatogenesis is invaluable. Valuable information for aquaculture and species management can be obtained by evaluating and manipulating environmental factors on reproduction in natural conditions as well as from experimental models (Schulz *et al.*, 2005; Miura and Kuwahara, 2006). Testicular histology and morphometry of such models provides sensitive indicators for determining the efficiency of spermatogenesis as well as the reproductive health of fish, especially when exposed to toxicants in their aquatic environment.

The aim of this study was, thus, to critically assess the effect of sublethal concentrations of *Lepidagathis alopecuroides* on the male of *Clarias gariepinus* with particular reference to its spermatogenesis, in an experimental setup in order to evaluate the impact of this toxicant in the streams and fresh water ponds. We report here the result on the spermatogenesis of this species of *L. alopecuroides*. This evaluation is critical in assessing the reproductive stability of the fish in the aquatic environment, particularly under toxicant stress.

MATERIALS AND METHODS

The experiment was carried out in the Postgraduate Laboratory of the Department of Applied and Environmental Biology, Rivers State University of Science and Technology, Port Harcourt, Rivers State, Nigeria between May-July, 2010. Fifteen gravid male *Clarias gariepinus* weighing between 1344-1808 g (mean total length 65±2.50 cm) and 14 months old were purchased from Tonebo Farms Ltd. in Rumuibekwe, Port Harcourt. They were transported in aerated aquaria to the Postgraduate Lab.

Fish management: The fish 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. Feed was withheld 24 h prior to the commencement of the experiment.

Known weight of air-dried leaves of *Lepidagathis alopecuroides* was ground into fine powder with Moulinex blender and stored in an airtight bottle. Stock solution of 4 g L^{-1} (4000 ppm) was constituted from which four sublethal concentrations previously established (Gabriel *et al*., 2010) were prepared at 0.00 ppm as control, 0.25, 0.50, 0.75 and 1.00 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. Three gravid male fish were used for control and 3 each for the treatment groups. Each aquaria was washed daily to remove waste matter and leftover feed and fresh water added to both control and test aquaria. All fish were treated according to the standard rules for laboratory animals throughout the duration of the experiment.

Histological methods: The fish were killed with a blow on the head after which the testes were removed by dissection and weighed for the calculation of the gonadosomatic index (GSI) as:

$$\frac{\text{Testes weight}}{\text{Body weight}} \times 100$$

Samples of the testes were fixed in 10 times their volume in Bouins fixative solution for 24 h, washed in 70% ethyl alcohol and thereafter dehydrated in a series of increasing concentrations of ethanol, cleared in three changes of chloroform and embedded in paraffin wax according to conventional techniques (Orlu and Egbunike, 2010). Histological sections 5 μm thick were stained according to the Periodic-Acid-Schiff technique and counter-stained with haematoxylin-eosin (Orlu and Egbunike, 2009). The slides were observed at 800x magnification with Tension Binocular microscope and photomicrographs taken with Samsung ES25 4X Zoom Lens digital Camera and downloaded into a computer.

Testicular morphometry: Testicular sections with cysts showing different stages of spermatogenic cells were analyzed for morphology of the testicular cells. Primary spermatocytes were identified by their nuclear diameter and the distribution of marginal heterochromatin (Schulz *et al.*, 2005). The spermatids were identified by a combined method of nuclear morphology, diameter and the acrosome development as revealed by haematoxylin- eosin sections (Orlu and Egbunike, 2009).

Cellular populations: Spermatogenic cysts and cellular populations were determined with 400-point graticule according to Berndtson (1977). Based on the proportion of germ cells, the following indices were estimated:

Mitotic index: This was estimated as the ratio of the primary spermatocytes at Preleptotene/Leptotene stage to Spermatogonia A. Meiotic Index was determined as the ratio of round spermatids per primary spermatocytes at Pachytene stage while spermatogonial efficiency was estimated as the number of round spermatids produced by each spermatogonia A.

Statistical analysis: Data were subjected to analysis of variance and Students' t-test according to Steel and Torrie (1996).

RESULTS

Macromorphological examination of the testes revealed paired elongated lobes milky white in color (Fig. 1a) and histological analysis of the testicular cross section showed seminiferous epithelium with cysts of different sizes at various stages of cellular division, differentiation and maturation intertubular space, interstitial cells and Leydig cells (Fig. 1b).

Spermatogonial proliferation, differentiation and maturation are shown in Fig. 2(a-*l*). Spermatogenesis occurred in cysts associated with Sertoli cells in the seminiferous epithelium of *Clarias gariepinus*. All stages of germ cells from spermatogonia A to matured spermatozoa and spermiation were observed and hereby presented in the control group (0.00 ppm) Fig. 2a-*l*.

There were qualitative (Fig. 3a-f) and quantitative (Table 1) changes in the germ cell populations in seminiferous epithelium of *Clarias gariepinus* kept in varying aqueous concentrations of *L. alopecuroides*. At 0.25 ppm the extract lowered significantly ($p<0.05$) mitotic proliferation with fewer populations of the mitotic cells seen (Fig. 3a). At 0.50 ppm (Fig. 3b) further significant ($p<0.01$) reduction in mitotic activity was observed with general decrease in the number of B-type spermatogonia committed to meiotic division at Preleptotene stage. With concentrations of 0.75 and 1.00 ppm (Fig. 3c-d) almost total mitotic inhibition was observed as accumulation of B-type spermatogonia without entry into meiotic division. Mitotic index reduction was significant ($p<0.01$) and concentration dependent, from 62.0 ± 2.50 to 12.40 ± 2.27%, meiotic index 3.67 ± 0.33 to 0.55 ± 0.08, spermatogenesis efficiency from 340.0 ± 25.96 to 51.0 ± 5.20 (Table 1).

Where division, differentiation, maturation and spermiation had occurred, A-type spermatogonia were observed scattered in the lumen along with spermatozoa with no evidence of any mitotic activity (Fig. 3f).

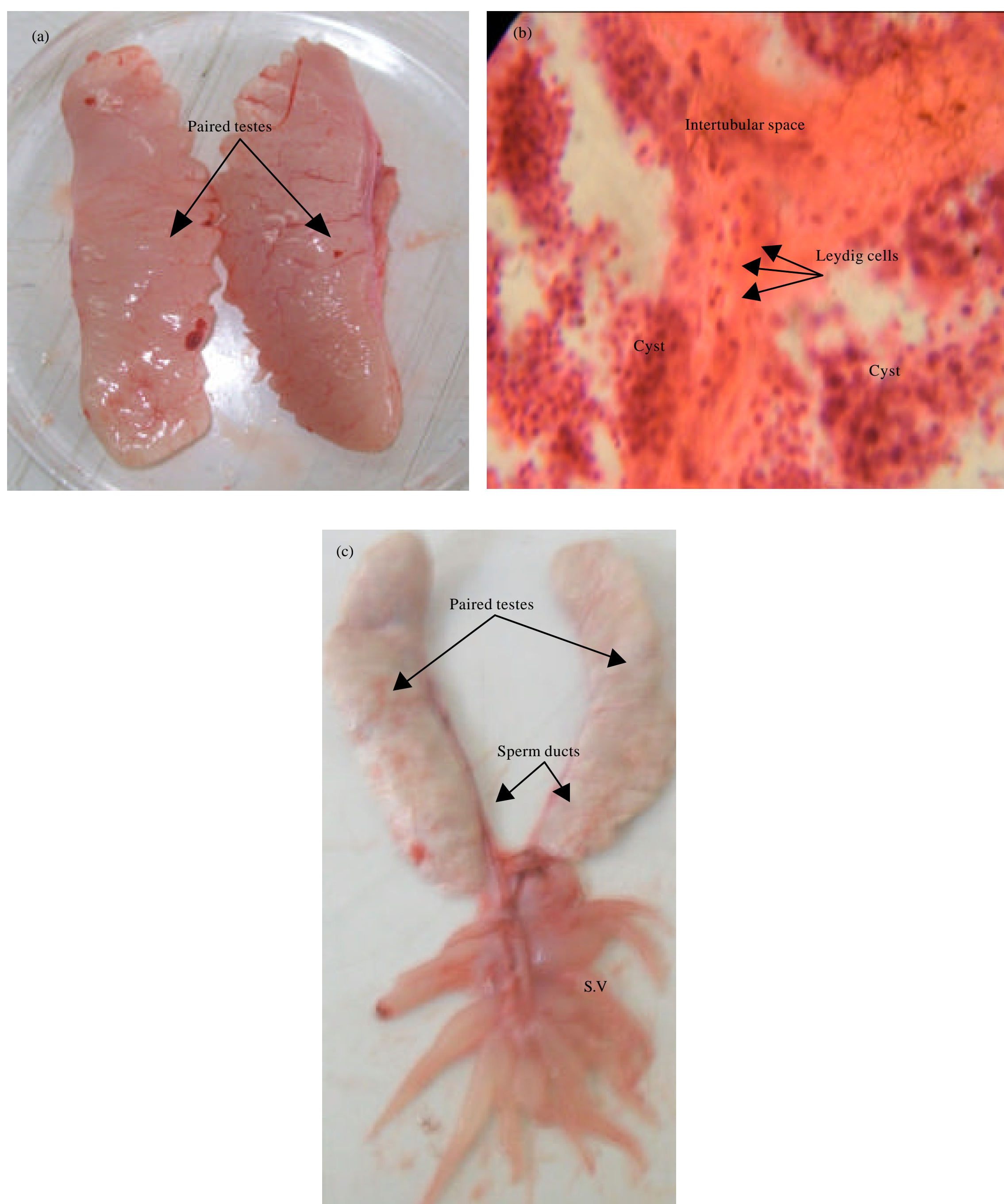

Fig. 1: (a) Paired Lobular testes of *Clarias gariepinus*, (b) shows a transverse section of the testis of *Clarias gariepinus* with Leydig cells, inter-tubular space and spermatogenic cysts containing germ cells at different stages of division, differentiation and maturation and (c) intact paired testes of *Clarias gariepinus* showing Seminal vesicles and sperm ducts

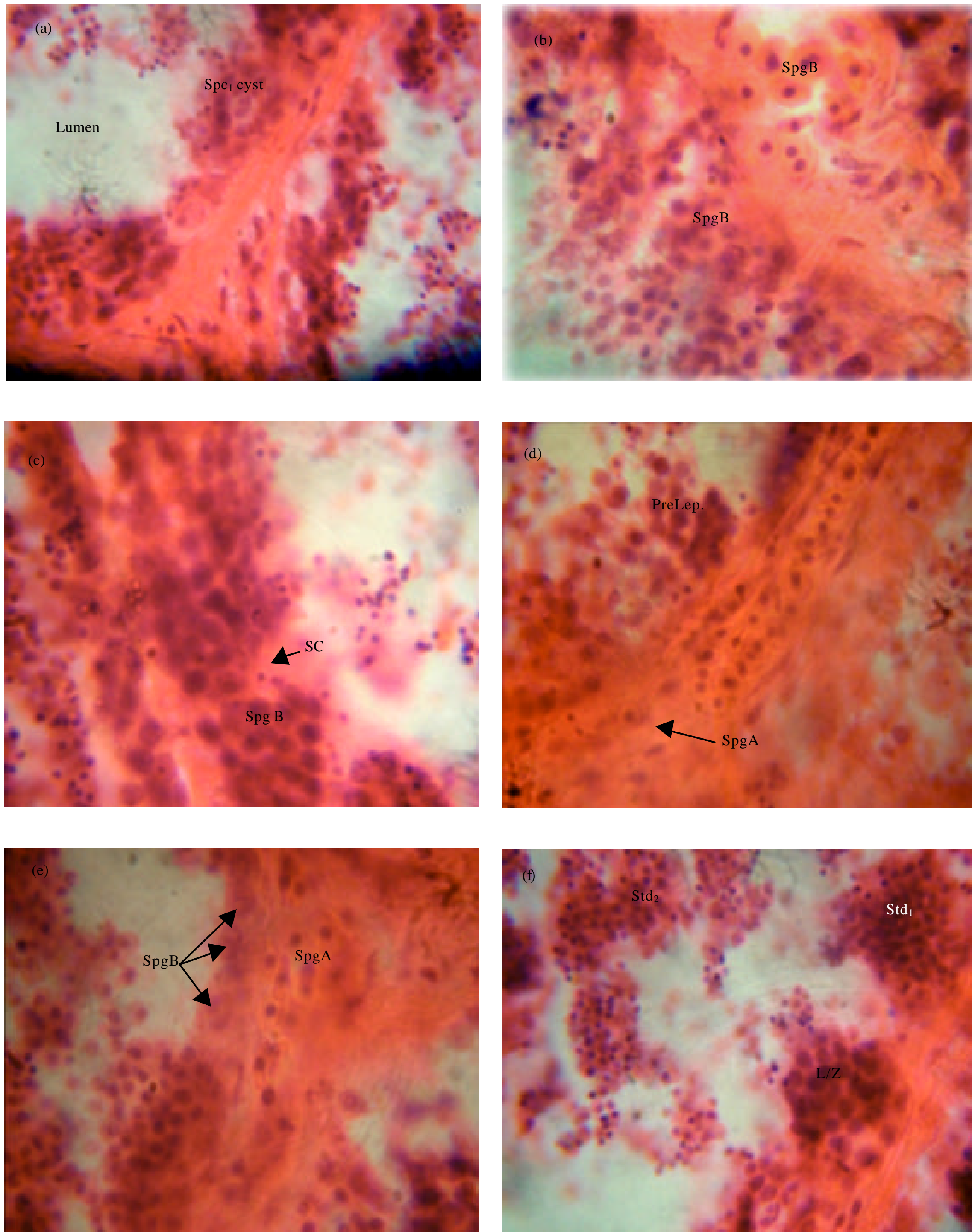

Fig. 2: Continue

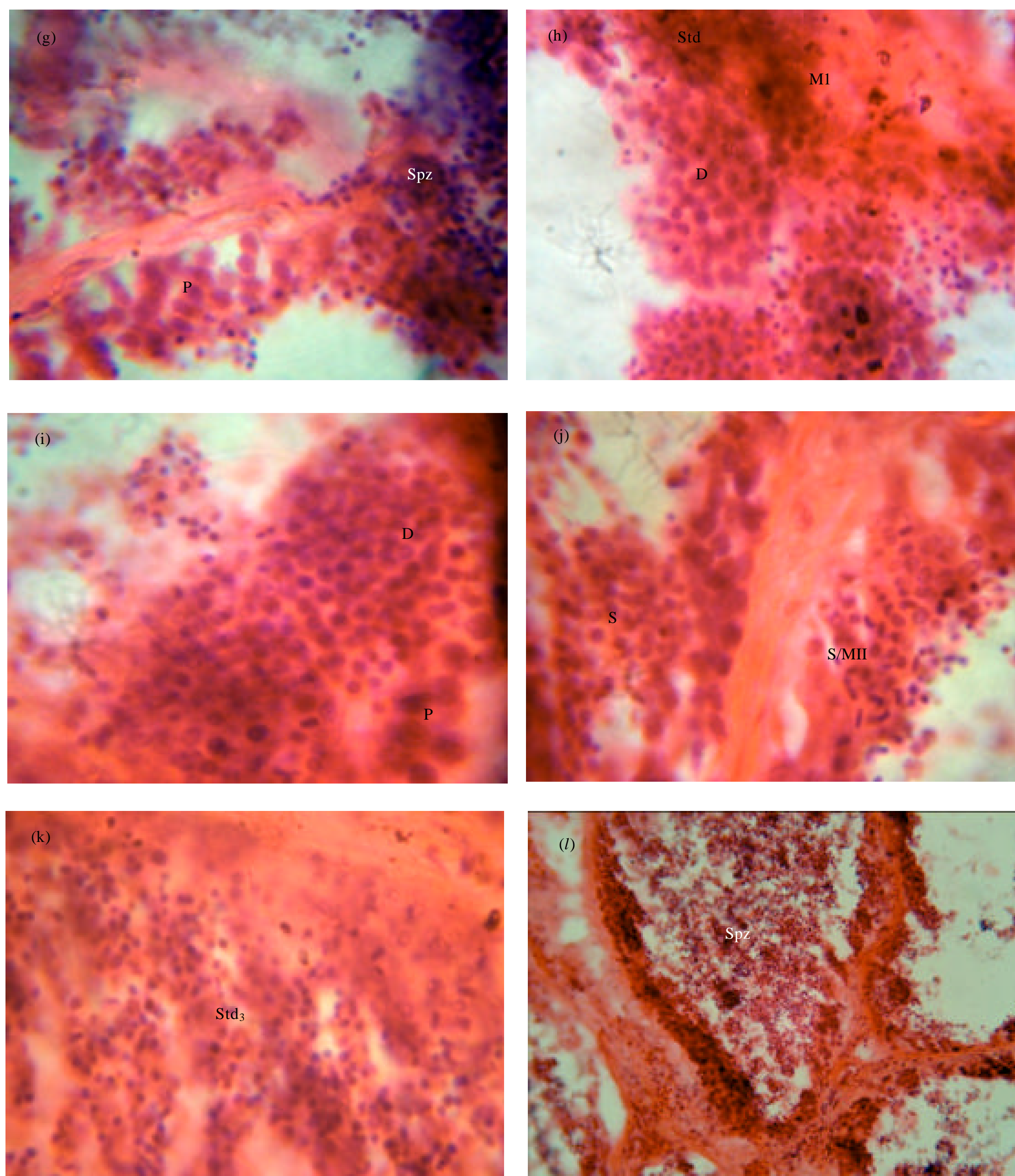

Fig. 2: The various germ cell cysts in *Clarias gariepinus* labeled a-*l*. (a) Cross-section with Lumen and cysts of Primary spermatocytes at Pachytene, (b) spermatogonia type B, (c) another generation of type B spermatogonia, (d) spermatogonia type A and Preleptotene spermatogenic cyst, (e) spermatogonia types A and B, (f) leptotene/zygotene primary spermatocytes with round spermatids at initial (std_1) and intermediate (std_2) stages of maturation, (g) pachytene cyst with matured spermatids, (h) diplotene, meiotic first maturation division (M1) and spermatids (std), (i) diplotene, (j) secondary spermatocytes undergoing second maturation division (M11), (k) spermatozoa and (*l*) A cyst undergoing spermiation

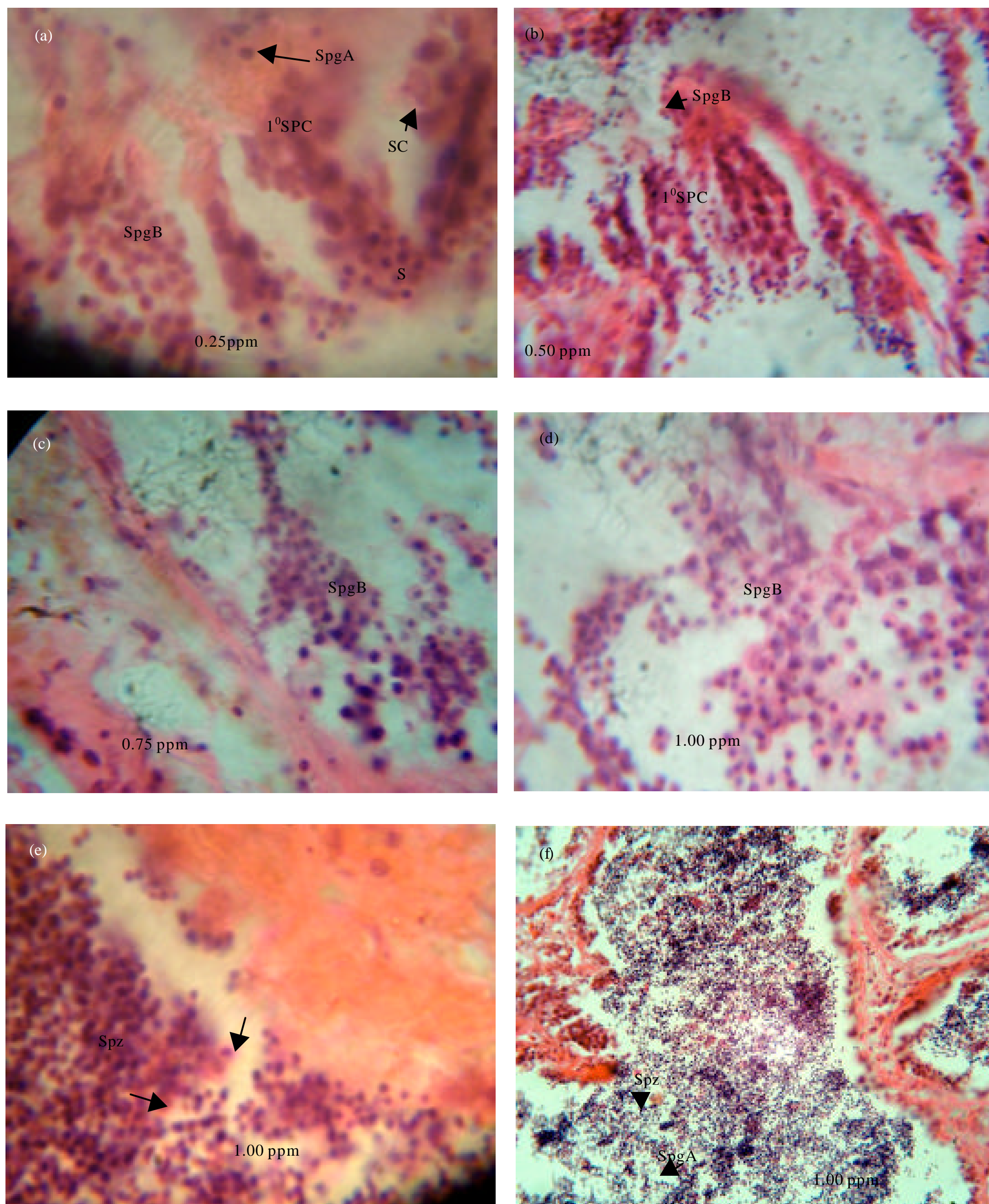

Fig. 3: (a-f) The effect of sublethal concentrations of *Lepidagathis alopecuroides* aqueous extract on spermatogenesis and germ cell regeneration in *Clarias gariepinus*. At concentrations 0.25 ppm. (a) are spermatogonia B, primary spermatocytes (1^0SPC), sertoli cell (SC) and secondary spermatocytes (S),0.50 ppm, (b) B-type spermatogonia and primary spermatocytes (1^0SPC), 0.75 ppm, (c) shows hyperchromatic B-type spermatogonia (SpgB) 1.00 ppm, (d) shows accumulated B-type spermatogonia and inhibition of germ cell regeneration after spermiation and (e-f) Arrows indicate scattered spermatogonia A in the lumen

Table 1: Effect of sub-lethal concentrations of *Lepidagathis alopecuroides* on germ cell numbers, mitotic indices and spermatogenic yield in *Clarias gariepinus*

	Concentrations of *Lepidagathis alopecuroides* (ppm)				
Spermatogenic elements	0.00	0.25	0.50	0.75	1.00
Spg A	1.00	1.00	1.00	1.00	1.00
Spg $B_1.B_6$	62.00±2.0	61.54±2.46	62.00±2.0	61.00±3.0	60.00±4.0
Prelep/L	122.00±6.0	81.74±3.67	49.86±3.18*	23.18±2.63**	25.62±4.21**
P	124.00±4.0	83.08±4.82	50.63±3.54*	24.80±4.66**	21.08±2.86**
Stds	491.00±16.0	328.97±8.10	220.95±5.62*	98.20±4.22**	73.65±5.41**
Mitotic index[1]	62.00±2.50	41.54±3.52	30.38±2.63*	12.40±2.27**	9.30±1.60**
Meiotic index[2]	3.67±0.33	2.46±0.21	1.80±0.11*	0.73±0.12**	0.55±0.08**
Spermatogenic yield[3]	340.00±25.96	227.80±20.12	166.40±10.26*	68.00±8.73**	51.00±5.20**

SpgA: Spermatogonia A, SpgB$_1$-B$_6$: Spermatogonia B type six generations, Prelep: Preleptotene primary spermatocytes, P: Pachytene primary spermatocytes, Stds: Round spermatids. [1]Mitotic index was estimated as the ratio of primary spermatocytes at Preleptotene /Leptotene to spermatogonia type A. [2]Meiotic index was the ratio of round spermatids to that of primary spermatocytes at Pachytene [3]Spermatogenic yield-was determined as the proportion of round spermatids produced per spermatogonia A. *Values significant ($p<0.05$), **Values significant ($p<0.01$)

DISCUSSION

The testes of gravid broodstock of *Clarias gariepinus* demonstrated gonadosomatic index (GSI) ranging from 0.70-1.00% (Mean= 0.80±0.20). The testes belong to the lobular type (Fig. 1a and c) previously reported (Billard, 1986), spermatogenesis occurred in cysts observed to be asynchronously developing at various stages of proliferation, differentiation and maturation (1b). Ostentatious seminal vesicles observed did not act as spermatozoa storage organs nor provide secretion toward sperm maturation, since as observed in this study; testicular sperm cells activated exhibited adequate motility quite unlike mammalian and avian testicular spermatozoa. However, the asynchronous development of germ cells during spermatogenesis in *C. gariepinus* indicates that spermatogenesis in this species is not seasonal hence continuous spermiation can occur even in captivity. This observation supports the report of Oteme *et al.* (1996) who made similar observation on *Heterobranchus longifilis*. The morphology of the spermatogenic cells observed in this study are quite similar to those described in mature Nile Tilapia (Schulz *et al.*, 2005). The A-type spermatogonia with pale ovoid nuclei (Fig. 2d) proliferated for self-renewal and to produce spermatogonia B (Fig. 2b-e) which after mitotic divisions became committed to meiosis at Preleptotene (Fig. 2d) primary spermatocytes. Following differentiation and recombination through Leptotene (L) Zygotene (Z) (Fig. 2f) Pachytene (P Fig. 2g) to Diplotene (D Fig. 2h-i) this germ cell undergoes the first maturation division (M1 Fig. 2h) to produce secondary spermatocytes (S Fig. 2j).When the second maturation division (M11 Fig. 2j) is accomplished the round spermatids undergo maturation and modification of the acrosome through stages Std$_1$ and Std$_2$ (Fig. 2f,k) before spermiation (Fig. 2*l*) releasing mature spermatozoa into the lumen of the seminiferous epithelium. This sequence of events demonstrates the presence of recognizable cycle of spermiation in *Clarias gariepinus*. This observation agrees with previous reports (Almeida *et al.*, 2008; Schulz *et al.*, 2005; Alvarenga and de Franca, 2009).

Lepidagathis alopecuroides aqueous extract exerted concentration dependent impact during spermatogonial proliferation resulting in the inhibition of mitotic division, leading to the presence of fewer germ cells at Preleptotene stage from 0.25 and 0.50 ppm to almost total inhibition of mitotic activity and the accumulation of hyperchromatic B-type spermatogonia at 0.75 and 1.00 ppm. The

observed changes in germ cell structure and population indicate the cytostatic and apoptotic effects of sublethal concentrations *L. alopecuroides* due to the presence of rotenones and saponins (Obomanu *et al.*, 2006) in this plant. This agrees with McClusky (2005) who had earlier reported apoptosis during spermatogenesis in the spiny dogfish.

Germ cell loss was recorded especially at 0.75-1.00 ppm of *L. alopecuroides* extract and particularly during the last mitotic division of B-type spermatogonia preceding the formation of Preleptotene primary spermatocytes. This result is in support of an earlier report in the teleost of germ cell loss during spermatogenesis in Nile Tilapia (Alvarenga and de Franca, 2009) and the variation in such losses said to be species-specific according to the spermatogenic phase (Chaves-Pozo *et al.*, 2005; Almeida *et al.*, 2008; Prisco *et al.*, 2003; Batlouni *et al.*, 2006). The germ cell loss was due to significant ($p<0.01$) quantitative decline in the spermatogenic activity shown as reduction in mitotic and meiotic indices as well as spermatogenic efficiency which resulted in delayed or outright inhibited regeneration of spermatogonia population following spermiation. However, spermatogonia A were observed scattered throughout the seminiferous epithelium of the cyst undergoing spermiation apparently without any mitotic activity (Fig. 3f). This is in line with the reports on Cardinal fish (Fishelson *et al.*, 2006) *Sparus aurata* (Chaves-Pozo *et al.*, 2005) that at low sublethal doses of toxicants, some spermatogonial stem cells might survive and repopulate seminiferous epithelium.

Based on the observed spermatogonial cysts, there are sub-populations of spermatogonia A after differentiation and several mitotic divisions before B-type spermatogonia produces premeiotic/Preleptotene primary spermatocytes (morphometric data yet to be published). The number of spermatogonial mitotic divisions preceeding meiosis is species specific in mammals and birds (De Rooij, 2001) 5-14 in teleosts prior to differentiation into primary spermatocytes (Ando *et al.*, 2000) while Schulz reported 8 for Nile tilapia.

Clarias gariepinus exposed to sublethal concentrations of piscicides like *Lepidagathis alopecuroides* even for short periods experience testis structure alterations and functions including delayed and inhibition of spermatogonia A renewal, inhibition of spermatogonia B from entry to meiosis resulting in production of fewer primary spermatocytes, hence reduction in spermatozoa production. This observation supports Jahan *et al.* (2009), who reported anti-fertility effect of *Abrus precatorius* seeds on spermatogenesis in male mice and concluded that *A. precatorius* tended to suppress spermatogenesis, induced reduction in germ cells and leydig cells as well as disrupting the seminiferous tubules and loosening the germinal epithelium. It is thus concluded that freshwater catfish *Clarias gariepinus* exposed to sublethal concentrations of *L. alopecuroides* would exhibit reduction in sperm production in the subsequent reproduction cycles. Thus, use of this biocide in aquatic environment requires regulation and control to avoid depletion in fish population and non-target aquatic fauna.

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