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# Morphological Effect of Dumpsite Waste Forage (*Calapogonium Mucunoides*) on the Reproductive Profile of Rabbit (*Oryctolagus Cuniculus*)

I.P. Solomon[1], *Oyebadejo S.A[2] and Akpan E.E.[1]
[1]*Department of Animal Sciences, Faculty of Agriculture, University of Uyo, Akwa- Ibom, Nigeria*
[2]*Department of Human Anatomy, Faculty of Basic Medical Sciences, University of Uyo, Akwa- Ibom, Nigeria*
*****E-mail: samsonoyebadejo@yahoo.com*

Subject: Biotechnology

## Abstract
Morphological effect of the feeding of dumpsite forage (Calapo -*Calopogonium mucunoides*) on the reproductive profile of rabbits (*Oryctolagus cuniculus*) was investigated. 24 rabbits; 20 females and 4 males were obtained and distributed randomly into two treatment groups of 10 females and 2 males. The forage, specifically Calapo (*Calopogonium mucunoides)* was fed to the two groups' *ad-libitum* with the non-dumpsite fed group serving as the control. After a period of 20 weeks, the rabbits were sacrificed, 5ml of Blood was taken out from the right ventricle, centrifuged, the serum was extracted and sent to the laboratory immediately for Hormonal analysis, while Testes were removed and cut into pieces in seminal fluid solution, they were squeezed gently, spermertocytes were allowed to flow out, after 5 minutes, semen analysis was carried out at room temperature. The semen analysis showed decrease in sperm concentration, sperm motility, morphology but increase in death ratio compared to non-dumped site where there is tangential progressive increased in all the sperm parameters including live and death ratio. Hormonal analysis results revealed decreased in Estrogen level, Luteinizing, follicle stimulating hormone and Prolactin level in dumpsite subjects compared to the non- dumpsite where these parameters appear normal within normal range, while Testosterone level for both sites were the same. In conclusion, feeding of dumpsite forages to rabbits could pose hazard and deleterious effect of reproductive morphology of rabbits.

**Keywords:** *Calapo (Calapogonium mucunoides), Rabbit (Oryctolagus cuniculus), Seminal fluid, Testes.*

## Introduction

Dumpsites are traditional methods of waste disposal to landfill method of waste management. Dumpsites are often established is disused quarries, mining or excavated pits away from residential areas (Abdusalam, 2009). Poor management of dumpsites could create a number adverse environmental impacts, one of these impact is due to location of dumping site in suitable areas. Locating a dumping site in a suitable area is a very time consuming process.

Soil is a vital resource for sustaining basic human needs, a quality food supply and a livable environment (Wild, 1995). It serves as a sink and recycling factory for both liquid and solid waste. Municipal solid waste has been found to contain appreciable quantity of heavy metals such as copper (Cu), Nickel (Ni), Iron (Fe), Lead (Pb), Arsenic (As) all which may end up contaminating the soil and even get to the forages growing on that soil. (Alloway and Aryes, 1997).

Reproduction inefficiency is the most constraint to efficient rabbit production in the tropical (Gbadamosi and Egbunike, 1999). The efficiency of sperm production, libido and quality of sperm tend to remain uniform throughout the reproductive life of an animal but may be significantly attend by age, nutrition, environment, health status, drugs and chemicals (Togun and Egbunike, 2006). Among these

factors mutation, drug and hormones are the most prominent. Sexual nutrition is known to be delayed by a poor nutrition requirement during growths (Omole, 1982). It also affects age at puberty and stimulation of hypothalamus indirectly produce interstitial cells stimulating hormone that acts in the testicular tissue (Cogan *et al*., 2004).

Calapo (*Calapogonium mucunoides* Desv.) is a vigorous, hairy annual or short–lived perennial trailing legume. It can reach several meters in length and form a dense, tangled mass of foliage, 30-50 cm deep. The root system is dense and shallow, at most 50 cm deep. The stems are succulent, covered with long, brown hairs. They are creeping in the lower parts, sometimes rooting at the nodes that come in contact with the soil. The upper part of the stem is twining. The leaves are up to 16 cm long and trifoliate. The hairy leaflets are 4-10 cm long x 2-5 cm broad, ovate to elliptical. The inflorescence is a slender hairy raceme that may be up to 20 cm long and bears 2 to 12 blue or purple small flowers. The Fruits are 3-8 seeded hairy pods, 2-4 cm long (FAO, 2011; Cook *et al*., 2005; Chin Chen Peng *et al*., 1997). It is mainly used as cover crop, alone or in mixture with other legumes, especially in rubber, oil palm or in young forest plantations. Calapo is used for green manure though its value for this use still needs confirmation. Calapo is a pioneer species: it provides soil protection against erosion, reduces soil temperature, improves soil fertility and controls weeds (Cook *et al*., 2005; Chin Chen Peng *et al*., 1997). Although not widely used, calapo is the most popular legume amongst Brazilian farmers and is the legume seed produced in greatest volume in Brazil (Pizarro, 2001).

*It* can be grazed and made into hay or silage. Animals especially cattle graze it during the latter part of the dry season (Cook *et al*., 2005). Its good persistence under grazing might be a way to improve overall pasture quality through enhanced soil fertility, subsequent higher pasture growth rate and weed control (Chin Chen Peng *et al*., 1997). One commercial cultivar, derived from plantation agriculture, has been developed in Brazil (Cook *et al*., 2005).

Heavy metals are natural components of the Earth's crust. They cannot be degraded or destroyed. To a small extent they enter the body through food, drinking water and inhalation of contaminated air. Heavy metals are considerably environmental concern due to their toxicity and accumulate behavior (Purves, 1985). Their uptake by plants from the soil is largely specified. Although all trace element are natural constituent of the soil, the dumping of waste on soil has been found to increase their heavy metal profile (Clarkson *et al*., 1983, Adeniyi *et al*., 1993 and Adeniyi, 1996). The effect of this, is that their concentration may reach toxic levels, resulting in increased health risk to animals especially if they are fed with forage on that soil. Studies on heavy metals in ecosystem have shown an indication of a silent epidemic of environmental metal poisoning of ever increasing metals in sub-tropical soils (Nriagu, 1988, Shuman, 1999). With increasing pressure on agricultural and proliferation of urban and peri-urban farming, waste dumpsites are becoming attractive because of their rich deposit of organic matter and plant nutrients.

Although the nutrient content of waste makes them attractive as fertilizer, land application of many industrial waste and sewage is constrained by the pressure of heavy metals, hazardous organic chemical, salts and extreme pH (Cameron *et al*., 1997). Animals studies have shown the reproductive toxicity of a number of heavy metals, but in this study only four of them will of interest which include; Lead (Pb), Mercury (Hg), Arsenic (Ar) and Cadmium (Cd).

Rabbit as a laboratory animal are very important in research institutes.. Rabbits are used as pets in homes and their feaces serves as biologically enhanced fertilizer. More recently, they have been used as source of meat. When feeding and management are of a high standard, the rabbit is one of the most efficient animal in the world at converting food for meat (Fielding, 1991).

They are being fed by human with forages grown on dumpsite which may contain heavy metals like cadmium, lead, mercury and arsenic etc. that can have adverse effect on the reproductive life of the animal. The efficiency of reproduction tends to remain uniform throughout the reproductive life of an animal but may be significantly altered by such factors as bio-climate, chemicals, hormones, drugs and nutrition (Togun and Egbenike, 2006).

Animal studies have shown that accumulation of heavy metals in the reproductive organs of animals like ovary and testes have resulted in infertility in females and reduced libido in males (Herbert et al., 2005).

The reported cases of infertility in females and reduced libido in buck rabbits fed dumpsite waste forage are sources of concern Herbert, 1997, 1998, Herbert *et al*., 2005; Joshi, 2007. The needed improvement in reproductive

performance of rabbit may be achieved by investigating the effect of heavy metals in dumpsite waste forages.

Making this study to be important because it will ascertain the effect of the dumpsite feed on the effects on hormones e.g. estrogen hormones, leuteinizing hormones, follicle stimulating hormones, prolactin hormones and reproductive performance in rabbits.

## Materials and Methods

### Drugs and Chemicals

Sodium chloride, formaldehyde, sodium trioxocarbonade V, sodium bicarbornate, xylene, 70% alcohol, 90% alcohol, absolute alcohol, distilled water, hutches, concentrate feed, syringe and hypodermic needles, EDTA treated bottles, latex hand glove, weighing scale, graduated vials, measuring tape, they were all procured from BDH Chemicals, England. All other chemicals were of analytical grade.

### Experimental Animal and Management

The animals were sourced from the University of Uyo Teaching and Research Farm, Use-Offot, Akwa Ibom, Nigeria. A 2- week experimental period was used to get the animals (rabbits) acclimatized with the experimental procedures. The experiment lasted 20 weeks (June, 2013 to November, 2013). The animals used in this study were 4 bucks and 20 does crossbred rabbits aged 6-7 months. The males weighed between 1350g and 1650g, while the females weighed between 1400g and 1800g.

Four bucks and twenty does respectively were divided into two groups of 12 animals each. When placing the animals into groups care was taken in order to balance the groups such that there were no significant differences between them on the basis of age and weight and the animals were identified individually with the aid of a permanent marker on their ears**.** The groups were randomly assigned to two (2) treatment diets: dumpsite fed animals and non-dumpsite fed animals.

The experimental animals were housed in a wooden hutch with a wire mesh floor and in-built waste trays. The management techniques employed for all the experimental animals included regular cleaning of the hutch, feeding and watering of the experimental animals on a daily basis. The experimental animals were managed well.

Drinkers and feeders were made of plastics and concrete with narrow but blunt mouth to discourage fed wastage and injuries. Forage (experimental diets) and clean water was also supplied *ad libitum*. Permission and approval for animal studies were obtained from the College of Health Sciences Animal Ethnics Committee, University of Uyo.

### Experimental Animal Health

The rabbits acquired were treated against internal and external parasites by subcutaneous injection of ivomec (0.2 ml per rabbit) and a broad spectrum antibiotic (Oxytetracyclin L. A.) was also administered at the rate of 0.2 ml per rabbit. Sulphur powder was given occurrences of mange and neomycin was given for diarrhea at the rate of 10g per four (4) liters of drinking water.

### Experimental Designing and Feeding Of Experimental Diets

Two treatments being the waste dumpsite fed and the non-dumpsite fed. Forages were obtained from two sites, one being the waste dumpsite within Uyo metropolis and the other being a land, which is the non- waste dumpsite. Forage used was *Calapogonium mucunoides* due to its palatability to the animals. The forages were supplied daily to the animals and fed *ad-libitum*. Alongside with the forage, the concentrate of pelleted poultry grower's mash meal (20% CP and 2700Kcal/kg) were fed routinely to facilitate the growth of the animals.

**Table 1: Showing the experimental designing of the study.**

| Group | Female | Male | Treatment (site) | Duration |
|---|---|---|---|---|
| 1 (NDS) | 10 | 2 | Non-dumpsite forage | 20 weeks |
| 2 (DS) | 10 | 2 | Dumpsite forage | 20 weeks |

## Seminal Analysis

**Specimen collection**
Testes (epididymis) were crushed into pieces in 5ml of normal saline and allowed to rest for 5-10 minutes to enable the spermatocytes to spread out into the diluents solution.

**Sperm motility**
Dilute the 1ml of seminal fluid in 20mls of Tris buffer solution or Buffer formol saline. Load 0.01 of the solution on a grease free slide, apply cover slip and observed microscopically. The result is expressed in percentage.

**Sperm morphology**
Dilute the 1ml of seminal fluid in 20mls of Tris buffer solution or BFS (Buffer formol saline)Load 0.01 of the solution on the grease free slide, apply cover slip and observe microscopically for Tail defect (TD), Neck defect (ND), Mid-piece defect (MD) and Head defect (HD).

**Sperm cell concentration**
Sperm concentration/ejaculate was calculated as: sperm concentration per ml × volume per ejaculate (Egbuka, 1995). Sperm concentration per ml of semen was evaluated using a visual count under the microscope using improved Neubauer haemocytometer. A small pipette with a dilution ratio of 1:100 was used. The sperm cells were killed (immobilized) using a 1% formaldehyde solution prior to counting.

**Sperm Live and Death ratio**
The differential staining (one drop of semen was mixed with two drops of eosin) on a slide observed under the microscope aided the determination of the total live sperm cells. The unstained cells represented the live cells while the stained cells showed the dead ones.

## Hormonal Analysis

**Testosterone Assay**
Serum testosterone was assayed from blood obtained from a left ventricular puncture. The samples were spun at 3000g for 10min in an angle head centrifuge at 25$^{o}$C. The samples were assayed in batches from a standardized curve using the Enzyme Linked Immunosorbent Assay (ELIZA) method. The microwell kits used were from Syntro Bioresearch Inc., Carlifornia, USA. With 10ml of the standard, the samples and control were dispensed into the number of coated wells to be used. 100ml testosterone conjucate reagent was added and then 50ml of anti testosterone reagent. The content of the microwell were thoroughly mixed and then incubated for 90 minutes at room temperature. The mixture was washed in distilled water and further incubated for 20 minutes. The reaction was stopped with 100ml of IN hydrochloric acid. Absorbance was measured with an automatic spectrophotometer at 450nm.

**Estrogen Assay**
Serum estrogen was assayed from blood obtained from a left ventricular puncture. The samples were spun at 3000g for 10min in an angle head centrifuge at 25$^{o}$C. The samples were assayed in batches from a standardized curve using the Enzyme Linked Immunosorbent Assay (ELIZA) method. The microwell kits used were from Syntro Bioresearch Inc., California USA. With 10ml of the standard, the samples and control were dispensed into the number of coated wells to be used. 100ml estrogen conjugate reagent was added and then 50ml of anti testosterone reagent. The contents of the microwell were thoroughly mixed and then incubated for minutes at room temperature. The mixture was washed in distilled water and further incubated for 20 minutes. The reaction was stopped with 100ml of IN hydrochloric acid. Absorbance was measured with an automatic spectrophotometer at 450nm.

**Luteinising Hormone Assay**
This was carried out using enzyme immune assay kits, catalog number: BC-1031. The assay utilizes two anti LH monoclonal antibodies. The process involves immunoextraction, labeled antibody reaction and colour development. A solid phase is incubated with a coloured enzyme substrate fer one hour at 37$^{o}$C. The process of alkaline phosphate causes a colour change from yellow to pink. The intensity of the pink colour is a measure of the labeled antibody hence LH. The LH concentration was measured off a calibration curve.

**Follicle Stimulating Hormone Assay**
This assay was carried out using double antibody radio immunoassay. A rabbit recombinant FSH {$1^{125}$} from Amersham, UK was used. The sensitivity of the assay was 0.9 ng/ml. A 96-well plate has been precoated with anti-Follicle Stimulating Hormone antibodies. Samples and standards and are added to the

wells, where Follicle Stimulating Hormone in the sample and standards binds to the precoated antibody. After incubation and washing, added Anti-Follicle Stimulating Hormone HRP conjugate binds to the antibody-Follicle Stimulating Hormone complex. After incubation, the wells are washed to remove unbound material and TMB substrate is then added which is catalyzed by HRP to produce blue coloration. The reaction is terminated by addition of Stop Solution which stops the color development and produces a color change from blue to yellow. The intensity of signal is directly proportional to the amount of Follicle Stimulating Hormone in the sample and the intensity is measured at 450 nm.

**Prolactin Hormone Assay**

This assay was carried out using double antibody radio immunoassay kit. A rabbit recombinant PL from Amersham, UK was used. The sensitivity of the assay was estimated subsequently. The GenWay Biotech Prolactin Quantitative Test is based on the principle of a solid phase enzyme-linked immunosorbent assay. The assay system utilized sheep anti-prolactin for solid phase (microtiter wells) immobilization and mouse monoclonal anti-prolactin in the antibody-enzyme (horseradish peroxidase) conjugate solution. The test sample is allowed to react simultaneously with the antibodies, resulting in the prolactin molecules being sandwiched between the solid phase and enzyme-linked antibodies. After a 45 minute incubation at room temperature, the wells are washed with water to remove unbound labeled antibodies. A solution of Tetramethylbenzidine (TMB) is added and incubated for 20 minutes, resulting in the development of a blue color. The color development is stopped with the addition of 1N HCl, and the resulting yellow color is measured spectrophotometrically at 450 nm. The concentration of prolactin is directly proportional to the colour intensity of the test sample.

## Results

### Seminal Analysis

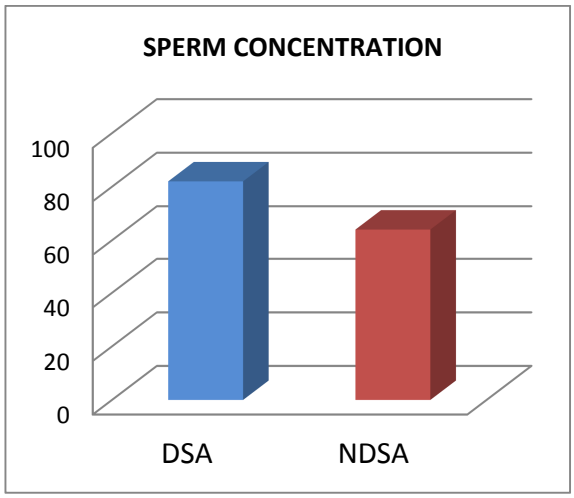


**Figure 1: Graph of sperm concentration of Rabbit fed with forage from Dumped (DS) and Non-dumped (NDS) sites.**

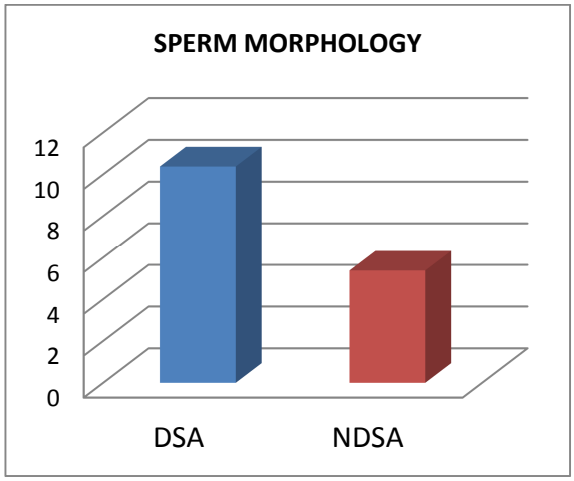


**Figure 2: Graph of sperm morphology of Rabbit fed with forage from Dumped (DS) and Non-dumped (NDS) sites.**

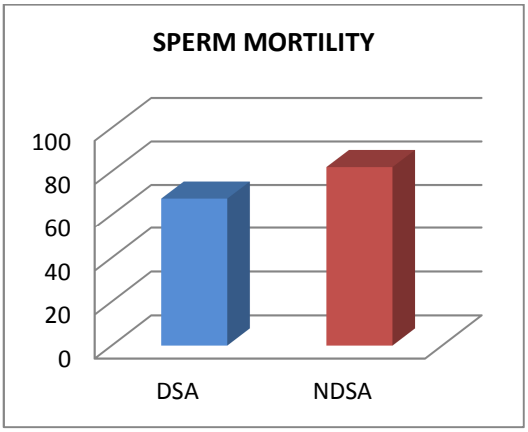


**Figure 3: Graph of sperm motility of Rabbit fed with forage from Dumped (DS) and Non-dumped (NDS) sites.**

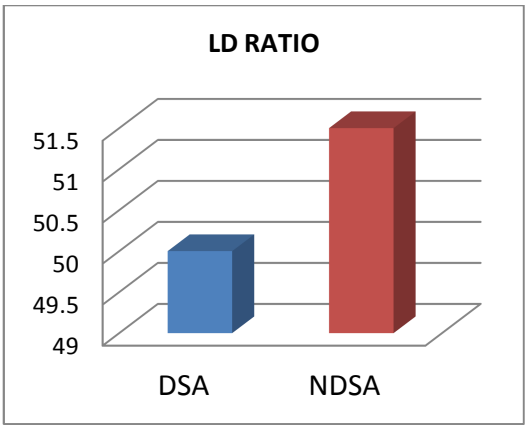


**Figure 4: Graph of Live and Death ratio of Rabbit fed with forage from Dumped (DS) and Non-dumped (NDS) sites.**

## Hormonal Analysis

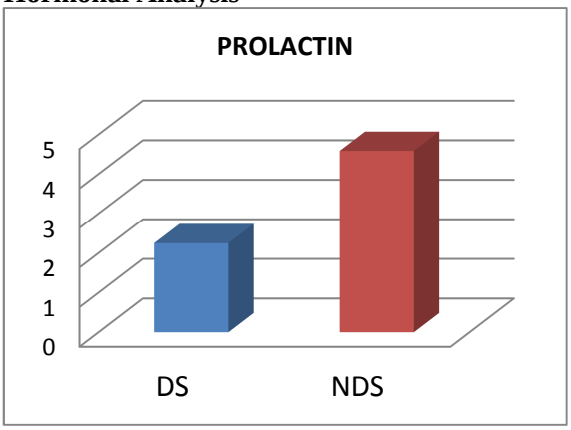


**Figure 5: Graph of Prolactin of Female Rabbit fed with forage from Dumped (DS) and Non-dumped (NDS) sites.**

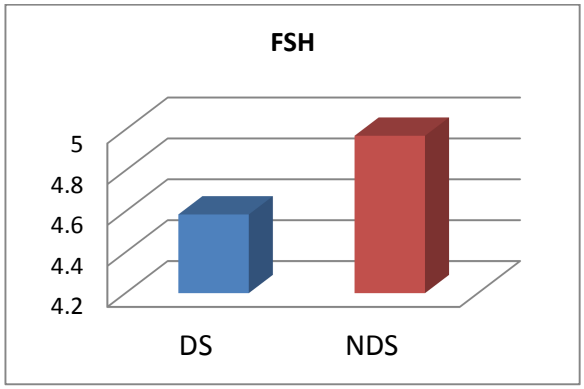


**Figure 6: Graph of Folicle stimulating Hormone of Female Rabbit fed with forage from Dumped (DS) and Non-dumped (NDS) sites.**

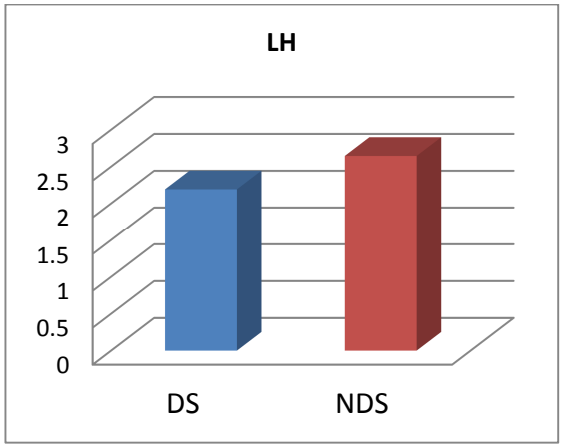


**Figure 7: Graph of Luteinizing Hormone of Female Rabbit fed with forage from Dumped (DS) and Non-dumped (NDS) sites.**

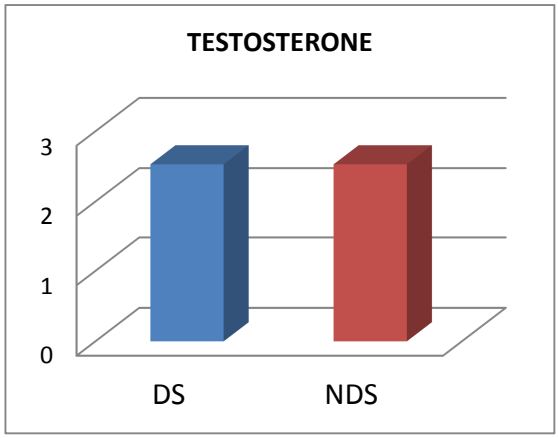


**Figure 8: Graph of Testosterone levels in Male Rabbit fed with forage from Dumped (DS) and Non-dumped (NDS) sites.**

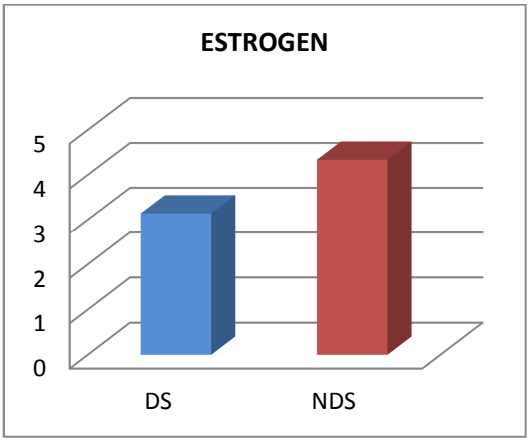


**Figure 9: Graph of Estrogen levels in Female Rabbit fed with forage from Dumped (DS) and Non-dumped (NDS) sites.**

## Discussion

Seminal parameters are considered to be valuable information for the evaluation of breeding ability and potential fertility of animals exposed to pollutants (Anonymous, 2006). Some seminal parameters Sperm concentration, Sperm morphology (Head, Neck, Middle and Tail defects), Sperm motility, Sperm progressivity, Live and Death ratio are used to access the semen condition used as indicators of heavy metals pollution.

From this study, it was observed that exposure of rabbits to heavy metals ingestion caused a significant decreased sperm concentration, sperm progressivity, sperm motility and live ratio, sperm morphology (head defect, neck defect middle defect and tail), death ratio increased with intake of contaminated dumpsite forages (Figure 2). The observed reduction and increased in the above parameters demonstrate and show reduced libido and infertility (Joshi, 2007) in the dumpsite treated rabbits as against the control (non-dumpsite). The observation in this study is similar to the findings of Maha Zaghlool *et al*., (1996) which shown the effect of increasing doses of mercuric acetate on semen quality and quantity in male rabbits. The result indicated that there was a significant decrease in semen ejaculate volume, sperm concentration, initial fructose concentration and osmolarity in rabbit treated with mercuric acetate compared with control animals.

Meanwhile, percentage of dead and abnormal spermatozoa and methylene blue reaction time were significantly increased in the semen of treated animals. These deleterious effects of forage from dumpsite on semen quality and quantity were dose-dependent.

Hormonal analysis is useful to know activities of hormones in the body, reproductive performance in animals could be altered by abnormality in the hormones (Omole, 1982). From this analysis it was observed that there was a decreased in prolactin level, luteinizing hormone level, follicle stimulating hormone level in the dumpsite treated animals. The testosterone level was the same for both dumpsite and non-dumpsite treated animals. This as a result of suppression of androgen production and reduction in sertoli cell number (Scott *et al*., 2007)

## Conclusion

From this study, it has been observed that prolonged feeding of dumpsite forage to animal's leads to negative effect on the semen and hormones which could bring significant pathological changes and biochemical alterations and physiological changes in the testis and ovary. It is advised that the breeder should avoid prolonged feeding with forages that contain heavy metals which are major component of dumpsite forages.

### Conflict Interests

The authors declare that they have no competing interests.

### Authors' Contributions

All the Authors contributed equally

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This research was not supported by any organization or funding body, however the authors contributed collectively to carry out the experiment.

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