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Meiosis of Germ Cells

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Highlights

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Discovering Thoughts, Inventing Future

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Control of Entry into Meiosis of Germ Cells Precursors in Chickens

By Trukhina A. V., Lukina N. A., Nekrasova A. A & Smirnov A. F.
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Abstract- This paper describes experiments on the induction of sex reversal in chickens by estrogen inhibitors (letrozole, tamoxifen), demethylating agent 5-azacytidine and retinoic acid (RA). Particular attention is paid to control entry into meiosis of germ cells. The obtained data indicates a partial overlap control of somatic and gametic sex.

Keywords: *sex reversal, aromatase, estrogen, 5-azacytidine, retinoic acid, meiosis, germ cells, chicken.*

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Control of Entry into Meiosis of Germ Cells Precursors in Chickens

Trukhina A. V. [α], Lukina N. A. [σ], Nekrasova A. A [ρ] & Smirnov A. F. [ω]

Abstract- **This paper describes experiments on the induction of sex reversal in chickens by estrogen inhibitors (letrozole, tamoxifen), demethylating agent 5-azacytidine and retinoic acid (RA). Particular attention is paid to control entry into meiosis of germ cells. The obtained data indicates a partial overlap control of somatic and gametic sex.**

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I. Introduction

Sex determination begins with colonization of the gonad (somatic nature organ) by the precursors of germ cells (PGCs) and followed by their entry into meiosis and the formation of the haploid gametes. PGCs are formed in embryonic development of birds and mammals (Murray et al, 2010). The source of the formation of gametes in birds is line of sex cells, which first appear in the early embryonic development. In the early blastula (stage X by Eyal-Giladi & Kochav, 1976) PGCs are located in the central region of the blastoderm. Then, as a result of morphogenetic movements prior to gastrulation the cells become localized in a limited region of the embryo blastodisk, so-called sexual crescent. On the third day of embryonic development PGCs begin migration via the bloodstream to the emerging gonads. The rudiments of the gonads appear on the third day of chicken embryogenesis (18-20 stages of development (Hamburger & Hamilton, 1951). From the beginning of the process of anatomical differentiation of gonads (7th day of development) reproduction rate of germ cells in the male and female gonads begin to differ. More intensive increase in the number of germ cells in the female gonad is accompanied by a significant increase in left ovary than in right ovary. In males both testes develop in similar rate. A higher rate of proliferation of germ cells is characteristic for representatives of heterogametic sex: female birds and male mammals. More intense proliferation of oogonia in the left ovary of birds leads to a considerable increase in their numbers when compared to the spermatogonia and then to an earlier entry into meiosis. Oogonia appearance characteristic of the stage of oocyte meiotic prophase leptoteny can observed at 13-14 days of incubation. In the testes the number of germ cells at similar stages of development is much smaller and at the time of hatching the population of male germ cells consists exclusively of spermatogonia (Ayers et al, 2013).

It is assumed that meiosis of female mammals is induced by retinoic acid (RA), which is inhibited in the males, by CYP26B1 (Bowles & Koopman, 2010). Unlike mammals, the molecular mechanisms of sex determination in birds remain largely unknown (Trukhina A. et al., 2013). In particular, the questions remain about the reasons why the sex cells of males and females enter meiosis during different stages of development, and what is role of sex hormones in this process.

Many epigenetic mechanisms of regulating the development of germ cells have been described in mammals. During entering into gonadal ridge genome of PGCs is undergoing methylation and chromatin remodeling (Martínez-Arroyo et al, 2014). In birds the role of epigenetic control of sex determination is less clear describe weaker for somatic gonad and PGCs (Trukhina & Smirnov, 2014). In particular, MHM locus (localized in Zp21 region) was described that consists of approximately 200 repeats of 2.2 kb and is being transcribed into noncoding heterogeneous RNA. This MHM region exhibits specific chromatin modification only in females (Itoh et al., 2011).

To confirm the possible role of retinoic acid (RA) in the induction of bird meiosis, we investigated the effect of the RA on meiosis of PGCs when it was administered to chicken embryos at different stages of development. The aim of this study was also to assay the effect of estrogen on the development of avian testicles and ovaries, as well on germ cell maturation. For this purpose, we modified the balance of sexual hormones by aromatase inhibition and altered the estrogen receptor sensibility to their hormone with corresponding modulators. Also we studied the impact of demethylation by treatment with 5-azacytidine (5-AC) embryos in the early stages of incubation.

II. Material and Methods

During the experiments, eggs of "Belaya Russkaya" breed chickens (experimental farm of All-Russia Institute of Farm Animal Genetics and Breeding, St.-Petersburg-Pushkin, Russia) or Highsex-white cross (Tosno, Lisii Nos, Russia) were used. All injections (at 100mkl into egg) were carried out into the air cells of eggs to under shell membrane in region of blastodisc. As control we carried out injections 1xPBS (at 100mkl into egg). Eggs were incubated for 17-19 days at 37,8°C

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and humidity 28%. For the first group of experiments incubated eggs were administered with RA in doses of 12.5 or 25mkg at 9th or 14th day of incubation. For the second group of experiments we have performed a number of experiments with aromatase inhibitor – letrozole (Novartis Pharma, Switzerlland) and modulator of estrogen receptors – tamoxifen (Ebeve Pharma, Austria) at concentration 1mg/ml. Injections of Tamoxifen or letrozole have been done once at the 4th day or twice at the 4th and 11th days. During one group of experiments, tamoxifen was injected first at the 4th day and then letrozole at the 11th day. At third group of experiments we carry out injections of 5-azacytidine (5-AC) at concentrations at 10, 12.5 and 15mkM at 1st or 4th day incubation.

Gonads isolated from 17 – 19 day old embryos after determination of their type (testicles or ovaries) were fixed with Clark mixture (ethanol mixed with glacial acetic acid in ratio 3:1). Gonads were then washed from the fixator, treated with alcohols of increasing concentration (ethanol–isobutanol–O-xylol), and embedded into paraffin according to the routine technique (Barikina et al., 2004) to prepare sections on the microtome. Sections were purified from paraffin, stained with Mayer's hematoxylin, dehydrated by alcohols with increasing concentration and embedded into Canadian balsam (DiaM, Russia). The meiotic marker SCP3 (Synaptonemal Complex Protein) was used for immunofluorescent staining (antibodies BDBioScience and AbCam, USA). After washing in 1xPBST (1xPBS buffer with 0.05% Tween 20), sections were incubated with secondary antibody at room temperature for 40 minutes. After that sections were stained by DAPI (12mkg/ml) (Sigma-Aldrich, USA) with adding Vectashield (Vector Laboratories, USA).

Gonadal histology was analyzed under a Leica DM6000B microscope (Leica Microsystems GmbH, Germany) equipped with a Leica DC500 CCD camera and Leica QWin v.1.2 software. We analyzed miniomum of 200 PGCs in each gonad from at least 3 embryos. Stages of meiosis prophase were determined visually. Comparison of mean values and errors at groups we calculated standardly, comparison of progressive average difference we carried out at Student t-test.

Genetic sex of each embryo was defined by polymerase chain reaction (PCR) using DNA isolated by the routine procedure (Griffiths *et al.*, 1998). Primers for the chicken *CHD1* gene were: CHD1F 5'-GTTACTGATTCGTCTACGAGA-3' and CHD1R 5'-ATTGAAATGATCCAGTGCTTG-3' (Elbrecht & Smith, 1992; Ellegren, 2002). The PCR conditions were: denaturation at 94°C for 5 min; gradual temperature decline from 60°C to 50°C, 1°C, 1 min; 35 cycles: 94°C for 30s, 50°C for 30s, 72°C for 60s. Last PCR step was performed at 72°C for 10min (Kamata et al., 2004). Amplified DNA was assayed in 2% agarose gel. Single fragment (380 bp) was identified in males and two fragments (500 and 380 bp) were observed in females.

III. Results

With the introduction of retinoic acid into eggs at 9 or 14- days of incubation in embryos on day 17 there was significlly more germ cells in the prophase stage of meiosis in left gonads of treated females than in controls. Oocytes I of control embryos were at stages of preleptotena and leptotena. At the same time most of experimentally treated embryos had oocytes I at stage of zygotena with specific arrangement of chromosomes condenseed in the characteristic form of bouquet. At a later period of incubation (19th day embryos development) control female embryos had oocytes at stage of preleptotena – zygotena. We did not find statistically significant differencesin quantities of gonocytes at the different stages of meiosis prophase between control and experimental female groups.

On 17th day of embryo's development control males had testis with only spermatogonias. At the same times experimental male had not spermatocytes I at stage of preleptotena. Quantities such cells were less than 14%. On 19th day of embryo's development control male also had testis with spermatocytes I at stage of preleptotena and their quantity was about 8.5%. In testis of experimental males number of such cells was nearly 16% (Fig.1). Number of spermatocyte I and oocytes I at various stages of meiosis prophese I (I, M, PL, L, Z) are presented in the diagram of Fig.2.

By fluorescence methods it was found that at 17th day of embryo's development germ cells of control females had meiotic SCP3 marker accumulating around their nucleus. At the same stage of development the germ cells of control males had not this marker. Gonads of experimental males had the germ cells with increasing intensive fluorescence such protein in the cytoplasm around nucleus.

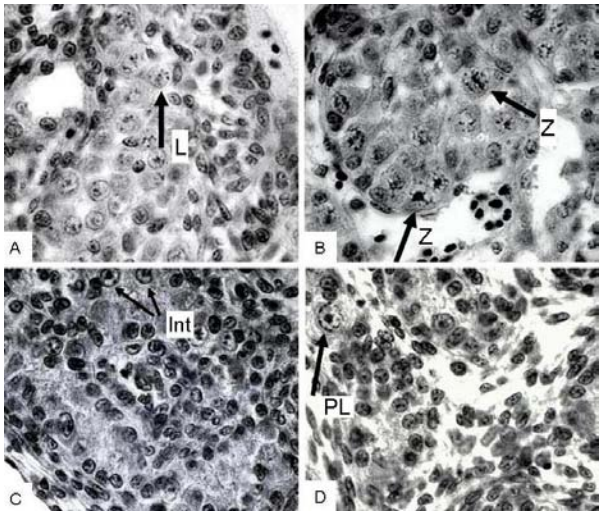

Figure 1 : Gonad sections of the experimental females (A, B) and males (C, D) at 17th day embryos development. It was injected 12.5mkg RA in egg at 9th (A, C) and 14th (B, D) day of incubation. On A and B it was showed the germ cells at stages of leptotena (L) and zygotena (Z), on C and D it was showed the germ cells at stages of interphase (Int) and preleptotena (PL)

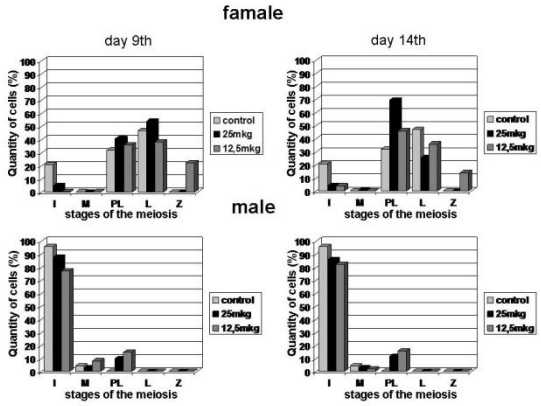

Figure 2 : The effect of RA into the entry for gonocytes I in prophase I of meiosis. At early terms of embryonic development RA in small dozes (12.5mkg) promotes to get over the first meiotic block in spermatocytes I and permit to entry into stage of zygotena for oocytes I when the synaptonemal complexes begin to form. But the effect of RA is insufficiently in order to get over the second meiotic block at the stage of diakinesis.I – interphase, M – mitosis, PL – preleptotena, L – leptotena, Z – zygotena

The effect of estrogen inhibitors (letrozole and tamoxifen) on gonad development in chicken embryos was assessed by anatomical (gonad pairs, their size and shape), histological and cytological (presence of seminiferous tubules, state of germ cells) standards. Control females have typical left gonads with medulla and cortex. Numerous germ cells at the stage of meiotic prophase were revealed in the cortex of control females. Control males of the same age had smaller testicles and a well developed system of seminiferous tubules with spermatogonia mostly not entered into the meiotic pathway. Genetic sex of control males and females fully corresponded to the phenotypic type.

The gonad morphology of experimental genetic females differed from the norm. The most apparent changes were observed after letrozole exposure: two well developed gonads, cortex thinning and structures resembling seminiferous tubules in medulla were observed. The germ cell number and their location in the cortex were unaltered. Most ovarian germ cells entered meiosis and were blocked at the meiotic prophase I. Genetic males exposed to letrozole (aromatase inhibitor) had no apparent changes in their gonads.

Single injection of tamoxifen into the egg did not produce changes in embryonic gonads of both sexes. A doubled tamoximen dose caused a slight modification: hypertrophy of the left gonad in males. Both gonads were preserved in most females; however, the right gonad was slightly smaller than the left one. Both gonads had an increased number of lacunae in medulla, thickened cortex, and rare structures resembling seminiferous tubules. The number of germ cells at the stage of meiotic prophase I in the left gonad increased. However, in the right gonad, germ cells did not enter the meiotic prophase. Simultaneous administration of tamoxifen and letrozole, but at various stages of development, increased the number of germ cells in seminiferous tubules of experimental males was observed. However, they also were blocked before the entry into meiosis. No similar changes were observed in genetic females.

In third experimental group the injections of the 5-AC at the 4th day of incubation (15mkM, V concentration) and at 1st day of incubation (10mkM, III concentration) did not change the sex of the gonads, however, it led to the entry into meiosis of males germ cells. In the developing testis the germ cells at stages of preleptotena and leptotena (Fig. 3, 4) were found and mainly in left gonad. Changes were not found in the right gonad.

IV. Discussion

Formation of the sexes begins with almost parallel derivation of the gonads of males or females and their settlement PGCs. Both of these processes are under different genetic controls (Murray et al., 2010). This paper examines the influence of the RA, aromatase and estrogen inhibitors (letrozole, tamoximen) and 5-AC on inversion of gender and entry into the meiosis in birds.

There is a view that sex of vertebrates is determined genetically and differentiation PGCs is influenced by the somatic gonad tissues (McLaren, 2003). It was suggested that the main gene responsible

for the induction of meiosis in mammals and birds is a gene STRA8, whose expression is regulated by RA (Bowles et al, 2010). We have shown that the administration of the RA in the incubated eggs at small concentration at the 9th or 14th days of development caused the emergence of the germ cells to the stage of preleptotena in the testis. Then prophase stops on this stage (Fig.2). Under the influence of RA the number of oocytes at the different stages of meiotic prophase I are increased in the female gonads. Only spermatogonia were present in the testis of control embryos. At later stages of embryo's development in the testis of 19th day embryos only a few spermatocytes appeared on stage proleptotene. In the same gonads affected by RA embryos the number of such cells reached 16% (Fig. 2). It was found that there were no meiotic gonocytes in the gonads of 20 day-old chicks,

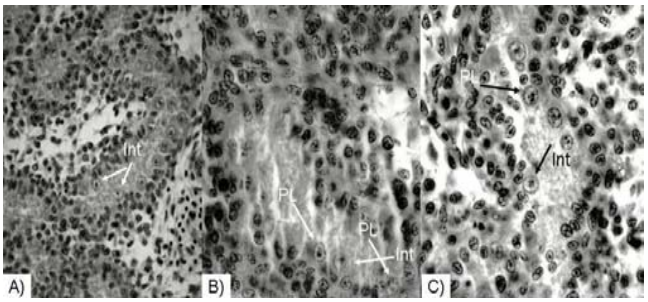

Figure 3 : The gonad sections of males at the 17th day development: A) control; B) the injection of 5-AC solution (10mkM) at the 1st day of embryos development; C) the injection of 5-AC solution (12.5mkM) at the 1st day of embryos development. It is notes spermatocytes I at the stages of interphase (Int) and preleptotene (PL).

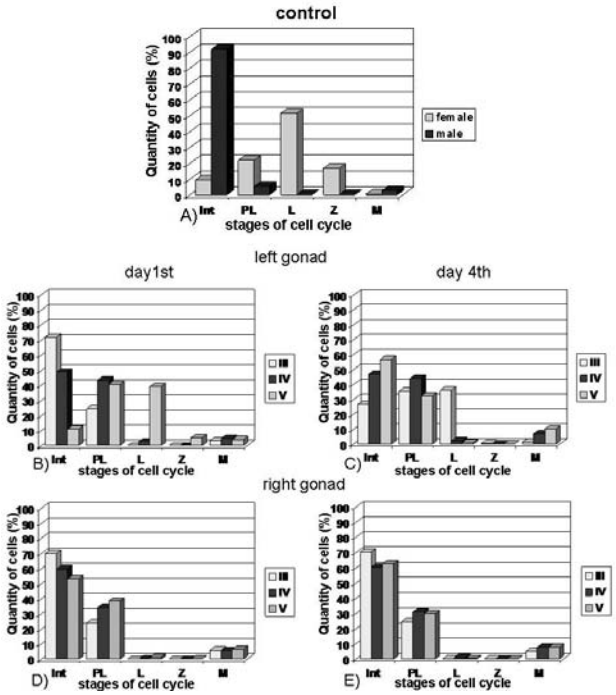

Figure 4 : The distribution of germ cells at different stages of meiotic cycle in the control female and male (A) and in the experimental male (B – E)

That were treated with RA on the 14th day of embryos development. This fact indicates that the RA is able to induce the entry into meiosis of the male cells. However, short-term exposure is not sufficient to advance the subsequent stages of meiosis and the entry into meiosis of all spermatogonia from the beginning meiotic transformations. The presence of the spermatocytes at the preleptotena stage of meiosis was confirmed by fluorescence protein SCP3 in the cytoplasm. We can assume that the RA takes part in the regulation of entry gonocytes into meiosis in birds. However, there are other factors involved. So masculinization of PGCs-supporting cells is initiated with gene *DMRT1* in some non-mammals, including chicken, *Xenopus laevis* and medaka, but in mice by induction gene *Sry* and *Sox9* expression (Yoshimoto & Ito, 2011). It is important that the injection of RA in chicken eggs during the incubation period studied is not accompanied by any sex inversion of testis.

Obtained data permits us to suggest that at early stages of embryonic development RA in small dozes (12.5mkg) allows to overcome first meiotic block in spermatocytes I and permit to entry into stage of zygotena for oocytes I when the synaptonemal complexes begin to form. During the studied terms of embryos development the germ cells of control females had not time to enter into this stage of prophase I. But the effect of RA probably is insufficient to get over the second meiotic block at the stage of diakinesis.

Earlier on the mutant of *am/am* maize it was was shown that switching over of the development program of sporogenic tissue cells from mitosis to meiosis takes place in last premeiotic mitosis. It is supposed that this mutant did not switch DNA synthesis type from mitotic type to meiotic partly delayed type of DNAsynthesis (Khvostova & Bogdanov, 1975). Very likely RA promotes this switching over. In consequence in germ cells of males first stages of meiotic prophase I begin. Moreover RA probably regulates the following events leading into meiosis: 1) delay of replication of genome DNA specific fraction (0,3%) and delay and asynchronic synthesis of all histone fractions; 2) the loss of ability to split centromeres in the next metaphase; 3) the coming prolonged meiotic prophase and the appearance spiralization of chromosomes typical for prophase type, determination of ability chromosome to the conjugation by pairs and acquisition them property to form chiasmas, forming meiotic morphology of chromosomes; 4) initiation of conjugation, synthesis of specific fraction of DNA.

According to one of hypothesis meiosis is induced by signals acting from the surrounding somatic tissues of gonad that stimulates or blocks the entry of gonocytes into meiosis in according with genetic sex of embryo (McLaren & Southee, 1997). The data about effect of RA to meiosis of mice germ cells testify in favour of the last hypothesis (Bowles & Koopman, 2010). Moreover sex hormones produced somatic cells of gonad may induce entry of germ cells into meiosis. Often it takes place at maturation time of germ cells in adult animal organism. How sex hormones influence on earlier terms of development so far is not clear.

Sex of embryos is determined chromosome set received him in the moment of fertilization. But at the stage of primary gonocytes male and female germ cells have not differences. Differences appear only after their entrance into the sex gland. In anlages of sex glands gonocytes proliferate for some time by mitosis. Oogonii cease proliferate in embryos period and turn into oocyte I. Then period of growth begins that is associate with accumulation in ovum nutritious substances from outside and with synthetic processes in ovum. The whole period previtellogenesis oocyte I gets ready for meiosis. After oocyte I entries in S-phase of reduction division, DNA amount is doubled and start prophase I of meiosis. Both oocytes and spermatocytes are in need of sex hormones those are produced by surrounding cells (Belousov, 2005).

Aromatase is a *CYP19A1* gene product revealed in both animals and plants and converse androgen to estrogen. Letrozole was used to suppress aromatase. It is less toxic to the organism (which is important for experiments with embryos) and is a nonsteroidal drug. We have shown that the sex hormone misbalance changed the female gonad differentiation into testicle formation (reduced cortex thickness, appearance of seminiferous tubules in medulla) and generation of a hermaphrodite gland, ovotestis. It should be noticed that the right gonad normally degrading during embryogenesis is preserved in genetic females with letrozole inhibited aromatase and morphologically similar to ovary. Androgen overproduction by progonadal cells abrogates the block in the development of the right gonad. The presence of the right gonad and left ovotestis in chicken genetic females creates a false impression of gonadal masculinization. This effect was also revealed by aromatase suppression by other of its inhibitors – fadrazole and vorazole (Vaillant et al., 2001; Yang et al., 2008; Li-xiu et al., 2013).

Sex hormones interact with their specific receptors (ESR1, AR), form complexes binding with regulatory regions in chromatin and initiate the transcription of particular genes (Jafarov et al., 2010). A number of chemical substances are able to limit hormone interaction with their receptors. Thus, tamoxifen is a modulator of the estrogen receptor and competes with estrogen for binding with the same receptor. As a result, estrogen penetrates into the cells of the developing female gonad in a very small, if any, amount. We suggest that the outcome should be gonadal masculinization. Indeed, we have observed this after single or double tamoxifen injection, but the effect was less apparent than after letrozole administration. A masculinization tamoxifen effect has also been observed by other researchers (Hutson et al., 1985). Also we showed that the sex hormones influence on cell differentiation of embryos gonads and formation of gonad tissues structure, physiology conditions for the future ovum and sperm. Alteration of sex hormone balance in genetic determinated embryos leads to gonad development anomalies and sterility.

In chicken meiosis of oocytes I starts in the second half of the embryonic development. At this stage in male gonads only spermatogonia are revealed. Chicken male germ cells enter meiosis only after hatching (Nakamura et al., 2013). We found that oocytes in the left gonad of ovotestis in masculinized females were at the zygotene – pachitene stage of the meiotic prophase, whereas, in the right retained gonad, germ cell differentiation was blocked. It was proposed that meiosis in females was induced by retinoic acid, the action of which may be blocked in males with cytochrome CYP26B1 (Nekrasova et al, 2011). In our experiments, the altered balance of sex hormones synthesized by gonadal cells did not influence the activity of retinoid acid and, accordingly, on the germ cell entering meiosis. The fate of germ cells in female gonads after sexual inversion requires further investigation.

An experimental approach to studying sexual inversion in birds is an important instrument for assessing the genetics of vertebrate sex determination.

It is still unclear when (on day of incubation 3, 4, 5, or 6) and where (air cavity, protein, yolk, or a "layer" of the drug on the yolk surface in the aria of the embryonic disk) it is best to carry out injections to improve drug penetration into the embryo through the blood system.

We also used an injection 5-AC as demethylating agent in the first days of incubation (1, 4 day) in order to cause the inversion of sex through the activation of MHM area of male Z chromosome and the inclusion of the two alleles of the supposed sex determining gene *DMRT1* 1. MHM area is hypermethylated in both Z-chromosomes of males and hypomethylated in the same one female region of Z. It is important that a particular methylation status is already set at 1 day of incubation (Teranishi et al., 2001). In a recent study of brazilian geneticists that used variant of RT-PCR was shown that the largest transcription of MHM for the analyzed period of incubation (4 - 14 days) is detected at 8 and 14. The males in the absence of transcription of this gene show an intense activity of DMRT1 (Caetano et al, 2014). In our experiments we used microinjection in the initial incubation period as especially important for sex determination. It turned out that the appearance of sex inversion quite rare. However, there was entry into meiosis of germ cells of males. Moreover, the cells were observed at the zygotene – pachytene stage that did not occur even with RA injection.

We want to draw attention to some interlocking control somatic and gametic sex in chickens. It is desirable to consider the role of the W chromosome in these processes (Graves, 2014).

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Isolation, Identification and Partial Characterization of Protease Producing Bacteria that Exhibiting Remarkable Dehairing Capabilities

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Abstract- A novel protease producing bacterium was isolated from the natural source. Gram staining & spore staining showed that the organism is gram positive and forms spore during adverse condition in the growth medium. After various tests it was suggested and the features agreed with the description of Bacillus subtilis in Bergey's Manual of Systematic Bacteriology [20]. It was also identified as B. subtilis with 99.9% identity by API 50 CHB and was also characterized and identified by using a bioinformatics tool PIB (Probabilistic Identification of Bacteria) that suggests the organism was B. subtilis (ID=0.9760).

Keywords: protease, identification, characterization, leather dehairing bacteria, isolation.

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Md. Ekhlas Uddin [α], Dr. Mustafizur Rahman [σ], Hossain Md. Faruquee [ρ], Md. Rezaul Islam Khan [ω], Md. Feroz Mortuza[¥], Mohammad Hafizur Rahman[§] & Pulak Maitra [χ]

Abstract- A novel protease producing bacterium was isolated from the natural source. Gram staining & spore staining showed that the organism is gram positive and forms spore during adverse condition in the growth medium. After various tests it was suggested and the features agreed with the description of *Bacillus subtilis* in Bergey's Manual of Systematic Bacteriology [20]. It was also identified as *B. subtilis* with 99.9% identity by API 50 CHB and was also characterized and identified by using a bioinformatics tool PIB (Probabilistic Identification of Bacteria) that suggests the organism was *B. subtilis* (ID=0.9760). In growth curve determination showed that the growth of the organism is increased with the increase of incubation period and the growth reached maximum at around 24 hours of incubation and the protease activity was the maximum of the 26 hours culture. Its optimum pH and temperature were 8.5 and 60°C. The enzyme hydrolyses a number of proteins including azocasein which suggests that it is an extracellular protease. Quantitative estimation has shown that 40mL of culture supernatant could dehair 2×1 cm of leather completely in 9 hours. In future the tanneries will use a combination of chemical and enzymatic processes. A number of companies such as NOVO chemicals started to produce NOVOzymes for tannery industries. The potential for use of microbial enzymes in leather processing lies mainly in areas in which pollution-causing chemicals are being used.

Keywords: protease, identification, characterization, leather dehairing bacteria, isolation.

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I. Introduction

Leather industries are one of the most promising fields for export to earn foreign currency in Bangladesh. Most of the tannery industries in Bangladesh use chemicals for Dehairing that led great environmental and health problem. Leather industry has contributes heavily to environmental degradation. The tannery pollutants are causing heavily damage to water resources, agriculture, fisheries and finally to avoid the deleterious effects of chemical agents in tannery industries [15].

Over the last few decades leather industry is based on large scale chemicals treatment which created worldwide environmental hazards. Recently government of People's Republic of Bangladesh has taken initiative to develop the industry from outside the city and modernize it. In the back drop of this scenario enzymes started replacing poisonous chemicals from tannery industries. A number of industries such as NOVO chemicals started producing NOVOzymes for the tannery industries. With the advent of enzymes leather processing in various countries has become environment friendly. Enzymatic dehairing is suggested as an environment friendly alternative to the conventional chemical process [13]. The use of proteolytic enzymes as an alternative to de-hairing skins has been investigated [14].

Cleaner leather processing biotechnology has been used in the tanning industry for several years. Enzymes can be used at all stages in the leather-making industry, with the exception, perhaps, of the actual tanning process. At present, biological methods are being used with relative success in soaking, de-hairing, BATING and, in part, degreasing. Enzymatic dehairing in tanneries has been envisaged as an alternative to sulfides [14]. Proteases could play an important part in biotechnological applications like enzymatic improvement of feather meal and production of amino acids or peptides from high-molecular weight substrates or in the leather industry [3, 12]. These enzymes could be applied for waste water treatment, textile, medicine,

cosmetic leather, feed and poultry processing industry as well as in the leather industry [12].

II. Materials and Methods

a) Isolation of bacteria from soil sample

The soil sample was collected from the poultry wastes in Savar, after serial dilution, culture were given in LB broth media from the sample for 16 h at 37°C. At the next day single colony was found. Among them few colonies were identified on the basis of different colony morphology. Each colony was inoculated into screw capped test tubes containing autoclaved feather with liquid broth media and incubated overnight at 37°C with shaking at 160 rpm. One media was used as negative control.

b) Bacterial identification

Gram's staining; morphological studies, physiological and biochemical characteristics of the isolate were investigated according to Bergey's Manuals [20]. A rapid bacterial identification test kit for Bacillus, API 50 CHB (BioMerieux, France), was used to identify species of bacteria.

c) Different Biochemical & Microbiological tests for the characterization of the organism:

To identify the biochemical properties of the organism different tests were performed. For correct interpretation of the results in every test Escherichia coli was taken as control. Fermentative capabilities of the isolated organisms were tested under anaerobic conditions in Durham tube. The carbohydrate tests that were performed are the Glucose, Lactose, Ribose, Sucrose, Mannitol, Adonitol, Arabinose, Sorbitol, and Maltose.

Others Biochemical tests that were performed are the Hydrogen sulfide test, Motility Test, Indole Production Test, Citrate Utilization Test, Nitrate Reduction Test, Oxidase test (young culture), Catalase Test, Urease test, Indole (SIM) test, Methyl Red (MR) Voges- Proskauer (VP) Test, Starch Hydrolysis Test and Gelatin Liquefaction Test. Some Microbiological tests that were performed are the Gram staining for the Bacteria, Spore staining, colony morphology and growth curve determination.

d) Isolation of Protease Enzyme and Determination of its Proteolytic activity

The microorganism was cultivated in sterile nutrient broth medium. The culture was grown overnight on a rotary shaker at 150 rpm and incubated at 37°C for 15-20 hours. The culture was then centrifuged at 10000 rpm for 10 minutes at 4°C. The supernatant was collected and used as crude enzyme sample. Proteolytic activities were assayed by Azocasein test, described by Kreger and Lockwood (1981) was done. Here azocasein is used as a substrate. Optical density was measured at 440 nm

e) Determination of effect of temperature on bacterial growth

The bacterial culture was grown in nutrient broth at various temperatures (25°C, 30°C, 35°C, 40°C, 50°C, 60°C) and was incubated for 48 hours to measure its growth profile.

f) Determination of the Effect of Temperature on bacterial growth and Protease Activity

The bacterial culture was grown in nutrient broth at various temperatures (25°C, 30°C, 35°C, 40°C, 50°C, 60°C) and was incubated for 48 hours to measure its growth profile.

For the determination of the effect of temperature, the culture medium was incubated at temperature ranging from 25-60˚C and the protease activity was determined at 37˚C using the usual methods.

g) Determination of the Effect of pH on Protease Activity

For determining the effect of pH on protease activity different buffer system with different pH were used. Azocasein was dissolved in different buffer solution and the enzyme assay was carried out within pH range (4.0 to 10.5) by azocasein assay method. All of them were used at 0.05M concentration.

Table 1 : Different buffer used and their p^H ranges

Buffer	P^H range
Acetate buffer	4.0-5.6
Sodium phosphate buffer	5.6-8.0
Tris HCl buffer	7.5-8.9
Glycine-NaOH buffer	8.6-10.5

h) Determination of the Effect of Temperature on Protease Activity

For the determination of the effect of temperature, the reaction medium was incubated at varied temperature and the protease activity was determined. For this purpose the enzyme preparation was added to a mixture of 1 mg 1 % azocasein solution, 0.1 ml of 0.06 M CaCl2 and buffer (0.2 M Tris-HCl buffer, pH 8.0) and incubated at 37°, 40°, 50°, 60°, 65°C temperatures.

i) Determination of effect of other effectors on Protease Activity

The activity of the isolated protease was tested in the presence of various known protease effectors (all

obtained from Sigma Chemical Co.), EDTA, 2-mercaptoethanol, potassium di-chromate, sodium thiosulfate. The azocasein assay was used with the addition of these effectors solution to achieve a final desired effectors concentration of 5mM. Control was taken where azocasein assay without these effectors was carried out.

j) Determination of effect of salts on Protease Activity

The protease activity was measured with adding different salts like ZnSO4, MgSO4 , CuSO4, NaCl, KCl at different concentration and then azocasein assay was performed.

k) Direct dehairing activity of the enzyme

For de-hairing studies, the organism was grown in nutrient broth at 37°C for around 20 hours. Then it was centrifuged at 4000 rpm for 8 minutes. The cell free supernatant was added on detergent washed goat skin to observed enzymatic dehairing capability of the organism. Sodium azide was used at 1% so that no organism can grow. Nutrient broth was used as control.

III. Results

a) Isolation and characterization of the organism

The main object of this work was to isolate and characterize thermophilic enzyme which could specifically be used for dehairing the hides and skins of cattle in the tannery industries. In this connection three ways were planned. One was to isolate thermophilic organism from different natural sources. The others is to characterize & identification of the isolated organism. The growth phenotype and some of the biochemical characteristics of the organism was determined. This organism was characterized and identified as a member of gram positive Bacillus family by several test. The features agreed with the description of *Bacillus subtilis* in Bergey's Manual of Systematic Bacteriology [20]. It was also identified as *B. subtilis* with 99.9% identity by API 50 CHB and was also characterized and identified by using a bioinformatics tool PIB (Probabilistic Identification of Bacteria) that suggests the organism was *B. subtilis* (ID=0.9760). So this bacteria is named here as a *Bacillus subtilis*. The results are presented here

Table 2 : Different morphological and biochemical test for the identification of *B. subtilis*

Test performed	Observations	Results
Streak plate isolation:		
NA at 37°C	milky colonies	positive
Gram stain	Small violate colonies singly	Gram positive rods
Spore stain	green color appeared	spore forms
Cultural characteristics:		
Nutrient Agar plates	growth on NA plates	small, non-pigmented, circular
Nutrient Broth	growth on NB	uniform fine turbidity
Nutrient agar slants	Growth on NA slant	moderate, non pigmented
Catalase test	bubbles formed	Positive for catalase production
Oxidase test	Black color formed	positive for oxidase production
Acid & gas production:		
Glucose	yellow	positive for acid and negative for gas
Sucrose	yellow	positive for acid only
Mannitol	red	Negative for acid and gas
Adonitol Arabinose Sorbitol Maltose	red yellow red red	Negative for acid and gas positive for acid only Negative for acid and gas Negative for acid and gas
IMViC test:		
Indole (SIM) test	bright red ring, growth away	Positive for indole and motility
H_2S test	from stab, black color	Positive for H2S production
Methyl red test	deep red ring formed	positive for mixed acid production
Voges-Proskauer test	weak red ring formed	positive for acetoin production
Citrate test	change in color	positive for citrate utilization
Urease test	no bright pink color	negative for urea catabolism
Nitrate test	no color change after zinc dust addition	positive for nitrate reduction
Gelatin test	remain liquefied at 4°	positive for gelatinase production
Starch test	bright zone	positive for starch hydrolysis

b) Determination of Proteolytic Activity of the Enzyme

Proteolytic activities were assayed by Azocasein test, described by Kreger and Lockwood (1981) was done. Here azocasein is used as a substrate. The proteolytic activity was found as 21.13 units for the sample. One unit of proteolytic activity is defined as the

amount of enzyme that produces an increase in the absorbance of 0.01 at 440nm.

c) Effect of temperature on bacterial growth

The aim of this experiment was to monitor the effect of temperature on the bacterial growth. For this purpose this organism was grown in nutrient agar medium at various temperatures (25°C, 30°C, 35°C, 40°C, 45°C, 50°C) for 48 hours and observed the growth profile of the bacteria.

Table 3 : Growth profile of the organism at different temperature

Temperature(°C)			
Time of incubation	Results	Absorbance at 600nm	
25°C	24	+	0.50
	48	+	Not done
30°C	24	++	0.745
	48	++	Not done
35°C	24	+++	0.950
	48	+++	Not done
40°C	24	+++	0.820
	48	+++	Not done
50°C	24	+	0.630
	48	+	Not done

- (NO growth) ± (Some colonies can be seen) + (Moderate growth) ++ (Good growth) +++ (Very good growth)

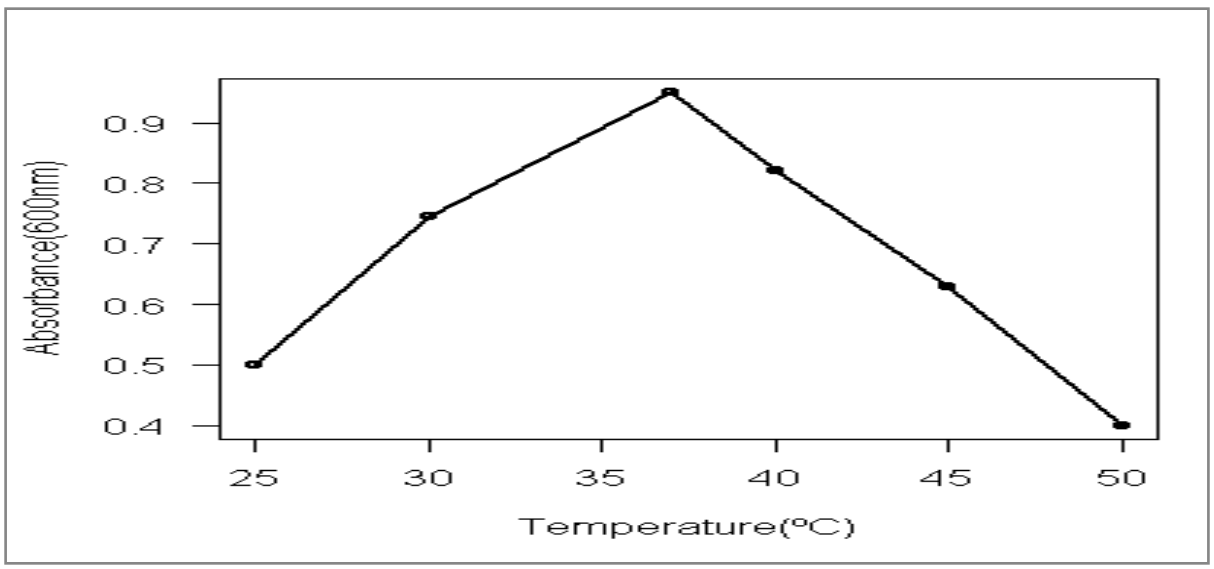

Figure 1 : Graphical presentation of effect of temperature on the organism growth

d) Growth profile and protease activity of the organism at 37°C

The organism was grown in nutrient broth at 37°C. Samples were taken at different time interval and absorbance was taken at 600nm to measure the growth profile. The growth profile of the organism showed that the organism showed optimum growth after about 24 hours and the protease activity was the maximum after 26 hours of incubation.

Table 4 : Growth profile and protease activity of the organism at 37°C

Time at hours	Absorbance at 600nm	Absorbance at 440 nm
4	0.562	0.030
6	0.756	0.052
8	0.864	0.141
10	0.978	0.185
12	1.132	0.212
13	1.197	0.403
14	1.257	0.569
15	1.357	0.578
16	1.393	0.844
18	1.432	1.108
20	1.604	1.497
22	1.731	1.612
24	**1.826**	1.836
26	1.75	**1.924**
28	1.728	1.735

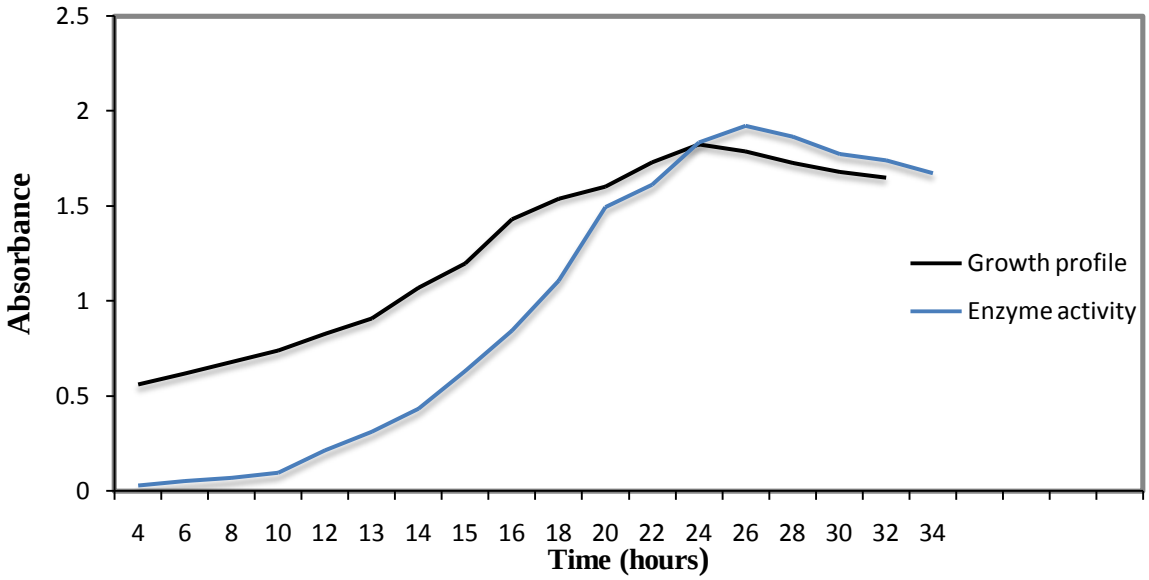

Figure 2 : Graphical presentation of growth of bacteria and protease activity at different time interval at 37° C

In the initial stage of growth there was basal level of extracellular protease which increased with the increase of time. The result showed that there was differential synthesis of enzyme with growth time.

e) Effect of temperature on enzyme activity

The activity of the enzyme was measured over a range of temperature (0°C, 4°C, 20°C, 30°C, 37°C, 40°C, 50°C, 60°C, 65°C, 80°C) and the result is presented in Table-5 and Figure-3.

Table 5 : Protease activity at different temperature (by Kreger and Lockwood method)

Temperature	Absorbance at 440nm
0°C	0.009
4°C	0.019
20°C	0.121
30°C	0.181
37°C	0.183
40°C	0.191

50°C	0.205
60°C	**0.250**
65°C	0.105
80°C	0.019

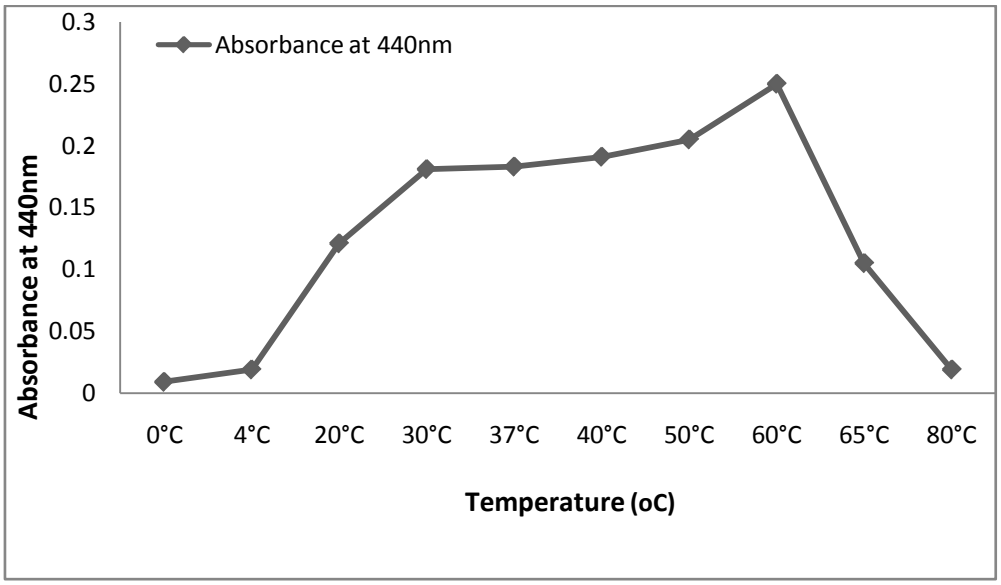

Figure 3 : Graphical presentation of protease activities at different temperature

The enzyme activity is increased with the increase of temperature. The experiment was reported 2 times and the result is reproducible. There was a significant increase in enzyme activity between 20°C to 55°C. The enzyme seems to be active at 60°C and its activity declines as the temperature increase beyond 60°c. At 80°c the enzyme has very little activity. This suggests that the enzyme might be a thermos table enzyme.

Fig-3 shows that the protease was active over a temperature range of 4°C ~80 °C, with an optimum at 60°C. Most proteases possess an activity optimum in the range of 30~80 °C, for example, protease from *B. pseudofirmus* AL-89 is of 60~70 °C [5] and a few have exceptionally high temperature optimum of 100 °C [13].

f) Effect of pH on protease activity from the organism

The pH of the reaction media can affect the protease activity. For this purpose the enzyme activity over a pH range between 4 and 11 was studied.

Table 6 : Effect of pH on Protease Activity

pH	Activity of Enzyme(unit)
4.0	28
5.0	36.5
6.0	48
7.0	63
8.0	68
8.5	**70**
9.0	66
10	60
11	48

The enzyme shows its maximum activity at pH 8.5. The activity decline at pH 8.0 or above 8.5. Therefore pH 8.5 might be the optimum pH for enzyme activity. Additionally, its optimum pH was similar to that of previous reports [18]. Most proteases are active in neutral to alkali conditions, from pH 7.0 to pH 9.5. For example, the activity optimum of protease from *Mycobacterium* kr10 is pH 7.0 [22], *B. pumilus* FH9 of pH 8.0 [4], *Fervidobacterium islandicum* AW-1 of pH 9.0 [13].

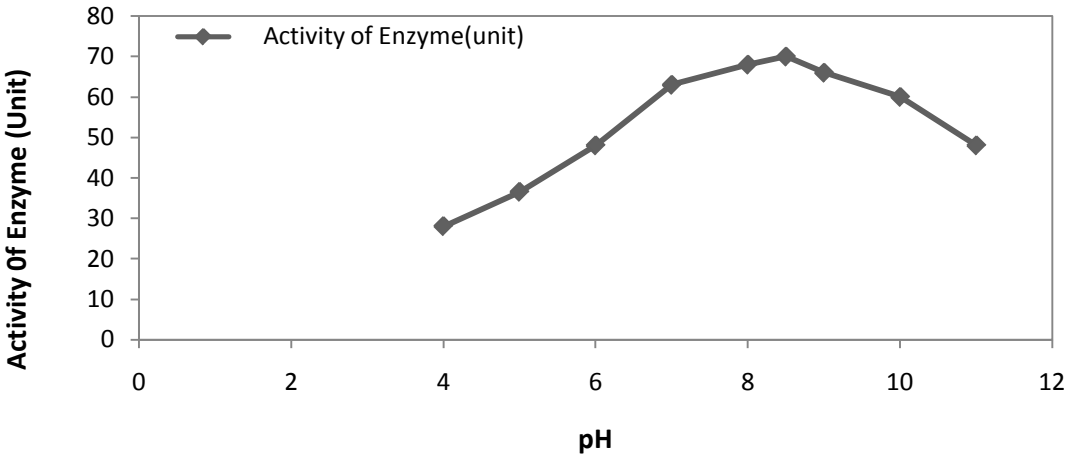

Figure 4 : Graphical presentation of effect of pH on protease activity

The fig-4 shows that the enzyme activity increase with the increase of pH of the media and the optimum pH is 8.5 for the activity of protease enzyme in Tris-HCL buffers. The results showed that the optimum pH of the protease enzyme was 8.5. Studies on growth temperature and pH suggest that the organism might be alkaline and thermophilic Bacillus.

g) Effect of salts and other effectors on the protease activity

The effect of different salts (MgSO4, ZnSO4, CuSO4, NaCl, KCl) and other effectors (EDTA, 2-mercaptoethanol, sodium thiosulfate) at different concentration was measured. MgSO4 increased the activity and β-Mercaptoethanol decreased the activity of the enzyme. NaCl didn't change the activity. Others had little deactivating effect.

Table 7 : Effects of salts and other chemicals on the activity of the protease

Compound (concentration in mM)	Caseinolytic activity (%)a
Control	100
$MgSO_4$ (5)	109
$ZnSO_4$ (5)	77.2
EDTA (5)	92.5
EDTA (5) + $ZnSO_4$ (5)	82.5
EDTA (5) + $MgSO_4$ (5)	102
EDTA (5) + $CuSO_4$ (5)	84.2
NaCl(100)	100
NaCl(200)	100
β-Mercaptoethanol(5)	44.9
Sodium thiosulfate(5)	78.9
Potassium per manganate(5)	96.7

a Caseinolytic activity is expressed as the percentage of the control value (with no addition).

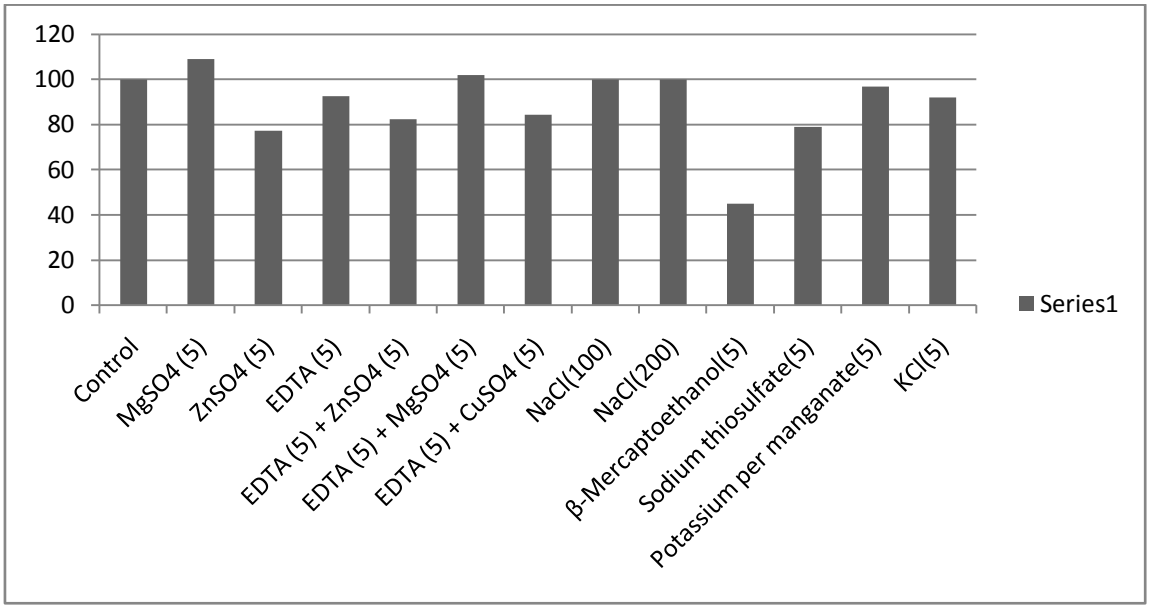

Figure 5 : Graphical presentation of effects of salts and other chemicals on the activity of the protease

The result shows that 5mM Mg^{++} ion slightly increased the activity of the enzyme while Zn^{++} showed slightly decrease. Other elements Na^{+}, K^{+} had no effect on the enzyme. EDTA showed no effect on the protease activity which suggested that the enzyme might not be metallo protease. The enzyme activity was significantly reduced by β- Merceptoethanol. β-Mercaptoethanol has been reported to stabilize cystein proteases by protecting the oxidation of sufhydral group in proteins [19]. No effect of EDTA was detected on enzyme activity suggesting that the metal might not be involved in enzyme activity [11].

h) Determination and Observation dehairing activity of the enzyme

For dehairing studies, the cell-free supernatants were used as sources of crude enzyme. The treated skins and controls showed visible differences after 9 h incubation. No color alteration was observed, although the presence of depilated areas was noticed in the skins treated with enzymes. When hairs were pulled with a forceps, they were very easily released after enzyme treatment.

After 9 h incubation intact hairs could be taken out of the skins easily by simple scraping. In controls, hair loosening was not observed, even by the mechanical action of a forceps. This result was much better than other different bacteria that also caused dehairing. Proteases have been used in the hide dehairing process, where dehairing is carried out at pH values between 8 - 10 [7].

Figure 6 : Enzymatic dehairing by *B. subtilis* on left, control is on the right (without enzyme)

Comparison of dehairing ability of Bacillus subtilis with other bacteria

Dehairing ability of the protease produced by our strain and other bacterial protease showed that our bacterial protease is very fast in dehairing compared to other three.

Table 8 : Comparison of dehairing ability of *B. subtilis* with other bacteria [1]

	Time of incubation for dehairing	Change of color of leather
Bacillus subtilis	9h	no change
Vibrio sp kr2	24h	no change
Flavobacterium sp kr6	24h	no change
Bacillus sp kr10	24h	no change

IV. Discussion

A protease producing bacterium isolated from local soil sample showing de-hairing activity of cow hides and skins both qualitatively and quantitatively. Gram staining & spore staining showed that the organism is gram positive and forms spore during adverse condition in the growth medium. After various tests it was suggested and was also characterized and identified by using a bioinformatics tool PIB (Probabilistic Identification of Bacteria) that suggests the organism was *Bacillus subtilis*. Biochemical characteristics, morphological tests indicate that the organisms might be *Bacillus. B. pumilis*, *B. licheniformis* [8]. The feathers agreed with the description of *Bacillus subtilis*. In Bergey's Manual of Systematic Bacteriology [20].

Azocasein assay developed by Kreger and Lockout is a well accepted method for the assay of wide variety of protease having overlapping specificity. This assay method is simple, easy and quick. A number of protease from different bacteria can be assayed at a time by using this method. Bacillus species have been reported to produce proteases [9, 21, 23]. Therefore, it may be called a very good method for the large scale screening of bacterial protease [6].The characteristics of the culture supernatant suggest that it contain an extracellular enzyme secreted by the bacterium. The enzyme hydrolyses a number of proteins including azocasein which suggest that it is an extracellular protease [2].

In growth curve determination showed that the growth of the organism is increased with the increase of incubation period and the growth reached maximum at around 24 hours of incubation and the protease activity was the maximum of the 26 hours culture. The enzyme seems to have an optimum temperature of 60°C. Most proteases possess an activity optimum in the range of 30~80 °C, for example, protease from *B. pseudofirmus* AL-89 is of 60~70 °C [5], *Nocardiopsis* sp. TOA-1 is of 60 °C and a few have exceptionally high temperature optimum of 100 °C [13].

The effect of different pH ranges on the proteolytic activity of the crude enzymes produced by *B. subtilis* was studied. The enzyme seems to have an optimum temperature of 8.5. Additionally, its optimum pH was similar to that of previous reports[17].The pH value of culture increased to about 8.5. *B. subtilis* strains had been widely utilized for enzyme production, including the proteases [9, 10]. Studies on growth temperature and pH suggest that the organism might be alkaline and thermophilic Bacillus. Preliminary studies on enzyme activity show that the enzyme might be thermophilic alkaline protease.

The effect of a number of ions on the activity of the enzyme was observed. Mg^{++} at 5-10mM level slightly enhances the enzyme activity while Zn^{++} ions slightly decrease the activity of the enzyme. β- Mercepto ethanol is an inhibitor of protease. β-Mercaptoethanol has been reported to stabilize cystein proteases by protecting the oxidation of sufhydral group in proteins [19]. No effect of EDTA was detected on enzyme activity suggesting that the metal might not be involved in enzyme activity [11].

Enzymatic de-hairing may be the ideal de-hairing process. Cow skin was qualitatively dehaired by overnight grown bacterial culture. The skin could be dehaired at room temperature within 8-12 hours. After 9h incubation intact hairs could be taken out of the skins easily by simple scraping. Quantitative estimation has shown that 40mL of culture supernatant could dehair 2×1 cm of leather completely in a 9 hours. This shows that the bacterial isolate moderate to high amount of enzyme for dehairing. Enzymatic dehairing in tanneries has been envisaged as an alternative to sulfides [15]. A significant feature of the enzymatic de-hairing process is complete hair removal and minimal usage of sulfide and the decomposition products formed from the tannery wastewater, with great improvement in wastewater quality as a result.

V. Conclusion

The results presented in this work indicate the bacterial isolates might belong to *Bacillus subtilis*. The

enzyme produced by the bacteria can be utilized in enzymatic dehairing of cow skin in tannery industry to control the environment from pollution. Circumstantial evidences are there to suggest that the enzymes might be proteases. The culture characteristics and biochemical tests of the organism suggest that it is a thermophilic, Gram positive, spore forming and aerobic bacteria. The characterization of protease so far showed that it is an alkaline protease, highly active at temperature near 60°C. The sequencing of the protein and identification of the gene is the future plan of the research work.

As the bacterial protease showed high activity in dehairing of cow skin and our next target is to introduce it to the tannery industries, so that they can use it instead of hazardous chemicals for better leather quality and most importantly for a better environment. Both the isolation, partial purification procedure set up and the characterization study of the protease were important to foresee potential production and uses of this enzyme.

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Demographic Structures of Gelada (*Theropithecus gelada*) in Guassa Community Protected Area, Ethiopia

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Abstract- In geladas, sociality and population structure are unusual because they establish highly differentiated bonds with other group members. Such bonds are particularly pronounced among female geladas, with female philopatry and male dispersal. In the present study, gelada band size and population structures varied between wet and dry seasons. The mean number of females per harem varied from 4.5 to 7.1. In addition, the mean harem, or reproductive female size in all study bands is not statistically significant within season, or between seasons ($P > 0.05$). The sex ratio did not also show any significant differences between seasons. However, the adult sex ratio and the mean number of reproductive females within the units were statistically significant ($t_{10=}$ -5.6, $P < 0.05$). Gelada population growth rate is relatively high (mean = 17.1 % per annum) in Guassa Community Protected Area, and it could be deduced by the differences in number of birth and death at a given period of time within the natural population. Ratio of females per male was relatively lower in the study bands of geladas in present study area, and it doesn't show a true relationship annual growth rate of gelada population. However, the number of births per female/year has brought a significant change on gelada population.

Keywords: *band, birth and death rates, gelada, growth rate, population structure, unit.*

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Eshetu Moges [α] & M. Balakrishnan [σ]

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I. Introduction

The gelada baboon (*Theropithecus gelada*) pose multi-female organization is known to have a multi-level (modular) social system (Le Roux *et al.*, 2011). Thus, although this species attests to the relationship between philopatry and stable dominance hierarchies (Dunbar, 1986). Among the Cercopithecine, gelada exhibits multilevel societies based on one-male unit system (Mori, 1979; Kawai *et al.*, 1983). Each one-male unit has a number of adult females and one leader male that has exclusive reproductive access to unit females. Females within one-male units are reported to have close female bonds (Johnson *et al.*, 2013) and are also thought to be philopatric with respect to the unit (Dunbar, 1993). Gelada units and bands are relatively stable in composition during stable environmental conditions (Ohsawa and Dunbar, 1984), although this stability may break down in more extreme environments (Mori *et al.*, 1999). Thus, geladas present an ideal example for examining the relationship between a multileveled society, male dispersal and female philopatry (Le Roux *et al.*, 2011).

Demographic structures of geladas more likely shaped by both dependant and independent factors (Ohsawa and Dunbar,1984). Population variables can also determine by a combination of environmental, demographic and social factors interacting in complex ways (Altmann and Altmann, 1970). Geladas birth rates and survivorship (growth) are adversely influenced by local climatic conditions. These independently influenced sex ratio, which in turn determine the proportion of male to female ratio and multimale reproductive units in the population (Hill and Lee, 1998).

II. The Study Area and Methods

a) The study area

The Guassa Community Protected Area (GCPA) is one of the high altitude ranges in the central highlands of Ethiopia located at a distance of 265 km from Addis Ababa, in the north-east direction, and 135 km from the zonal capital (Debre Birhan) in the north direction, this area lies between 10° 15'–10° 27' N latitude and 39° 45'–39° 49' E longitude (Fig.1). The GCPA with a total area of 111 km^2, forms part of the western edge of the Great Rift Valley, at an altitude range of 3, 200–3,700 m asl. Rainfall of the area is characterized by a bimodal pattern. The major wet season occurs during June and September and a short rainy season during February and April. The annual rainfall in the area ranges from 1,200 to 1,600 mm. Temperatures of the area is characterised by mild days and cold nights. In the driest months (December–February), day time temperatures can rise upto 25°C, while night time temperatures may fall to –7°C (a diurnal fluctuation of 32°C). The area is characterized by high altitude vegetation types. Traditional indigenous management of natural resources in the area has helped the survival of various species of endemic fauna and flora that are locally extinct in similar parts of the country (Zelealem and Leader-Williams, 2005; Zelealem *et al.*, 2012).

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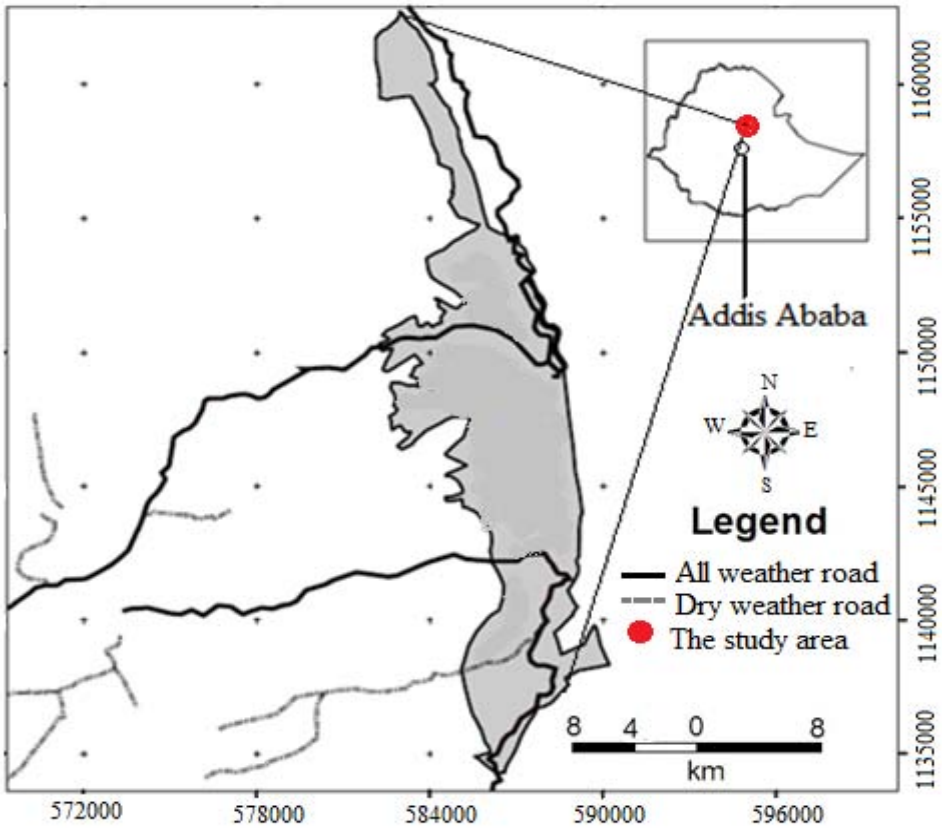

Figure 1 : Map of the study area

III. Methods

In the beginning of the study, an intensive effort was made to obtain a complete census of all the units in the main bands. Intact units in the band were randomly selected in order to collect data about population structure and behavioural activities. The geladas were followed walking slowly from a distance of around 5 m and data were collected by means of focus group sampling (Altmann, 1974). Data were collected five days a week, between 07:30 h–18:30 h. For population estimation and growth change of geladas in the study area, sweep census technique was used (Beehner *et al.*, 2008) regularly at least once per month in each of the study sites across the study period, covering both wet and dry seasons.

IV. Results

a) *Demographic structure of the gelada population*

A total of 1502 individuals of geladas were recorded in the six study bands in GCPA during the present investigation. The population size of each these bands and their structure varied between wet and dry seasons. Results on demographic structure of geladas have indicated that there was no difference in the mean reproductive unit size between seasons (Kruskal-Wallis H test, $P > 0.05$) (Table 1). Kruskal-Wallis H tests showed that there were significant differences in the band size of study population of Tsewo, Baltegra, Sefedmeda, Wochanka, Dejameda and Atsewuha between seasons ($P < 0.05$).

Table 1 : Demographic structure of geladas in dry and wet seasons

Study site	Season	Band size	Number of Units	Number of AMU (x̄)	Repro-ductive unit size(x̄)	Adult sex ratio (F:M)	Adult females/ reproductive unit (x̄)	Sex ratio (F:M)	Multi-male units (%)
Tsewo	Dry	185	13	1	13.7	3.5	5.4	1.4	13.3
	Wet	233	14	1	15.2	3.7	6	1.7	28.6
Baltegra	Dry	249	16	2	14.1	2.3	6.1	1.5	37.5
	Wet	260	17	2	14.1	2.2	5.9	2.2	41.2
Sefedmeda	Dry	203	18	2	10.5	2.4	4.6	1.6	16.7
	Wet	260	19	2	12.9	2.5	4.6	1.3	21.1
Wochanka	Dry	532	24	3	20.1	3.2	7.1	1.8	25
	Wet	229	20	3	11.4	3	4.1	1.4	20
Dejameda	Dry	174	14	2	11.4	2.8	6	1.7	14.3
	Wet	246	16	2	14.1	2.7	5.8	1.7	18.5
Atsewuha	Dry	209	16	1	12.5	4.7	6.5	2.3	12.5
	Wet	235	17	1	13.4	4.3	6.1	2.1	17.6

The mean number of females per harem varied from 4.5 to 7.1 for the 6 bands. The mean harem, or reproductive female size in all study bands is not statistically significant within season, or between seasons (Kruskal-Wallis H test, $P > 0.05$). However, the mean size of the reproductive unit (all ages and sex) of the geladas was significantly larger at Wochanka ($P < 0.05$).

The sex ratio (females per male) was not statistically significant between seasons (Kruskal-Wallis H test = 0.01, df_1, 3.34, $P > 0.05$). There was no significant difference in the sex ratio within season across the study bands ($P > 0.05$). However, the adult sex ratio and the mean number of reproductive females within the units were statistically significant ($t_{10=}$ -5.6, $P < 0.05$). In most cases, the reproductive unit has one adult male. However, some reproductive units contain more than one adult male (multimale units). The percentage of multimale units differed significantly between seasons (Mann-Whitney U test, $P < 0.05$).

Population growth rate per annum for the six bands are given in Table 2. The growth rate based on the number of births and deaths relative to the band size, (however migration was not been included due to high fusion and fission rates). Populations of geladas were increased (overall mean = 17.1 % per annum). There is a close correlation between annual growth rate and birth rate. The overall mean growth rate was higher at Tsewo (20.3 % per annum, n = 47) than at Baltegra (12.7 % per annum, n = 39). The overall mean mortality rate was low (1.4 % per annum), and the lowest mortality rate was recorded at Deja Meda (0.78 % per annum), and the highest mortality rate was recorded at Wochanka (1.99 %).

The number of females per male was relatively lower in the study bands of geladas (Table 2). The lowest ratio of females to male was recorded in Baltegra and Sefed Meda bands (2.4:1). However, females to male ratio in Atsewuha were higher (4.5:1) as compared to the other studied bands.

Table 2 : Estimates of annual growth rates for the six gelada bands in Guassa Protected Area

Bands	Seasons	Females/ male	Birth/ female/ year	Gross annual growth rate	Mortality (n)	Mortality rate (%)	Net annual growth rate
Tsewo	Dry	3.5	0.6	+23.2	5	2.7	20.5
	Wet	3.7	0.6	+21.0	2	0.86	20.1
Baltegra	Dry	2.3	0.5	+18.1	6	2.41	15.7
	Wet	2.2	0.3	+11.2	4	1.54	9.7
Sefedmeda	Dry	2.4	0.5	+19.2	3	1.47	17.3
	Wet	2.5	0.7	+23.2	1	0.49	22.7
Wochanka	Dry	2.8	0.5	+21.3	5	0.93	20.4
	Wet	3.2	0.5	+17.3	7	3.05	14.3
Dejameda	Dry	2.8	0.3	+16.1	2	1.15	14.9
	Wet	2.7	0.5	+19.5	1	0.41	19.1
Atsewuha	Dry	4.7	0.3	+14.8	3	1.43	13.7
	Wet	4.3	0.4	+16.5	1	0.43	16.1

** Migration and emigration of each band have been not considered to this data.*

As the regression line shows in (Fig. 2) the value of R-squared on the graph for female per male with an equation $y = - 0.004x + 3.162$, $R^2 = 0.000$, the slope is not significantly greater than 0 ($t_{22} = 1.72$, $P > 0.05$), hence, it doesn't show a true relationship between the number of female per male and annual growth rate. However, the number of births per female/year has brought a significant change in the population of gelada, and hence the regression line, $y = 0.029x - 0.033$, $R^2 = 0.726$. From the analysis, the value of R is closer to 1.0, better the fit of the regression line ($P < 0.05$), i.e, the closer the line passes through all of the points. Eventhough, information such as the number of data points to make an accurate statistical prediction as to how well the regression line represents the true relationship, the equation two represents a better relationship of birth per female/year and annual growth of gelada (Fig. 2).

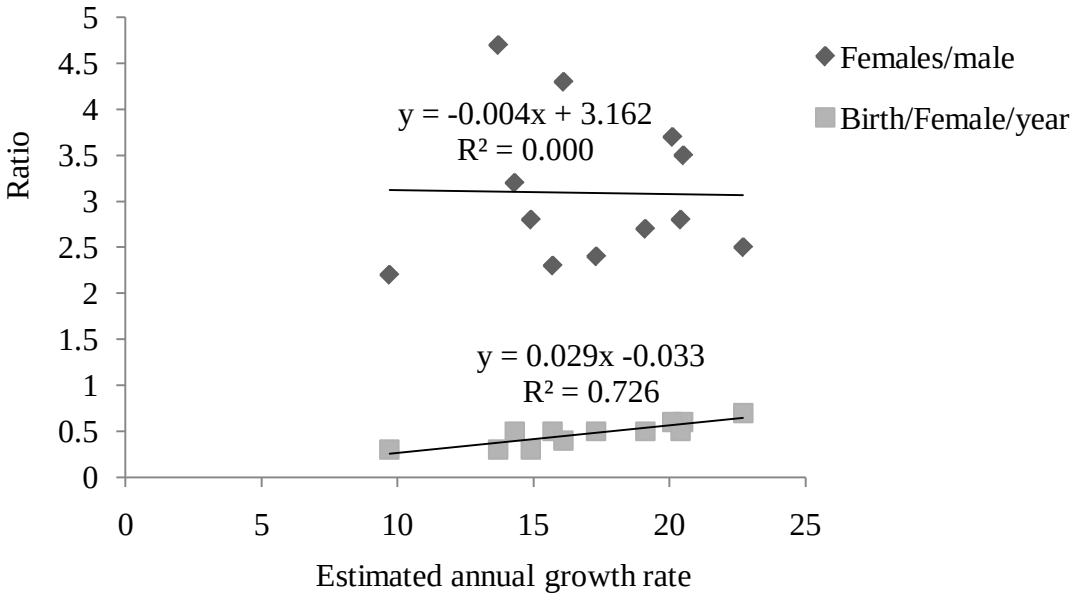

Figure 2 : Annual growth rate of geladas in Guassa Community Protected Area showing females to male and birth per female/year

V. Discussion

In the present study area, the size of the reproductive unit of gelada was not significantly different between seasons. However, there was a difference in the band size between seasons. This might be in relation to the variations in food availability between seasons. The variation of unit size within the band is probably due to the demographic processes related to the unit. Variations in band size between the study sites may be due to the dynamics of individual bands driven by demographic processes. Studies in Semien Mountains National Park in Ethiopia have also revealed similar variations of the band size across populations of geladas due to internal changes (Ohsawa and Dunbar, 1984) that can not be determined by generalized environmental factors such as rainfall and temperature. Moreover, Dunbar (1980) also stated that internal processes like rates of birth and death could affect band sizes of geladas differently over time.

The mean number of reproductive females in the study bands did not show a significant change between seasons. This might be due to the fact that gelada females undergo long period of time in the category of harem, or reproductive females. The sex ratio of the study population also did not show major changes between seasons. This is probably due to sex ratio in the study population is insensitive to environmental variations between seasons. Similar findings were also reported by Ohsawa and Dunbar (1984), who stated that the sex ratio at birth is independent to changes in environmental conditions. In the present study, the number of multimale units varied between seasons. This might be due to the instability of units in relation to food availability. During the dry season, resource availability usually decreases, agonistic interactions become intense, and the follower adult and sub-adult males leave their natal unit and join the peripheral males. In contrary, during the wet season, some members of all male unit system may join reproductive units, after aggression is getting decrease within the unit. Similarly, Dunbar and Dunbar (1974) and Mori (1979) noted that acquisition of harems occurs through the entry of males from all male groups into reproductive units, thereby forming multi-male units. It is predicted that many of the variables correlate with each other in a predictable way due to the effect of increasing competition as more males are excluded from reproduction or holding harems. Furthermore, Grueter and Zinner (2004) noted that the change in multi-male units over time is due to 'freelancers'. They are adult male geladas appear to represent prospect future member of the all male unit, and sometimes move with an AMU. They have also close relationship with specific units.

The population growth rate of the present study bands of geladas is little higher than that in the Semien Mountains National Park (Ohsawa and Dunbar, 1984). The mean growth rate of the study population was 17.1 %. This high annual growth rate of the gelada population may be due to the better conservation practices undertaken in the area through the GCPA. The number of reproductive females per male was minimial, and the average size of females to the male enables to decrease intense competition of females to access male so that it has a great contribution for rapid growth of population of geladas in the study area. Other probable reseason for the high annual growth rate of the gelada population might be due to low mortality rate. However, the mean annual growth rate varied from one study band to the other. This might be due to the variation in the overall environmental factors such as food, human effect and predators, and internal demographic processes of the band concerened. Similarly, Ohsawa and Dunbar (1984) have reported that the mean growth rate varied from one band to another in the Semien Mountains National Park. It was associated with the variation in climatic conditions between the study bands. Mortality rate also varied from one study band to another. Similarly, in the present study area, mortality rate varied from one study band to another. This is probably due to different factors, such as disease caused by taenia worm. Exceptionally, gelada population in the Guasssa area, as it was observed, has been seriously affected by this infectious diseases and the animal will not survive more than six months after infection. Moreover, killing or trapping by human and predators mainly in the case of the Wochanka band may increase the mortality rate of geladas in GCPA.

In the present study population, the number of adult females per male varied from one band to another with a range of 2.4:1 to 4.5: l. This might be due to the variations in the composition of bands, which results in differences in the ratio of females per male. Dunbar (1980) also noted similar sex ratio of adult female to male in the Semien Mountains National Park. However, studies carried out by Zewdu *et al.* (2013) in Wonchit Valley, Ethiopia showed relatively high sex ratio of adult females to males that varied from 6.5:1 to 6.7:1. The present study has revealed that annual growth rate has no direct relationship with the number of females per male. This might be due to the fact that the growth rate is directly determined by the number of birth per female/year, which in turn is influenced by the habitat quality and demographic processes. Similarly, Dunbar and Sharman (1983) have reported that birth rate (number of births per mature female per year) contributed for the population growth, and against the adult sex ratio. However, sex ratio is a causal precursor of birth rate (Ohsawa and Dunbar, 1984).

VI. Conclusion

The present investigation provides useful information on gelada population structure and growth rate of the species in the area. Average reproductive unit size in geladas did not show a significant difference between seasons in each study bands. Variations of unit size in the band between seasons have no significant change on the average size of the reproductive unit. Band size of geladas varies between seasons. The study also revealed that gelada population growth rate is high in the study area. This shows that threats of predators in the area are minimal. The area with good ground vegetation shows the positive effect of better conservation practices undertaken during the study period.

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Systematic Values of Foliar Anatomical Features in some Members of Nigerian Clusiaceae

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Abstract- Foliar epidermal features of four Nigerian species of the family Clusiaceae were studied. This was with a view to exploiting their systematic and taxonomic values to aid their taxonomy. Representatives of the four species were obtained from various parts in Southern Nigeria and passed through standard treatments to make permanent anatomical slides for the study by light microscopy (LM). Micrographic evidences of distinguishing and affinity taxonomic features were recorded. Variations in stomata, epidermal and guide cells attributes were obvious and they could be used as systematic evidence to taxonomically delineate these taxa even at generic levels. Data accruing from this study could help to resolve the taxonomic problems in this family and confirm their identity.

Keywords: *epidermal features, trichome; anatomical, clusiaceae, nigeria.*

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I. Introduction

The Clusiaceae formerly recognized as Guttiferae includes herbs, shrubs, trees with sap resinous and oil glands present in most species. Leaves are opposite or whorled, rarely alternate and exstipulate. Flowers are usually unisexual, sometimes bisexual on the same plant and functionally polygamodioecious, actinomorphic with sepals of 2-10 or more (Keay 1954). Petals range from 4-12 usually imbricate, subvalvate or contorted. Stamens are few or numerous, hypogenous, distinct and variously united. The anther is 2-celled and dehiscing longitudinally with 1 pistil and superior ovary (Robson, 1961).

The family is distributed mainly in the temperate and tropical regions of the world (Keay *et al*., 1964; Kokwaro, 1976). Robson, (1961) and Matig *et al.* (2007) opined that, they occur commonly in humid lowland rainforest or gallery of West and Central Africa sub regions, extending from Congo to Sierra Leone, Madagascar and the Mascarene Islands. The major centers of diversity and species richness of this family in Nigeria are mainly in the Southern and Southeastern parts of the country (Keay, 1989).

In Nigeria, the family is traditionally represented by 16 species distributed in 5 genera, consisting of *Symphonia* L., *Allanblackia* Oliv., *Pentadesma* Sabine., *Mammea* L. and *Garcinia* L. (Keay, 1954; Gill, 1988). However, the reclassification by Keay (1989), to include other genera such as *Vismia* Vand., *Harungana* Lam *ex* Poir. and *Endodesmia* Benth makes them vulnerable to much debate.

Gustafsson *et al*. (2002) started the phylogeny reconstruction of the Clusiaceae using the chloroplast gene rbcL. The analysis provided support for the monophyly of three clades viz., Kielmeyeroideae, Clusioideae, and Hypericoideae + Podostemaceae, except for *Clusiella*, that they traditionally placed in Clusioideae, when it initially appeared in Kielmeyeroideae. Sharma *et al* 2013) noted that the Clusiaceae, even with the removal of the Hypericaceae (from the traditional Guttiferae Juss.), remains a heterogeneous agglomeration partly due to their taxonomic works which are confined to restricted geographical areas and unclear mode of reproduction Malaysia (Whitmore, 1973) and tropical Africa (Robson, 1961; Bamps *et al*., 1978). In the same line Nnamani and Nwosu, 2013a & b) used pollen morphology and distribution of secretory canals respectively to verify their taxonomic status.

The use of data generated from leaf epidermal studies in resolving the taxonomic problems in plants have gained much recognition for a very long time (Aworinde *et al,* 2009). They reiterated that the epidermal and cuticular traits of plants could serve as vital tools exploitable in the systematics of the present day angiosperms. Leaf epidermal features in systematic botany is now popular just like the use of other markers like DNA sequencing and chemical compositions in providing valuable data for taxonomic affinity (Edeoga and Ikem, 2001; Mbagwu and Edeoga, 2006). It is the second most important character after cytology for solving taxonomic problems (Folorunso and Olaniyan, 2009).

Foliar epidermal features particularly the stomata have proved very useful in the delineation of doubtful families (Yasmin *et al.,* 2009). The major aim of this work is to present a more precise characterization of the foliar epidermal features using light microscopy to identify the taxonomic potential in some members of Nigerian Clusiaceae. The main objectives were to 1) determine the various types of epidermal features and 2) utilize the data occurring from the stomata to aid the classification of Nigerian Clusiaceae.

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II. Materials and Methods

a) Study Location

This study was conducted in Southeastern Nigeria that covers an area of 95,488 sq km. It lies between Latitudes 4° and 7° North of the equator and Longitudes 3° and 15° East of the Greenwich Meridian (Iloeje, (1999) Ofomata, (1975). These regions comprise of Abia, Anambra, Ebonyi, Enugu and Imo States of Nigeria.

b) Selection of Taxa

Plants used for this study were from four genera out of the eight genera recorded in Nigeria by Keay (1989). Representative species examined were, *Harungana madagascariensis, Garcinia kola Allanblackia floribunda* and *Pentadesmia butyracea.* This decision was based on the availability of samples, considering the fact that *Garcinia kola, Allanblackia floribunda* and *Pentadesma butyracea* are listed as "threatened to near endangered species" in (Cheek, 2004; Isichei, 2005).

c) Leaf Epidermal Structures

Leaves for epidermal studies were collected fresh from samples growing in the field. To avoid and minimize spatial heterogeneity effects on foliar epidermal characters, sample materials for slide preparations were taken from the same internodes of each branch. The method used is the Impression Technique, where a thin layer of colourless nail vanishes was spread over the leaf surfaces and allowed to dry for 1 hour. Peels were made from both adaxial and abaxial surfaces of each leaf with the aid of a transparent adhesive cello tape on these regions. These strips were mounted on glass slides.

d) Microscopy

Both qualitative and quantitative micro morphological foliar characters were observed using Olympus CH Trinocular Microscope (LM), fitted with 650 IS Cannon Digital Camera. Four slides were prepared for each of these samples. The epidermal features studied include: Nature of the epidermal cells and cell wall, types of trichome (if present or absent), distribution and types of stomata, stomata frequency, length and breadth of stomata. Other dimensions of stomata considered were, length and breadth of guard cells, shape of stomata, size of the stoma, pore length and breadth, nature of subsidiary cells and stomata index (SI) Metcalfe and Chalk (1957) which was given as:

$$SI = \frac{S}{S + E} \times 100$$

Where: S = the number of stomata per field of view,

E = the corresponding number of epidermal cells.

e) Statistical Analysis

Data obtained from quantitative assessments were synthesized and presented in tables and figures. These values were tabulated with the species description citing mean standard errors only for all the morphological features. Statistical analyses on anatomical features were based on 20 measurements of each feature per slide by the four slides per sample. Values derived were subjected to statistical analysis using the General Linear Model (GLM) procedure in Statistical Analysis System (SAS), SAS Institute (2000), version 9. Means of those traits which show significant differences between taxa were separated by Least Significant Difference (LSD) tests at P = 0.05.

III. Results

a) Epidermis

Epidermal cell walls were irregular in outline with a conspicuous presence of pipillose cells on the abaxial surface partially obscuring the epidermal cells in *H. madagascariensis*, irregular for *G. kola*, straight for both *A. floribunda* and *P. butyracea* (Plate 1A-D).

b) Trichome

There were the presence unicellular stellate hairs of about 189.02 ± 5.7 μm long which have leaf-like structures attached to the body of the epidermis in *H. madagascariensis*, while in *G. kola trichome were* unicellular non-glandular of about 109.2 ± 1.07μm long, and an elongated multicellular trichome with branched tips of about 197.89 ± 4.09 long in *A. floribunda* (Plate1E-G). However, there was complete absence of trichome on both surfaces of *P. butyracea* Qualitative evaluation of stomata are strictly hypostomatic mainly paracytic (Rubiacous type) surrounded by four to six subsidiary cells. Their shapes were oblate spheroidal for *H. madagascariensis*, oblate for *G. kola* and prolate for *A. floribunda* and *P. butyracea* (Table 1).

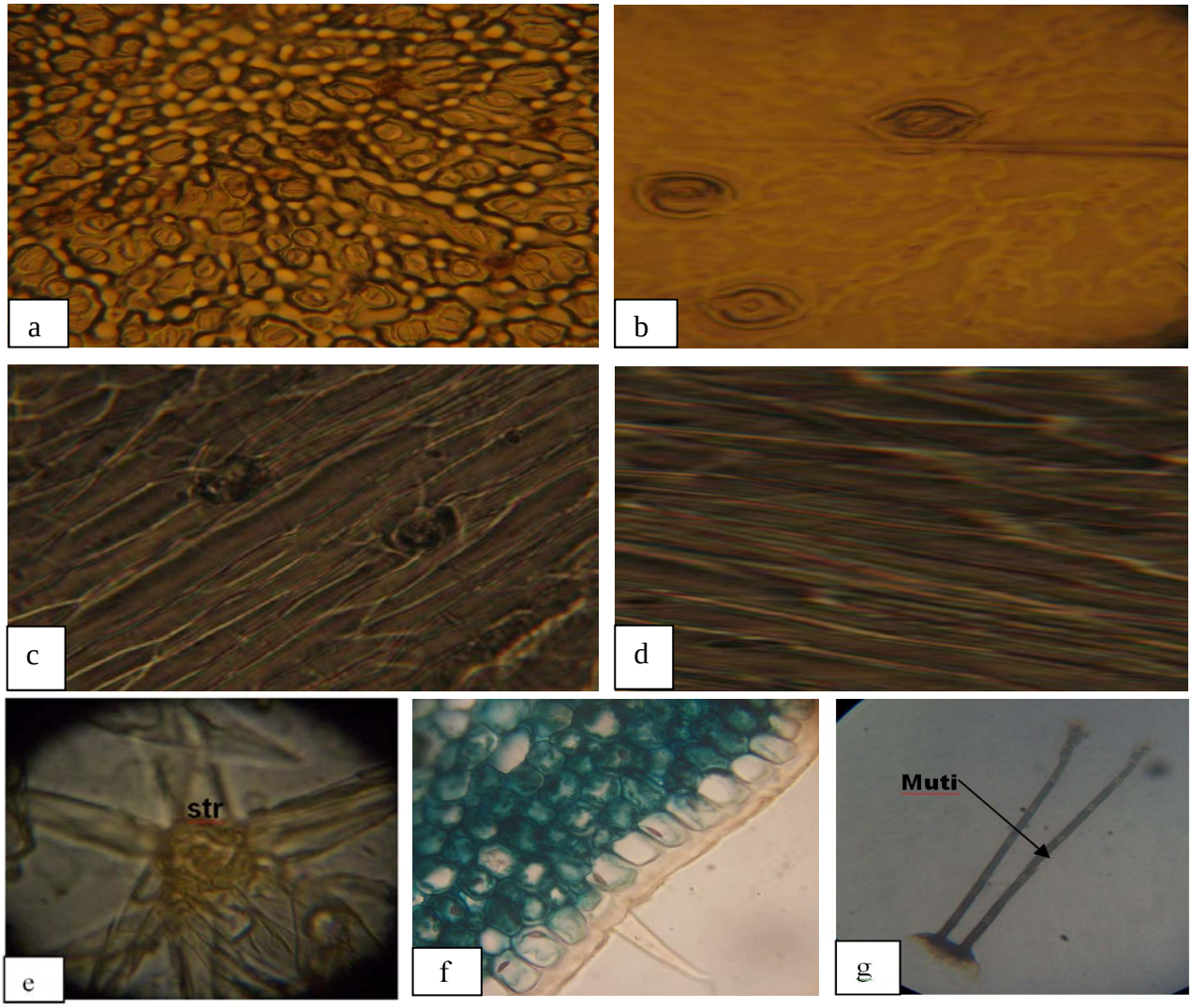

Plate 1: Foliar epidermal features of, a= H. madagascariensis, pc-papillose cell , Abaxial surface devoid of stomata. b.= A. floribunda, c= G. kola, d= P. butyracea icw- irregular cell wall, e= str- stellate trichome with crystal base in H. madagascariensis, f= unicellular trichome in G.kola, g= multicellular trichome in A. floribunda

Table 1 : Qualitative Values of Stomata Features of the Four Species of Nigerian Clusiaceae

Species	Stomata			Trichome	Nature of Epidermal Wall	
	Distribution	Type	Shape	Nature	Abaxial	Adaxial
H madagascariensis	Hypostomatic	Paracytic	Oblate Spheroidal	Stellate	Irregular	Irregular
G. kola	Hypostomatic	Paracytic	Oblate	Uniseriate	Irregular	Irregular
A floribunda	Hypostomatic	Paracytic	Prolate	Multicultural	Straight	Straight
P. butyracea	Hypostomatic	Paracytic	Prolate	Glabrous	Straight	Straight

c) Stomata Features

Stomatal features vary significantly in *H. madagascariensis, G. kola, A. floribunda* and *P. butyracea* in most of the features, but they were statistically the same in *A. floribunda* and *P. butyracea. H. madagascariensis* and *G. kola* varied in all their features except in their stomata breadth (Table 2). Statistical analysis of some of the attributes of stomata showed that, there are very high significant differences ($p < 0.05\%$) between stomata. lengths, pore lengths and breadths, number of stomata per field of view, guard cell lengths and breadths among the four species

with LSD of 0.1, 0.13, 0.08, 0.15, 3.21, 0.11 and 0.04, respectively (Table 2).

Table 2 : ANOVA on Stomata Features of the Four Species (mean standard error in μm x 400)

Species	STB	STL	PL	PB	NSPV	GCL	GCB
H. madagascariesis	0.41c	1.31b	0.77b	0.11b	132.25a	0.83c	0.11c
G. kola	1.81a	1.40b	1.15a	0.37a	32.85b	1.1b	0.25a
A floribunda	1.26b	1.95a	1.15a	0.12b	18.5c	1.29a	0.21b
P. butyracea	1.23b	1.86a	1.15a	0.12b	17.95c	1.26a	0.21b
LSD	0.1	0.13	0.08	0.15	3.21	0.11	0.04

Note: Means followed by the lower case letters were not significantly different, but were significantly different from mean of a different case letters.

Legend: STB - somatal breadth, STL- stomata length, PL - pore length, PB - pore breadth, NSPV- number of stomata per field of view , GCL- guard cell length, GCB- guard cell breadth

d) Stomata Density, Number of Epidermal Cell and Stamata Index

Stomata densities per a field of view varied in the four species from 133.1 ± 19.0, 32.6 ± 4.10, 17.00 ± 1.50 and 17.00 ± 1.50 in *H. madagascariensis, G. kola, A. floribunda* and *P. butyracea,* respectively (Fig 1).

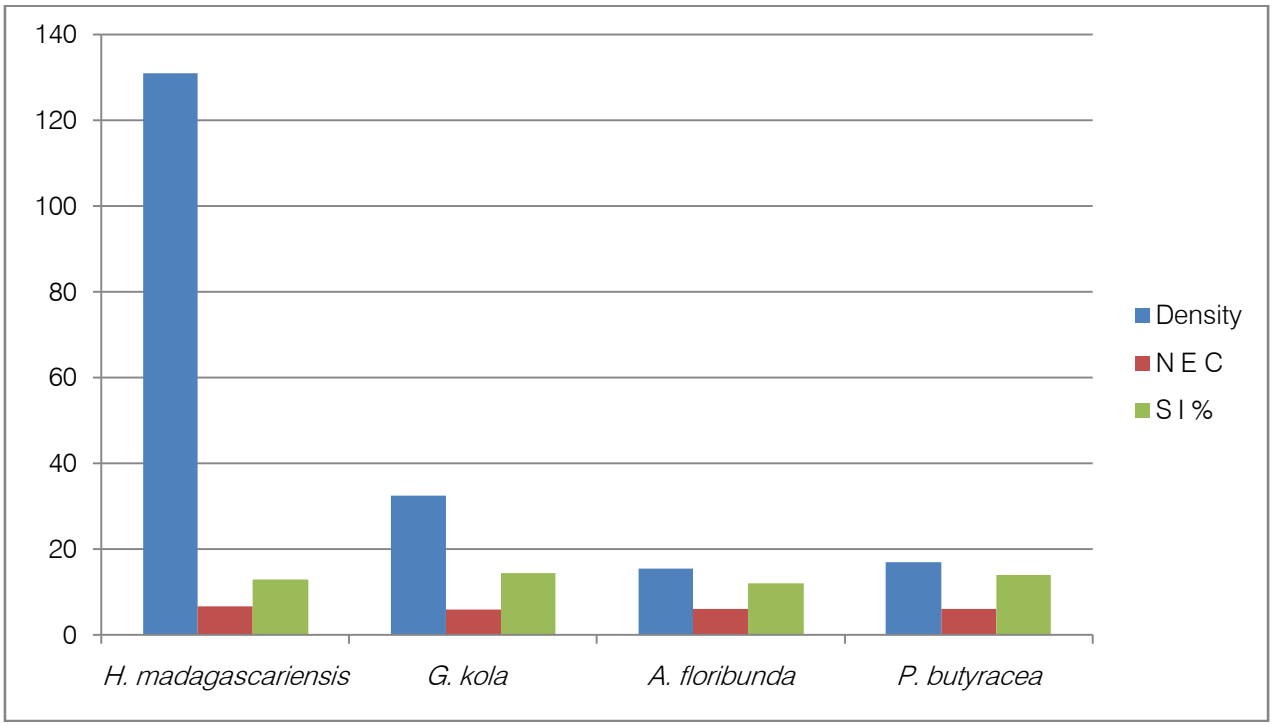

Figure 1 : Stomata Density, No of epidermal cell and Stomata Index (μm, x 400). Where NEC=No of epidermal cell, SI= Stomata Index

IV. Discussion

a) Foliar Epidermal Features

Foliar epidermal features from the four species showed that, the shape of the epidermal cell walls were highly variable in outline among these species studied. Cell walls were irregular with palpilose cells on the abaxial surface in *H. madagascariensi*, irregular in *G.kola,* but straight in *A. floribunda and P. butyracea.* These findings are in line with the findings of Ahmad *et al.,* (2009), who reported that epidermal cell shapes and anticlinal cell wall patterns are highly variable from taxon to taxon and even within the same taxon. Stace (1965) stressed that curves or irregular walls of plant epidermal cells are more of mesomorphic character and that environmental conditions such as temperature and

relative humidity play significant roles in determining the patterns of these cell walls. He pointed out that irregular walls are features of open vegetation while straight walls are associated with forest environments.

This present work is in conformity to some extent with the above reports, as *H. madagascariensis* and *G. kola* with irregular cell walls are found in and around open environment, while *A. floribunda* and *P. butyracea* with their straight cell walls are associated with forest environments, where the relative humidity and temperature are more of constant.

Bearing the above opinions in mind, the number of epidermal cells and the presence of palpilose cells in *H. madagascariensis* seem to have more taxonomic value. These tend to suggest the uniqueness of this taxon from others. The above diagnostic features could serve in the delimitation of the genus *Harungana* from the rest of the genera in the Clusiaceae. These findings are in line with the report by Metcalfe and Chalk (1957) who observed some of these features in some exotic members of the Hypericaceae where they placed *H. madagascariensis*.

b) Trichome

Three basic types of trichome were encountered in this study; stellate types in *H. madagascariensis* (Plate 1e), unicellular type in *G. kola* (plate 1f) and multicellular elongate type with branched tips in *A. floribunda* (Plate 1g). The presences of these diverse types made it difficult to infer their taxonomic value. Moreover, their taxonomic value in this work is greatly limited by the complete absence of trichome in *P. butyracea*. The above findings were in accordance with the reports of Pandey (2004), who stated that a whole family may be recognized by the occurrence of one or more types or by the presence or complete absence of one distinctive type of trichome or the other in members of the same family.

Although the significance of these hairs as revealed in this study is not yet fully understood because of the above facts, Metcalfe and Chalk (1957) held that trichome frequency, size and types were environmentally controlled. They reiterated that it was therefore possible that each species responds to its environment in a specific way, by modifying the basic plan of certain features to improve its adaptation to such environment.

c) Stomata

The relevance of stomata in the taxonomy of angiosperms has severally been emphasized by (Edeoga and Ikem, 2001; Edeoga and Ogbobor, 2001). These authors mostly considered epidermal features at the species and genus levels, thus making it worthwhile to investigate its usefulness at the generic levels within this Nigerian Clusiaceae. A look at (Table 2) shows that *A. floribunda* and *P. butyracea* are the same in all their stomata features. This could be an indication of their close phylogenetic relatedness.

However, the size and density of stomata have been reported by several authors to be correlated in the sense that small stomata often have a high density while large stomata are associated with lower density (Dessine *et al.,* 2005). They further stated that small stomata are particularly present in plants with microphyllous leaves often having thick cuticle and/or are densely hairy. Similarly, Abdul rahaman and Oladele (2012) reported that the rate of transpiration affects stomata type and size, according to them, leaf having higher rate of transpiration are with larger stomata sizes than those having lower rate of transpiration. The above observation was taxonomically relevant in the classification of these taxa. This present study is in conformity in some respect with the above reports, wherein *H. madagascariesis* that had the highest stomata density of 131.1± 19.0 was characteristically in domentous and had smaller stomata as against others with lower densities.

V. Conclusion

Conclusively, data from this study could help to resolve the taxonomic problems in this family, confirm the identity and supports the observation of earlier worker that micro morphological characters and other epidermal features could be employed for species delimitations.

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Determinants of Aquatic Plant Community Structure, Diversity and distribution in Wetlands of Northern Region (Ghana)

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Abstract- The drivers of community structure, diversity and distribution, has generated a lot of interest among many scientists, giving the complex environmental and biological interaction, at different scales. This prompted the application of multivariate techniques to explain the factors influencing variations in wetland plant community structure, diversity and distribution, in Northern Region of Ghana. A total of 40 species were sampled and separated into four community structure(swamp forest community; shrub land community; grassland community and herbaceous community), using DCA. Herbs, grasses and trees/shrubs constituted 72.72%, 27.27% and 0.01% respectively. Change in plant community distribution was marginal, as the first two axes only explained 1.18% of the variance along a longitudinal profile of environmental gradient. Species turnover was low, indicating a far more unimodal responses of some species to a gradient of disturbances than others. Plant diversity was moderate(H′ = 1.86 - 2.66).

Keywords: community structure, canonical correspond- dence analysis, environmental gradient, diversity, spatial distribution, detrended correspondence analysis.

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Keywords: community structure; canonical correspondence analysis; environmental gradient; diversity; spatial distribution; detrended correspondence analysis.

I. Introduction

Aquatic plants perform a vital role in wetland ecosystems, as primary producers, providing food and habitat for aquatic and terrestrial organisms (Rolon & Maltchik, 2006). Because of their intimate contact with physico-chemical parameters of water, aquatic plants are considered as one of the key indicators of wetlands health status. Many scientists have recognized a complex interaction of environmental and biological factors as the drivers of community distribution, composition and diversity, thus prompting the application of multivariate techniques to explain variations in wetland plant community (Toivonen & Huttunen, 1995; Lenssen *et al.* 2000). The factors controlling the distribution of aquatic plants have been of historical interest (e.g. Moyle, 1945) and influenced by a matrix of climatic (Walther et al. 2001; Klanderud 2005) and environmental factors (Kotze & O'Connor, 2000). Altered plant community through degradation of water quality (Heegaard et al. 2001; Seilheimer, Mahoney & Chow-Fraser, 2009), wet and dry periods (Kath, Brocque & Craig-Miller, 2010), fire (Gboloo, 1998; Smith et al. 2001), soil condition and air pollution (Wild, Neuha¨uslova´& Sofron, 2004) and altitude (Heegaard 2004) have been documented at different spatio-temporal scales. But changes in plant community along a disturbance gradient, varies at different scales because of species-specific responses and inherent ecological conditions. Although extensive research have been carried-out on some wetlands in Ghana (e.g., Attuquayefio & Wuver, 2003), environmental predictors influencing community structure, diversity and the spatial distribution is much less studied in Northern Region of Ghana. Wetlands in Northern Region of Ghana are severely exploited by communities within the catchment and as such, are of high conservation concern. It is therefore, important to model aquatic plants in relation to environmental variables, so that the effects of environmental changes can be predicted. Furthermore, few surveys have shown evidence of species range shift, linked to environmental disturbances (e.g., Nsor, Obodai & Blay, 2014). Therefore, identifying the main environmental mediating factors influencing the community structure and distribution will be important in deciding the type of conservation and management intervention to employ in order to sustain the functioning status at the local scale.

II. Methods

a) Study area

The study was carried out in six wetlands located in the Northern region of Ghana, with their co-ordinates as follows: (i) Wuntori (N09º 08.335' W00º1 09º.685'); (ii) Kukobila (N10º 08.723' W000º 48.179'); (iii) Tugu (N09º 22.550' W000º 35.004'); (iv) Bunglung (N09º 35.576' W000º 47.443'); (v) Adayili (N09º 41.391' W000º 41.480') and (vi) Nabogo (N09º 49.941' W000º.51.942') (Fig. 1).The six sites lie on the extensive floodplain along the course of the White Volta River, which has overtime

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become incised and modified through meandering and aligning along various topographic features. All six wetlands comprised of marshes (Wuntori, Tugu and Kukobila wetlands); riparian wetlands (Adayili and Nabogo) and artificial wetland (Bunglung). The hydrological regimes of the six wetlands under study were typical of permanent wetlands, whose depth at low tide did not exceed 2 m on average. Sizes of the wetlands were as follows: (a) Wuntori = 7.7 ha; (b) Kukobila = 5 ha, Tugu (c) 2.7 ha; (d) Nabogo = 7.9 ha; (e) Adayili = 6.7 ha and (f) Bunglung = 11.5 ha.

Annual rainfall is in the range of 1000-1,300 mm/p.a. Average temperature varies between 14°C and 40°C. Altitude ranges between 108 – 138 meters above mean sea level. The vegetation cover is a mixture of grassland dominated by *Lersia hexandra* and woodland dominated by Mahogany (*Khaya senegalensis*) and shea tree (*Vitellaria paradoxa*) interspersed with shrub communities (*Mitragyna inermis*).

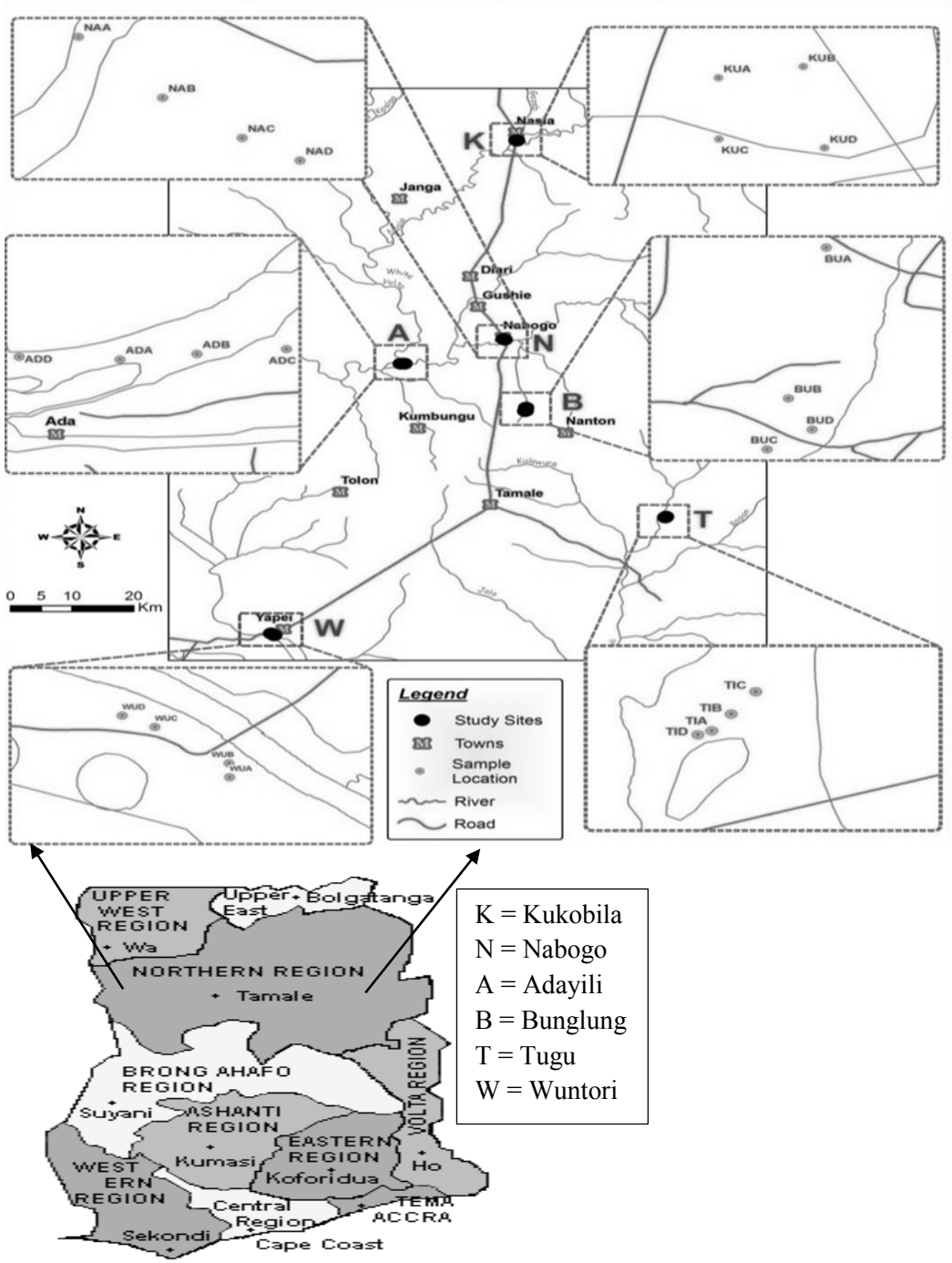

Figure 1 : Map of the study areas, showing the location of the wetlands in the floodplains of the White Volta River catchment, Northern Region

b) Vegetation sampling procedure

In order to cover a wide taxonomic range, we used the broad definition of aquatic plants, which include submerged, floating and emergent plants (herbaceous cover, shrubs and trees). Sampling of aquatic plants was carried in each of the 24 Modified-Whittaker plots (Stohlgren, Falkner & Schell, 1995) over a 2-year period. The Modified-Whittaker plot is a vegetation sampling design that is used to assess plant communities at multiple scales. Four Whittaker plots were randomly laid in each of the six wetlands, bringing the total to 24 plots. The plot measures 20 m x 50 m (1000 m^2) and contains three different sizes of nested subplots. A 5 m x 20 m (100 m^2) subplot was placed at the centre of the plot, while two 2 m x 5 m (10 m^2) subplots were placed in opposite corners of the plot. The remaining ten of 0.5 m x 2 m (1 m^2) subplots are placed at the edges of the main plot. Plots were laid along an environmental gradient of the vegetation type sampled, in order to register majority of species heterogeneity. The Domin-Krajina cover abundance scale was used to estimate ground cover (see Mueller-Dombois & Ellenberg, 1974). Plants were identified up to species level, with the aid of manuals developed by Johnson (1997), Okezie & Agyakwa (1998) and Arbonnier (2004).

c) Assessment of environmental factors

Random soil samples were collected with a soil augur at a depth of 15 cm, using the zigzag sampling method (Carter & Gregorich, 2006), on each Modified-Whittaker plot (Stohlgren *et al.* 1995). Three composite samples were taken from three different 25 cores in each plot. Samples were put in transparent polyethylene bags and labeled according to the code assigned to each plot and taken to the laboratory to analyze the presence of Nitrogen, Phosphorus, Potassium, Magnesium, Calcium and soil pH, using atomic absorption spectroscopy (AAS) techniques (Murphy & Riley 1962; van der Merwe, Johnson & Ras, 1984). Organic carbon was determined using the Walkley-Black method (Walkley & Black, 1934). All analyses were carried out at the Savanna Agricultural Research Institute (SARI) at Nyankpala in the Northern Region. A score ranging 1–4 was used to assess the scope and severity of every threat. A ''scope'' hereby referred to as the percentage ratio of the study area affected by a specific threat within the last 5 years (where 100% correspond to total site area: χ ha) (Battisti, Luiselli & Teofili, 2009). The scores were assigned as follows: 4 = the threat is found throughout (50%) the site; 3 = the threat is spread in 15–50% of the site; 2= the threat is scattered (5–15%); and 1, the threat is much localized (<5%). Assessment of the area disturbed was carried out within 1.2 km radius, starting from the hydric delineated zone of the wetland. This is because all land use activities assessed were observed within the stated radius following a preliminary survey of the wetlands.

d) Statistical methods

Shannon-Weiner index was performed to determine the current status of aquatic plants, fish and bird community composition. Shannon-Weiner index equation expressed as:

$$H' = -\sum_{i=1}^{s} p_i (\ln p_i) \text{ (Shannon-Wiener, 1963)}$$

Where s is the number of species and P_i is the proportion of individuals or the abundance of the *ith* species expressed as a proportion of the total cover and *ln* is a natural logarithm (Shannon & Wiener, 1963).

A one-way ANOVA test was applied to test for the differences in species diversity/evenness and species richness from one wetland to the other, using SPSS version 16. Kruskal-Wallis test was applied to test the differences in the mean of the diversity index.

To determine the influence of environmental drivers of change on variations on community structure, diversity and spatial distribution, a canonical correspondence analysis (CCA) was performed (ter Braak, 1986), using Environmental Community Analysis (ECOM.exe) ver. 1.4 package (Henderson & Seaby, 2000). CCA is a constrained ordination method where axes are created through linear combinations of environmental variables, which makes it a useful method for detecting key variables that explain variation in species data (ter Braak, 1995). Prior to CCA, species identified and registered at <5% of the 24 sample plots were omitted from canonical correspondence analysis, because rare species typically have a less influence on results of multivariate statistics and are treated as outliers in ordinations (Gauch, 1982).

A Monte-Carlo permutation test with 9999 iterations was performed to evaluate the significance of eigenvalues for both axes 1 and 2 and the sum of all the eigenvalues (ter Braak & Verdonschot, 1995). This was to determine the significant contribution of each environmental driver of change in influencing macrophyte community structure and distribution pattern. Only axis that were statistically significant ($p<0.05$) were interpreted. Detrended correspondence analysis (DCA) (Hill & Gauch, 1980) was performed primarily to determine the compositional variation of mean ground cover, using Community analysis package version 1.41 (Henderson & Seaby, 1999). DCA techniques have the ability to handle large, complex data sets and uncover long ecological gradients, as well as help in data reduction and data exploration (Kent & Coker, 1992). Only plants sampled in the 10 of the 1m^2 sub-plots in each of the 24 Whittaker plots were subjected to CCA. One-way ANOVA was performed to

determine if environmental variables differed significantly from one wetland to the other, using Statistica version 10.0.

III. Results

a) Plant community distribution gradient (DCA)

A total of 40 plant species were registered across the six sites, with Kukobila wetland recording the highest mean number of species (29.5±1.9) predominantly herbs and grasses. While Bunglung (constructed) wetland was the least in species abundance (23.5±1.04). Detrended correspondence analysis (DCA) separated aquatic plants into four floristic associations, based on variations in plant community structure and wetland type. They included swamp forest community; shrub land community; grassland community and herbaceous community (Fig. 2, Table 1). The change in plant community distribution was marginal, as the first two axes only explained 1.18% of the variance along a longitudinal profile of environmental gradient. Of the species captured in the ordination diagram, herbs, grass and shrubs/trees constituted 72.72%, 27.27% and 0.01% respectively. Plant species in group 1 (Bunglung wetland), was dominated by grassland community alongside pockets of herbaceous species (Fig. 2). *Cynodon dactylon* (Linn.) Pers. and *Deplachne fusca* (L.) P.Beauv.ex Roem were the dominant grass species, while *Polygonum salcifolium* Brouss. ex. Wiild and *Neptunia oleracea* Lour. constituted the herbaceous cover. Previous burnt tree stumps and grass tussocks affected species turnover in some of the plots. Transformed portion of the site was more than the area with cover abundance.

Species distribution in the three improved sites (Wuntori, Kukobila and Tugu marshes) in group 2, constituted a mosaic of grass and herbaceous communities. The three sites were typically closed systems, as their surface were virtually covered with emergent/floating aquatic plants (e.g., *Pistia stratiotes* Linn. and *Nymphaea micrantha* Linn.). Systematic distribution of *Ceratophyllum demersum* L., *P. stratiotes* Linn., *N. micrantha* Linn., *Cyperus spacelatus* (Rottb) and *Schizachyrium sanguinum* (Retz.) Alston. respectively, reflects a gradient of assemblage change in the longitudinal wetness profile. *C. demersum* L. was the only submerged species and formed a dense intertwined mat. Few shrubs namely; *Mitragyna inermis* (Wiild.) Kuntze. *Mimosa pygra* L. and *Salacia recticulata* were sparsely distributed along the fringes of less wetness condition.

In group 3 distribution diagram, *S. reticulate* L., *Vitex crysocarpa* (Planch. Ex Benth), *Ziziphus abyssinica* Hochst. ex A. Rich. and *M. inermis* (Wiild.) Kuntze. represented typical trees and shrub communities from the Adayili and Nabogo riparian systems (Fig. 2). Spatial distribution of species reflects a gradient of assemblage change in a vertical profile of wetness condition. For instance, *Syzygium guineense* (Wild) D.C and *S. reticulate* which had a ¼ of their stems below surface water level, were found at the foothills of stream bank, while *V. crysocarpa* (Planch. Ex Benth), *Z. abyssinica* and *M. inermis*, were at a much higher ground of less saturation. Pockets of grass and herbaceous species such as *D. fusca* (L.) P.Beauv.ex Roem., *Leersia hexandra* Sw. and *Scoparia dulcis* L., formed the undergrowth. Total species turnover was low (0.026±0.058), indicating that some species exhibited far more unimodal responses to a gradient of environmental disturbances than others. This observations led to the use of CCA, to deduce the major environmental factors that influenced variations in species assemblage.

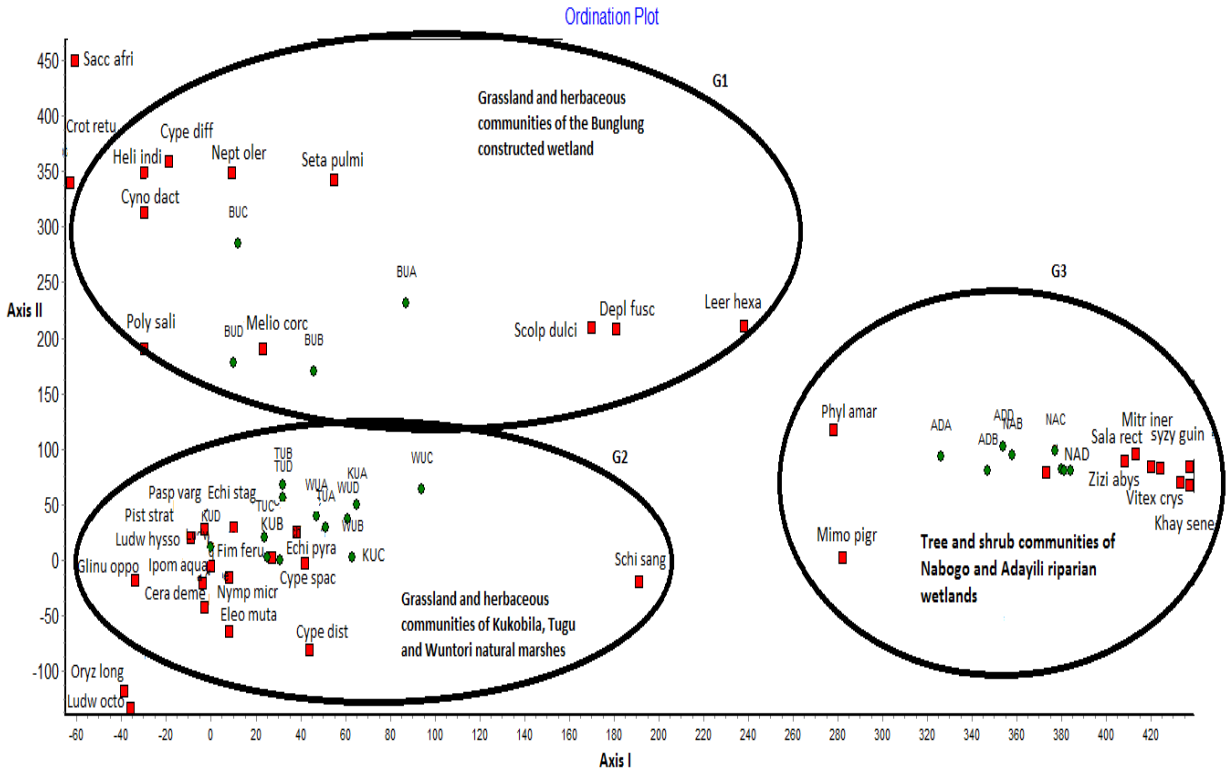

Figure 2 : Species-site DCA ordination showing ground cover distribution pattern, separated into three groups along axes I & II. The red squares represent abbreviated plant species (e.g., *Cype distans* = Cype dist), the green circles represent sample sites and the arrows represent each of the environmental variables plotted pointing in the direction of maximum change of explanatory variables across the six wetlands. The abbreviations denote different sample plots in the six wetlands. WUA-WUD = Wuntori wetland at Yapei; TUA-TUD = Tugu wetland; KUA-KUD = Kukobila wetland; BUA BUD = Bunglung wetland; ADA-ADD = Adayilli wetland and NAA- NAD = Nabogo wetland. Mean = 0.026±0.05

Table 1 : Socioecological classification of plant species into three community structure across the six sites. Species assigned with asterics (*) are typical aquatic plants

N = 40 species

Grassland community	Herbaceous community	Trees and shrubland community
Brachiaria mutica (Forsk.) Stapf.	*Ceratophyllum demersum* L. *	*Khaya senegalensis* (Desr.) A. Juss
Cyperus difformis Linn.*	*Pistia stratiotes* Linn. *	*Mitragyna inermis* (Wiild.) Kuntze
Cyperus distans L.F. *	*Nymphaea micrantha Linn.* *	*Mimosa pigra* L.
Cyperus spacelatus (Rottb) *	*Ipomea aquatica* (Forssk) *	*Salacia reticulata* L.
Cynodon dactylon (Linn.) Pers.	*Ludwigia octovalvis* (Jacq.) Raven*	*Syzygium guineense* (Wild) D.C.
Fimbristylis ferruginea (L.) Vahl.	*Ludwigia hyssopifolia* (G. Don) Exell.	*Vitex crysocarpa* (Planch. Ex Benth.)
Imperata cylindrica (L.) P. Beauv.	*Neptunia oleracea* Lour. *	*Ziziphus abyssinica* Hochst. ex A. Rich
Leersia hexandra Sw.	*Polygonum salicifolium* Brouss. Ex.Wiild*	
Pennisetum polystachion (L.) Schult	*Helioptropium indicum* Linn.	
Oryza longistaminata A. Chev.&Roehr*	*Phyllanthus amarus* Schum.&Thonn.	
Paspalum varginatum L. *	*Scoparia dulcis* L.	
Echinochloa stagnina (Retz.) P. Beauv.	*Crotalaria retusa* L.	
Deplachne fusca (L.) P.Beauv.ex Roem	*Glinus oppositifolius* (L.)	
Echinochloa pyramidalis (Lam.) Hitchc. & Chase	*Meliochia corchorifolia* L.	
Setaria pumila (Poir.) Roem & Schult	*Mormodica chrantia* Linn.	
Sacciolepsis Africana C.E. Hubb. & Snowden		
Scirpus grossus Linn. F.		
Schizachyrium sanguineum (Retz.) Alston.		

b) Variations in species diversity along a disturbance gradient

Overall, plant diversity was moderately high across the six sites and ranged between (H′ = 1.86 - 2.66) (Fig. 3). Although, species diversity was higher in the wet season than the dry season (*F-test* = 4.318, p<0.064), variations among the sites, did not differ significantly (One-way ANOVA test p>0.05). On average, all the three marshes (Kukobila, Wuntori and Tugu) tended to be the most diverse (H′ = 2.231±0.110 - 2.656±0.079), followed by the two riparian wetlands – Adayili (H′ = 1.865±0.110) and Nabogo (H′ = 1.895±0.078) and Bunglung wetland (H′=1.857±0.260). Variations in diversity across the six sites, simultaneously followed a disturbance intensity gradient and differed significantly in their evenness distribution (*t* = -16.511, p<0.05). Heavily impact sites such as the two riparian systems and Bunglung constructed wetland, largely reflected in their low diversity index.

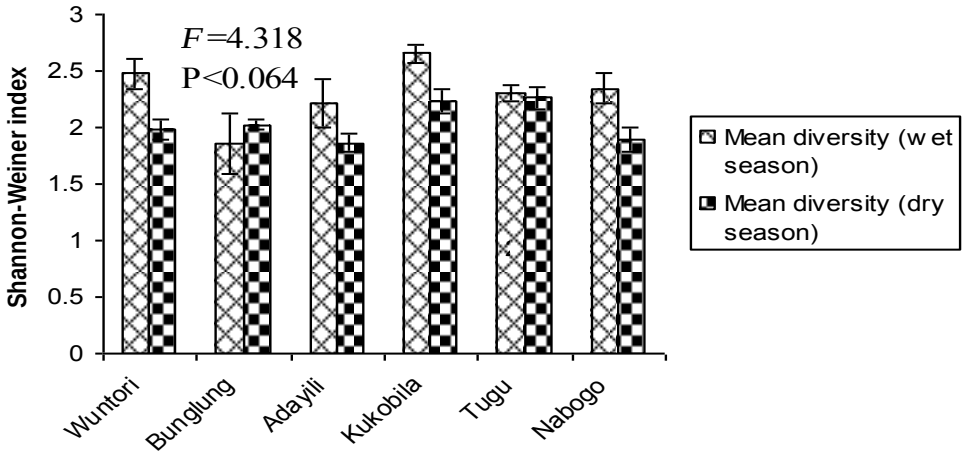

Figure 3 : Plant community, showing seasonal variations in diversity index among the six wetlands in the dry and wet seasons

c) Predictors of aquatic plant community structure, composition and distribution (CCA)

Canonical correspondence analyses revealed that the impact of environmental factors on plant distribution in each of the six sites differed significantly (p < 0.05), following Monte Carlo permutation test. The inherent environmental conditions in each of the six wetlands, jointly explained 61.29% (R^2 = 0.61, p<0.05) of variability in plant distribution pattern, by the two axes (axis I = 24.84 and axis II = 36.45) (Fig. 4, Table 2). Since axes I and II accounted for more than 50% of the variation in ground cover data, as recommended by ter Braak (1986), axis III. Magnesium and bushfire were highly correlated in axis I and constituted the most important environmental gradients on plant community distribution, while in axis II, farming activities, potassium and nitrogen were the key environmental factors. CCA analysis diagram showed the various groups of plant communities' distribution according to the type of environmental mediating factors and the type of wetland sampled. We noticed that *S. guineense* (Wild) D.C. *Salacia reticulate* and *Z. abyssinica* from the riparian systems, on the left half of the ordination diagram strongly correlated with fire and erosion intensity, while *V. crysocarpa* Planch. Ex 10 Benth, *Khaya senegalensis* (Desr.) A. Juss., *Pennisetum polystachion* (L.) Schultes, *Imperata cylindrica* (L.) P. Beauv. and *Phyllanthus amarus* Schum. & Thonn were rather influenced by soil pH. Erosional features and channel incision, were prominent in portions of the stream bank that had less ground cover and mostly invaders. Although erosional features were prominent, they were not widespread as this was evident in the weak correlation on both axes I and II (Table 3). Patchiness from previous burnt undergrowth were common, as farmers periodically used fire for land preparation along the stretch of the wetlands.

High magnesium concentration, intense farming and grazing activities was observed in Bunglung constructed wetland found on the right lower half of the diagram. With the exception of obligate species like *Cyperus difformis* Linn. and *P. salicifolium* Brouss. ex. Wiild, the rest of the species were of derived savannah origin and mostly associated with disturbed terrestrial areas. For instance, we observed a strong correlation between *Sacciolepsis Africana* C.E. Hubb. & Snowden., *Crotolaria retusa* L. and *Helioptropium indicum* Linn. and farming activities (p<0·05) along axis I, while *P. salicifolium* Brouss. ex. Wiild., *L. hexandra* Sw. and *N. oleracea* Lour rather showed a strong association with magnesium on the same axis (p<0·05). The natural marshes of Kukobila, Wuntori and Tugu on the upper centre of the CCA diagram, exhibited optimal levels of nutrient loads and hence were not polluted. This was evident from the abundance of *P salcifolium* Brouss. ex. Wiild. which is an indicator of a less polluted wetland.

Majority of species not represented in the ordination diagrams grew in habitats with average conditions of the environmental factors investigated.

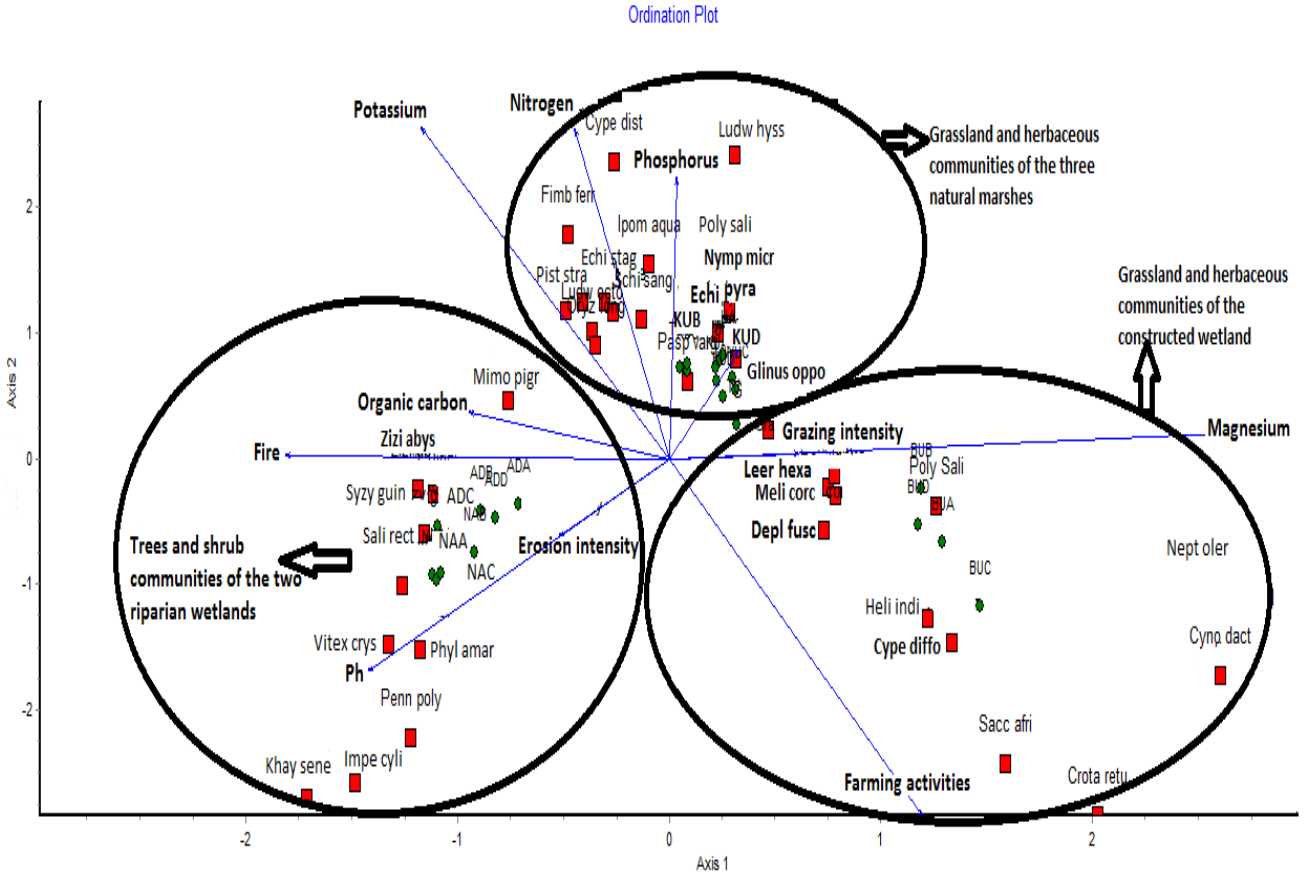

Figure 4 : Canonical correspondence analysis (CCA) ordination diagram, showing the influence of environmental factors on species range shift, explained by the first two axes (Axis I =24.84 & Axis II =36.45) and accounted for 61.29%cumulative percentage variance across the six sites (R^2 = 0.61, p<0.05). The red squares represent abbreviated plant species (e.g., *Cyperus difformis* = Cype diff, *Leersi hexandra* = Leer hexa), the green circles represent sample sites and the arrows represent each of the environmental variables plotted pointing in the direction of maximum change of explanatory variables across the six wetlands. The abbreviations denote different sample plots in the six wetlands. WUA-WUD = Wuntori wetland at Yapei; TUA-TUD = Tugu wetland; KUA-KUD = Kukobila wetland; BUA-BUD = Bunglung wetland; ADA-ADD = Adayilli wetland and NAA- NAD = Nabogo wetland

Table 2 : Canonical coefficients and the correlations with the first three axes of the environmental variables of the canonical correspondence analysis (CCA) for the six sites. Percentage variance of species, explained by the first two axes of explanatory variables. Inter-set correlations were significant ($p < 0.05$) for the two axes, following Monte Carlo permutation test

	Axis I	Axis II	Axis III
pH	-0.367*	-0.261	0.065
Organic carbon	-0.244	0.056	0.153
Nitrogen	-0.115	0.405*	0.163
Phosphorus	0.009	0.346*	0.233
Potassium	-0.303*	0.406*	0.045
Calcium	0.085	0.132	-0.139
Magnesium	0.772*	0.035	-0.101
Fire	-0.469*	0.002	-0.197
Grazing intensity	0.157	0.005	0.167
Erosion	-0.134	-0.095	0.127
Farming activities	0.311*	-0.441*	0.075
Canonical eigenvalues for cover	0.733	0.342	0.198
Pearson correlation sp-envtal scores	0.81	0.88	0.84
Cumulative percentage variance	24.84	36.45	43.16
% variance explained (61.29%)	24.84	11.6	6.71
Number of species (response variables)	40		
Number of environmental variables	11		
Total variance in species data	2.951		

IV. Discussion

The global loss of wetlands has driven interest in the number of research aim at understanding the patterns of biodiversity in wetlands, including aquatic plants (Lougheed *et al.* 2001; Smith & Haukos, 2002; Heegaard *et al.* 2004). This survey represents one of the most complete dataset that attempts to determine the effects of environmental factors on the aquatic plant community in wetland systems at a relatively local scale in Northern region of Ghana. The findings in this study showed that environmental factors namely; magnesium, phosphorus, nitrogen, fire and farming activities, were the predictors of species diversity and spatial distribution across the six sites. This was confirmed by the CCA explanatory variables accounting for 61.29% cumulative species variance across the six sites (R^2 = 0.61, $p<0.05$). Altered plant community through degradation of water quality (Heegaard et al. 2001; Seilheimer *et al.* 2009), wet and dry periods (Kath *et al.* 2010), fire (Gboloo, 1998; Smith *et al.* 2001), soil condition and air pollution (Wild *et al.* 2004) and altitude (Heegaard *et al.* 2004) have been documented at different spatio-temporal scales. Al though climatic variability (Walther, Burga & Edwards, 2001; Klanderud, 2005) have historically contributed in controlling the distribution of aquatic plants (e.g. Pearsall, 1920; Moyle, 1945), environmental factors (Kotze & O'Connor, 2000) have been shown to be a major determinant influencing community distribution. Species response to disturbance scenarios are varied on the basis of inherent ecological conditions and species type. For instance, while species like *P. stratiotes*, *C. difformis* Linn. and *P. salicifolium* were susceptible to fire, *S. guineense* (Wild) D.C. *S. reticulate* and *Z. abyssinica* from the riparian systems, were rather resistant to fire. Incidences of fire and farming activities in the marshes and Bunglung (constructed) wetland, affected the turnover of resident species, while at the same time encouraged the establishment of species of neighbouring derived savannah such, as *S. Africana* C.E. Hubb. & Snowden. *Crotolaria retusa* and *H. indicum* Linn. This suggest the competitive advantage of derived savannah species over aquatic species in the utilization of limited below and above ground resources. With the resultant change in community structure and composition, the wetlands are probably on a transformational trajectory. This observation was confirmed by the findings of Nsor *et al.* (2014) in their study of species range shift dynamics in the same sites. The authors found that of the 40 species sampled, facultative (40%) and obligate upland species (27.5%) were in excess of 67.5%, compared to 35% obligate species (typical aquatic plants). Assessment of

environmental impact among the six sites, suggest that Bunglung (constructed) wetland may undergo a rapid transformation in community structure and diversity, as a result of the weak responses of species to disturbances. Studies in the Everglades wetlands have shown evidence of wetlands fires completely destroy vegetation and resident seedbank (Smith *et al*. 2001).While in some cases, species like saw grass (*Cladium jamaicense*) have persisted under fire, by periodically eliminating successional species (Loveless, 1959). Over the last 50 years, agricultural activities in have caused the extinction of wetlands plants (Kopeć *et al*. 2008). And one of the causes of agricultural activities in altering the ecosystem of wetlands, is through nutrient enrichment (Matson *et al*. 1997). But the findings in this study, indicated that farming activities did not correlate with N and P concentration. Thus the optimal levels of the nutrient loads especially in the three marshes, possibly explained the appreciable abundance of typical aquatic plants and their structural distribution. Phosphorus availability is a critical factor regulating plant species distributions in the (Davis, 1994). The observed disturbances gives an indication that future intensification of farming activities and bushfire, could potentially increase the rate of species extinction of typical aquatic plants sensitive to slight disturbances. This phenomenon could consequently impair the functional status of the marshes, unless strict conservation and management measures are put in place to check on human-led activities.

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- Reason of the study - theory, overall issue, purpose
- Fundamental goal
- To the point depiction of the research
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	A-B	**C-D**	**E-F**
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Introduction	Containing all background details with clear goal and appropriate details, flow specification, no grammar and spelling mistake, well organized sentence and paragraph, reference cited	Unclear and confusing data, appropriate format, grammar and spelling errors with unorganized matter	Out of place depth and content, hazy format
Methods and Procedures	Clear and to the point with well arranged paragraph, precision and accuracy of facts and figures, well organized subheads	Difficult to comprehend with embarrassed text, too much explanation but completed	Incorrect and unorganized structure with hazy meaning
Result	Well organized, Clear and specific, Correct units with precision, correct data, well structuring of paragraph, no grammar and spelling mistake	Complete and embarrassed text, difficult to comprehend	Irregular format with wrong facts and figures
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References	Complete and correct format, well organized	Beside the point, Incomplete	Wrong format and structuring

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