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Original pictures on front cover: Cephalic capsule of *Eriogaster catax* caterpillar © Alexandra Ciorîță & Cristian Sitar

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Conservation status of the rare species *Betula nana* in the peatbog Tinovul Luci (the Harghita Mountains, Romania)

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Abstract. The aim of the study was to assess the conservation status of the boreal relict plant species *Betula nana* in one of the southernmost site of its range - the Natura 2000 site ROSCI0246 Tinovul Luci (Harghita county, Romania). Data collected in the field were confronted with that published in 1930 and 1960. Our findings show that *Betula nana* is critically endangered (CR) in the studied site, with a high risk of becoming extinct in the near future. The main threat is the change of habitat, which may be reversed, in part, by decreasing the cover of the tree layer.

Keywords: *Betula nana*, biodiversity conservation, Natura 2000, peatbog.

Introduction

Batula nana L. (dwarf birch) is a small shrub, up to 1m height, with circumpolar distribution and several isolated occurrences in the mountains of temperate zone. In Romania it is a glacial relict, preserved in only two peatbogs of the Eastern Carpathians (Pop, 1928; Rațiu, 1967) – Găina-Lucina (SV) and Luci (HR). It is listed in the Red Book (Dihoru and Negrean, 2009) as critically endangered (CR) at national level (IUCN, 2012). The peatbog Tinovul Luci, at 46°12’ N, is at the southernmost rear edge of global range of *Betula nana*. This peatbog is laying on the place of an old volcano, located in the Harghita Mountains of Eastern Carpathians, at an altitude of 1080 m. With its 120 ha, it is one of the largest peatbogs in Romania (Pop, 1960; Șofletea and Curtu, 2008). It was declared botanical reserve in 1995 and Natura 2000 site in the year 2008 (ROSCI0246). The forests inside are managed by two forest districts, F.D. Miercurea Ciuc (190 ha) and F.D. Tălișoara (89 ha).

The state of art regarding the dwarf birch on this protected area dates from 1926-1928 (Nyárády, 1926; Pop, 1928), and the newest came from 1973 (Dihoru and Negrean, 2009).

The aim of this study is to assess the conservation status of *Betula nana* in the peatbog Tinovul Luci in order to establish the proper management measures. The objectives were: (i) to establish the population's size and range, (ii) to analyze species habitat and the main driving factors, (iii) to investigate the regeneration capabilities, (iv) to identify actual and potential threats on species and/or its habitat.

Materials and methods

Data from forest management plans and literature (Nyárády, 1926; Pop, 1928, 1960) was processed and a map with potential habitat of *Betula nana* was created, using QGis software (www.qgis.org). The species range was mapped as grid-cells of 50x50 m.

Six field trips were carried out in 03.06, 24.06, 08.07, 16.07, 21.10, 02.11.2017. Observations were made along transects covering 377 grid-cells out of 503, i.e. 75% of the area of the peatbog.

The following habitat parameters were measured: the distance between *Betula nana* groups and the nearest water pond or stream, water temperature in the peatbog near *Betula nana*, soil temperature in the 2 forest types – pine and spruce dominated, soil reaction in the A horizon with a Hellige pH-meter, depth of watertable at 4-5 m away from *Betula nana* grups using a drill and a tape line. The temperature was read around 12 o'clock, in 2 repetitions on 3 June and 8 July, on an electronic device with 1% accuracy. The plant communities in which *Betula nana* grows were described by 2 relevés with an area of 25 m^2. The climate aridity was expressed through the Ellenberg quotient: $EQ=T_{VII}/P\cdot 1000$, where T_{VII} is the temperature of warmest month (July) and P the annual sum of precipitations (Ellenberg, 1992). The yearly values of T_{VII} and P were extracted from ROCADA grids (Dumitrescu and Bîrsan, 2015).

Population parameters were: number and height of shoots, shoots diameter at ground level, vegetative growth, capability of sexual reproduction (inflorescences, fruits and seedlings). Threats and pressures were assessed by personal observations and information gathered from local foresters. The number of individuals was not counted but inferred since we try to limit the stress upon the species, i.e. we didn't check the underground connections between stems. An individual was regarded as a bunch of shoots close enough (in a range of 1 m) to form a genet. A shoot was considered the aerial part of a plant emerging separatedly from the ground. Further, a group refers to all neighbouring individuals (as defined above) clearly distant (>10 m) from other conspecific plants.

The species names follow Sârbu *et al.* (2013) for vascular species and Frey *et al.* (2006) for bryophytes.

Results and discussions

There were identified 6 groups of *Betula nana* spread within only three grid-cells (Fig. 1). The biggest distance between groups is 220 m. There were counted 139 shoots with a range of 8-47 shoots/group. We assume that were encountered no more than 20-22 individuals. The height of *Betula nana* shoots varies between 5 and 65 cm; the diameter of shoots at the ground level is at most 1.5 cm.

Soil temperature under pine stand reaches 11-12 °C during summer; it exceeds by 2-3 °C that of spruce stand and by 4-5 °C that of the neighbouring stream. The peatbog is very strong acidic, with pH=3.5-4.3.

The water table was at 40-50 cm depth during summer.

The vegetation around *Betula nana* is a Bog woodland (91D0*), a priority Natura 2000 habitat (Gafta and Mountford, 2008). It may be classified into the association *Vaccinio uliginosi-Pinetum sylvestris* Kleist 1929 em. Matusz. 1962 (Coldea and Plămadă, 1989; Coldea *et al.*, 2015). The canopy is dominated by *Pinus sylvestris*, with scarce admixture of *Picea abies* or *Betula pubescens*. On the herb layer prevail *Vaccinium myrtillus* and *V. vitis-idaea*. The moss layer is a continuous and thick blanket of *Sphagnum* species. Overall, the floristic composition is typical for the habitat, free of non-native, ruderal or invasive species. Among characteristic species are *Andromeda polifolia, Betula pubescens, Empetrum nigrum, Eriophorum vaginatum, Polytrichum strictum, Sphagnum capillifolium, S. fallax, S. fuscum, S. magellanicum*.

Relevé 1 – Altitude 1080 m, Slope 0°, Area 25 m^2; Tree layer (40%): *Pinus sylvestris* 3, Shrub layer (2%): *Betula nana* +, *Pinus sylvestris* (juv.) +, *Picea abies* (juv.) +, *Betula pubescens* (juv.) +, Herb layer (40%): *Vaccinium myrtillus* 2, *Vaccinium vitis-idaea* 2, *Andromeda polifolia* +, *Eriophorum vaginatum* +, Moss layer (100%): *Sphagnum capillifolium* 5, *Polytrichum strictum* 1, *Sphagnum girgensohnii* 1, *Pleurozium schreberi* 1, *Dicranum scoparium* +, *Sphagnum magellanicum* +, *Sphagnum angustifolium* +.

Relevé 2 – Altitude 1080 m, Slope 0°, Area 25 m^2; Tree layer (40%): *Pinus sylvestris* 3, *Picea abies* 1; Shrub layer (5%): *Picea abies* 1, *Betula nana* 1; Herb layer (60%): *Vaccinium myrtillus* 3, *Vaccinium vitis-idaea* 3, *Eriophorum vaginatum* 1, Moss layer (95%): *Sphagnum angustifolium* 3, *Sphagnum capillifolium* 2, *Sphagnum girgensohnii* 1, *Sphagnum magellanicum* 1, *Polytrichum strictum* 1, *Pleurozium schreberi* 1, *Dicranum scoparium* +.

The previous descriptions of *dwarf birch* on Luci mentioned large bushes and frequent fruit production (Nyárády, 1926; Pop, 1928, 1960). Later on, vegetation surveys from 1976 of Coldea and Plămadă (1989) reveals a lower frequency for this species.

During our survey in 2017, only a few individuals of *Betula nana* were identified, without inflorescences or fruits. Probably the population ceased the sexual reproduction, being exposed to low genetic diversity. The main cause of species decline may be the change of habitat conditions. As a light demanding, hygrophilous, pioneer species (Ellenberg, 1992; De Groot, 1997; Sârbu *et al.*, 2013; Drzymulska, 2014) it may suffer in a shady forested peatbog. One argument is given by species physiology. *Betula nana* requires at least 2300 lux during summer to achieve the photosynthetic compensation point (photosynthesis greater than respiration); comparatively, the Scots Pine, a light demanding species too, can reach this point at only 1000 lux (Ellenberg, 1992). Moreover, the importance of light on *Betula nana* survival is confirmed by past observations on Luci and the current state at Găina-Lucina where *Betula nana* is in better conditions, as the habitat is more open – 7110* Active raised bogs (Pop, 1928; Ursu *et al.*, 2017). During interwar time the Luci peatbog had patches without or with very low cover of trees, where *Betula nana* was most abundant; on the contrary, the species grew seldom, as disperse, isolated stems under the pine canopy (Pop, 1960).

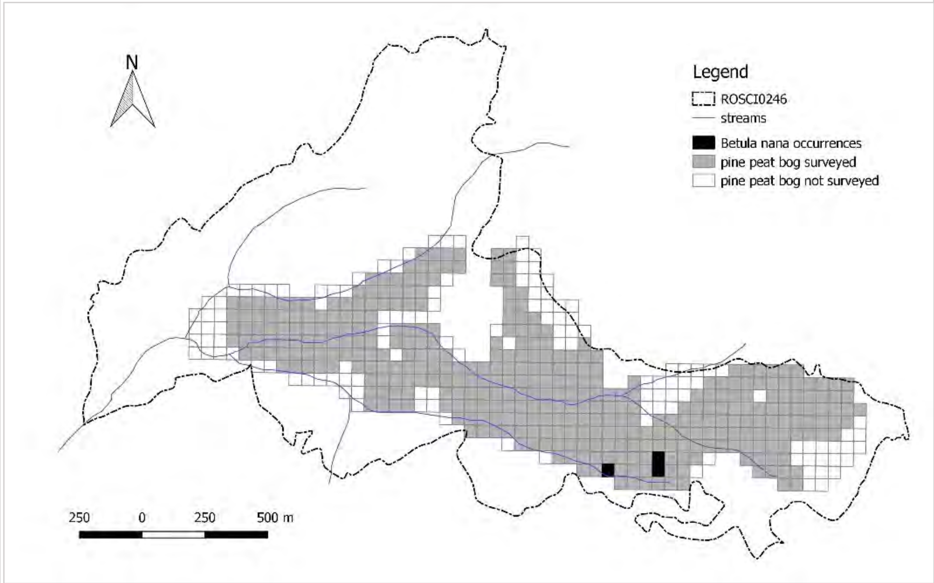

Figure 1. The occurence of *Betula nana* and its potential habitat, peatbog with *Pinus sylvestris*, in Tinovul Luci Natura 2000 site (ROSCI0246)

Nowadays, the light availability on the ground level decreases as pine trees' height and crown increase. On its subarctic range *Betula nana* can grow inside forests, but at lower latitudes the shading effect is more severe as the daylight during growing season is smaller. In former times, the forest gaps inside Luci bogland could have had secondary, man-made origins. At that time, the peat was used as bed for livestock; the forest was felled; sometimes fires were started by local villagers or shepherds in order to run away the bears. Obviously, the peat removal had a negative impact on all plants, but later on *Betula nana* was favored by reduced competition stress of pine, spruce and bilberry. Nowadays, the light regime and competition became unfavourable for the dwarf birch as disturbances diminish and the pine grows. Indirect effects may be on development of *Sphagnum* layer and evapotranspiration.

The time series of aridity index at Luci area for 1961-2013 shows occurrences of drought (EQ>30) on the last 25 years (Fig. 2). Comparatively, at Găina-Lucina the ombrothermic regime is more favorable and could maintain the habitat open. It is known that in the sub-continental climate of Eastern Europe the precipitation amount is usually insufficient to prevent peat bogs afforestation (Ellenberg, 1992). Therefore, in such sub-optimal conditions the favorability for trees may be rapidly shifted either by disturbances or by small climatic changes/ fluctuations. It is challenging to know if the passive preservation regime (non-intervention) in Tinovul Luci counted for such changes on *Betula nana*, i.e. favouring the natural succession toward bog woodland. Analysis of long-term vegetation dynamic in Luci area reveals the role of human impact in the modern age: the increase of pine is related with a sharp decline of beech (Tanțău *et al.*, 2003).

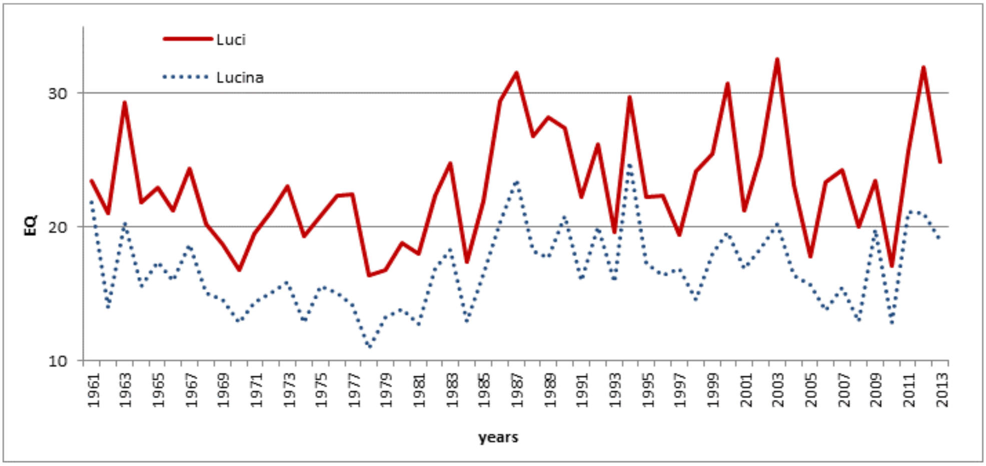

Figure 2. Variations of aridity Ellenberg quotient (EQ) during 1961-2013 for the neighbouring of Luci (HR) and Găina-Lucina (SV) peatbogs

Some human pressures upon reserve are encountered nowadays. The most obvious is the berry harvesting, that leads to trampling, brushing and, the worst of all, accumulation of plastic waste. Accidentally, livestock passage can occur, without significant impact. The effect of these pressures upon the dwarf birch may be inferred only, since no direct relationship was observed. The use of combs for berries harvesting may cause injuries to branches, leaves or twigs of *Betula nana.* Nevertheless, this pressure may not have a significant impact, since the species is resistant/resilient to tearing or grazing (De Groot *et al.*, 1997).

Overall, the conservation status of *Betula nana* on the Natura 2000 site Tinovul Luci was assessed as unfavourable bad (Tab. 1).

The following management measures are recommended:

- monitoring the population every year, due to its severe scarcity.
- improving habitat condition by decreasing canopy cover and creating small gaps in the pine stands around *Betula nana*; although the pine forest is a priority habitat, 91D0*, the EU regulations allow or even recommend to remove trees in order to restore the true raised bogs of type 7110*, 7130*, 7140* (Gafta and Mountford, 2008).
- planting saplings of dwarf birch obtained from seeds collected from other sites; the aim is to diminish genetic decline of local population.
- conducting controlled fertilization in order to increase shoots meristematic activity of dwarf birch (Bret-Harte *et al.*, 2001).
- regulating berries harvesting inside the protected area, harmonized with the objectives of Natura 2000; being a traditional activity it can’t be simply banned, but it may be bind with conservation awareness.

Table 1. Assessment of the conservation status of *Betula nana* on Luci peatbog

Parameter	**Description**	**Assessment**
Area of ocuppancy	7500 m^2	unfavourable bad
Area trend	decreasing	
Population size	< 25 indiv.	unfavourable bad
Population trend	decreasing	
Habitat area	125 ha	unfavourable bad
Habitat quality	poor, in terms of light conditions	
Habitat trend	stable	
Threats	competition for light; low genetic diversity	unfavourable bad
Overall		unfavourable bad

To the above assessment it should be add that the Luci's population of dwarf birch is geographically and genetically isolated. The nearest known conspecific population is more than 155 km away, at Găina-Lucina. Thus, *Betula nana* is facing an extremely high risk of extinction at the site level, corresponding to the IUCN category critically endangered (CR) (IUCN, 2012).

Conclusions

The dwarf birch in Luci peatbog is in a severe decline, having a very small, isolated population, with limited area of occupancy, habitat favourability and amphimixis. At site level this species is critically endangered (CR) and dedicated management actions are urgent. The current absence of a conservation strategy may be explained by the lack of a custodian and the division of forest management between two forest districts.

This study aimed to point out the vulnerability of a relict species and the related unfavourable effects of non-intervention strategy. As a key species of the Luci peatbog ecosystem, the dwarf birch deserves special attention and pertinent planning of protective actions.

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REFERENCES

Bret-Harte, M.S., Shaver, G.R., Zoerner, J.P., Johnston, J.F., Wagner, J.L., Chavez, A.S., Gunkelman, R.F., Lippert, S.C., & Laundre, J.A. (2001). Developmental plasticity allows *Betula nana* to dominate tundra subjected to an altered environment. *Ecology*, *82(1)*, 18-32.

Coldea, G., Indreica, A., & Oprea, A. (2015). Les associations végétales de Roumanie. Tome 3 – Les associations forestiéres et arbustives. *[in French]. Presa Universitară Clujeană* Cluj-Napoca, pp. 213-218.

Coldea, G., & Plămadă, E. (1989). Vegetația mlaștinilor oligotrofe din Carpații românești (Cl. *Oxycocco-Sphagnetea* Br.-Bl. et Tx. 43). *[in Romanian]. Contrib. Bot.*, *29*, 37-44.

Dihoru, G., & Negrean, G. (2009). Cartea roșie a plantelor vasculare din România. *[in Romanian]. Ed. Academiei Române* București. pp. 105-106.

Drzymulska, D. (2014). Postglacial occurrence and decline of *Betula nana* L. (dwarf birch) in northeastern Poland. *Estonian Journal of Earth Sciences*, *63(2),* 76-87.

Dumitrescu, A., & Bîrsan, M.V. (2015). ROCADA: a gridded daily climatic dataset over Romania (1961–2013) for nine meteorological variables. *Nat. Hazards*, *78(2)*, 1045-1063.

Ellenberg, H. (1992). Vegetation ecology of Central Europe. 4th ed. *Cambridge University Press*, pp. 153, 194, 340-341.

Frey, W., Frahm, J.P., Fischer, E., & Lobin, W. (2006). The liverworts, Mosses and Ferns of Europe. *Harley Books*.

Gafta, D., & Mountford, J.O. (2008). Manual de interpretare a habitatelor Natura 2000 din România. *[in Romanian]. Risoprint* Cluj-Napoca, pp. 69.

De Groot, W., Thomas, P., & Wein, R. (1997). *Betula nana* L. and *Betula glandulosa* Michx. *Journal of Ecology*, *85(2),* 241-264.

IUCN (2012). Guidelines for Application of IUCN Red List Criteria at Regional and National Levels: Version 4.0. *IUCN* Gland, Switzerland and Cambridge.

Nyárády, E.I. (1926). Adnotațiuni la flora României. *[in Romanian].* II. *Bul. Gr. Bot. Muz. Bot. Cluj*, *6(3-4)*, 102-107.

Pop, E. (1928). *Betula nana* L. și *Betula humilis* Schrk. în România. *[in Romanian]. Bul. Gr. Bot. Muz. Bot. Cluj*, *8(1)*, 1-7.

Pop, E. (1960). Mlaștinile de turbă din R.P. Română. *[in Romanian]. Ed. Academiei R.P. Române* București.

Rațiu, F. (1977). Corologia migroelementelor glaciare *Betula nana* L. and *Betula humilis* Schrank în Romania. *[in Romanian]. Studii și Comunic. Ocrot. Nat.*, Suceava, *4*, 177-187.

Sârbu, I., Ștefan, N., & Oprea, A. (2013). Plante vasculare din România. *Pteridophyta et Spermatophyta*. *[in Romanian]. Victor B Victor* București.

Șofletea, N., & Curtu, L. (2008). Dendrologie. 2nd ed. *[in Romanian]. Pentru viață* Brașov, pp. 197.

Tanțău, I., Fărcaș, S., Reille, M., & de Beaulieu, J.L. (2003). L'analyse palynologique de la sequence de Luci: nouvelles donnees concernant l'histoire de la vegetation tardiglaciaire et holocene de Monts Harghitei. *[in French]. Contrib. Bot.*, *38(1)*, 155-161.

Ursu, T.M., Fărcaș, S., Coldea, G., Stoica, I.A., & Proorocu, M. (2017). Review of habitat distribution, conservation status and human impact: the case of one Natura 2000 site in the Eastern Carpathians (Romania). *Contrib. Bot.*, *52*, 105-118.

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The relation between natural and sexual selection – study case on the phenotypic characteristics that influence mate choice in a North-West Romanian human population

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Abstract. The force of sexual selection acts upon the mating process of all beings, this great force is influenced by different factors as phenotypic characteristics. Related to mate choice, in humans, recent studies have shown that, along with eye color, hair color is considered a relevant physical trait (Frost, 2006). Thereby, the purpose of this study is to reveal how these two factors influence mate choice by analyzing the hair color preference of a small population from Romania. Additionally, we explore how different types of selection influence each other and assume that both contribute in choosing a partner. In order to obtain basic data, a socio-demographic survey was addressed to adult male and female individuals. Based on their answers, disregarding the gender, a considerable number of individuals are brown-haired, as we expected from this area of the globe. Among the results obtained, the following considerations are worth mentioning: the most common hair color in Romania is brown, individuals with this trait are considered the most attractive for the opposite sex and parents' hair color represents a predictor for future partners. These preferences are both in concordance and in contrast with natural selection. However, much more research is needed, approaching other areas such as genetics and forensics to determine how all these mechanisms, as well as others, affect the process of mating.

Keywords: clinal variation, hair color, parental imprinting.

Introduction

Evolution is influenced by both sexual and non-sexual interactions of certain physical traits (Janif *et al.*, 2014; Zinnia *et al.*, 2015). Even so, individuals' secondary characteristics are the basis for any type of selection and their evolution has an essential role in the survival of the species (Darwin, 1874).

When it comes to a partner, since Darwin's first observations, from insects to humans, all have a preferential behavior, related to sexual selection (Darwin, 1874). For example, a whole group of males will be in a competition to create ideal conditions, according to the requirements and expectations of the females. The male’s goal and all his intense work comes from the ambition to pass its genes to the next generation. It has also been noticed that, if males compete for areas rich in food, females may develop a certain affinity for this type of males with wide territories and rich in resources (Buss, 2006).

In nature, fighting and defending have the role of generating food, territory and continuity of generations. Thanks to these behaviors, besides better preserved individuals in the population, organisms with small variations of characteristics also appear, which increases the chance of personal safety, reproduction and evolution, as a result of cumulative natural selection over time. Even changes in texture or color of the skin, fur, plumage or hair can affect their safety and bring changes in behavioral development. As a result of new varieties in species, the predecessors less adapted to the existing living conditions are gradually replaced. A certain variation, more or less different from the wild form creates a slow and balanced evolution restricted by environmental conditions required for survival. Selective pressures act upon organisms in an unfavorable manner, as long as they do not adapt to the conditions imposed by the environment in which they live (Darwin *et al.*, 1930; Lyon and Montgomerie, 2012).

Recently, types of selection should not be classified or isolated into two different categories, sexual or natural selection. They intertwine and have the same end-purpose: the continuity of species (Lyon and Montgomerie, 2012). As we know so far, the process of choosing a partner is complex, determined by phenotypic characteristics, some influenced by parental imprinting and framed in a varied clinal range.

Parental imprinting phenomenon explains individuals' preferences and affects, to some extent, sexual selection, based on the early distinction of parental traits. It has two complementary forms of manifestation, a positive one and a negative one. First category includes a form of sexual selection, based on the correlation of phenotypic characteristics, valid for both sexes, that exist between the two partners or between the individual’s partner and parents and explained by familiarity formed in early years of life. Wilson and Barrett determined that the similarities between the eyes of a daughter's partner and the eyes of her father are not at all accidental, while the resemblances with the mother's eyes are insignificant (Wilson and Barrett, 1987). Negative imprinting is in opposition to the first model, so the individual is rejecting a potential mate with physical traits which are similar to his parents. This repulsion is also developing towards other individuals encountered in the first part of their life, such as siblings. Although at first it was thought so, research results do not demonstrate the existence of sexual aversion for the same-sex parent physical qualities (Westmarck, 2000; Little *et al*., 2003). None of these two types of imprinting abolish each other, but, on the contrary, each one has a well-established evolutionary role and influence how sexual selection acts upon individuals. The attractiveness of parents' general features determines the transmission of genes to the next generation, while the rejection is an intuitive behavior in order to avoid an inappropriate pairing, that leads to genetic and evolutionary imbalances (Bateson, 1980, Little *et al*., 2003).

Clinal variation refers to the geographical position, more precisely the latitude, which forms a variety of phenotypical characteristics observed in hair, eyes and skin color. It is an evolutionary process which has the role of modeling sexual selection by changing the way that parental imprinting works. All these modifications are maintained on a genetic level, along generations, until nowadays. In humans, latitude creates a globally chromatic diversity. Around the Equator, the color of the skin, and both eyes and hair are darker and become brighter as we reach the poles. This phenotypic variability began in Ancestral Europe, when modern humans left Africa. More severe climate and low temperatures have led to a decrease in melanin and vitamin D synthesis followed by brightening of skin, eye and hair color. All of these changes are happening in order to adapt to low UV radiation (Darwin *et al*., 1930; Parra, 2007; Vaughn *et al*., 2008; Frost, 2014). Hence, the preferences related to this phenotypic marker are differently expressed spatially and geographically, and depend to some extent on the socio-cultural factors (Ayton, 2005).

Finally, aspirations for certain qualities in a partner, with respect for sexual selection, create an evolutionary revolution by increasing the frequency of a desired quality and/or decreasing an unwanted characteristic. Ultimately, this phenomenon leads to an evolution of genetic diversity and diminishes the effects caused by the cline (Janif *et al*., 2014).

In this context, this study evaluates the percentage of the population from North-West part of Romania with relevant characteristics for the biogeographical area in which they are located. We also evaluate the number of volunteers in which parental imprinting determines preferences for the companion. Moreover, preferences for partners with different hair pigmentation have been exploited and analyzed. These preferences, belonging to sexual selection, are within the range of clinal variation. Last but not least, it has been attempted to explain a situation where sexual selection determines a compatible behavior or attitude, in contrast with the clinal variation belonging to natural selection.

In order to accomplish this, participants answered questions about their hair color, both parents and current partners hair colors and they also were given the possibility to choose from relevant pictures, illustrating individuals of each gender with different phenotypical characteristics.

Materials and methods

During October 2017 until February 2018, 107 volunteers, randomly chosen, 57 women and 50 men, aged 45 to 78 from Cluj-Napoca city of Romania participated in this study. The volunteers were part of a socio-demographic survey synthetically presented in Fig. 1. Initially, according to the participants' answers, we evaluate sex and age of each subjects. In the next step, four photos were presented (Tab. 1 and Fig. 1), two for each gender, which illustrate two women with opposite facial features: one of them with dark hair and brown eyes, and the other one blonde with blue eyes. Male photos were chosen following the same pattern. In order to make the interpretation of the data easier, each picture was marked by a letter: dark-featured female (A), light-featured female (B), dark-featured male (C) and light-featured male (D). Each participant was asked to choose the favorite photograph, males chose between A and B, and females between C and D, then to classify how attractive the chosen individual is, on a scale from 1 (I do not like it) to 7 (I really like it). Following, four questions were addressed, in order to find out the natural hair color of the subject, of both parents and the current partner. Descriptions of hair shades have been restricted for statistical reasons to four types of hair color: brown, black, blond and red.

Table 1. Survey format

Name initials: ____________________
Sex: M F
Age: ____________________
Which individual presented do you preffer? (A, B, C or D)
How much do you like her/him? (1 2 3 4 5 6 7)

1.Natural hair color of your mother is:
2.Natural hair color of your father is: ____________________
3.Your natural hair color is: ____________________
4. Do you have a partner? If yes – natural hair color of your partner is:

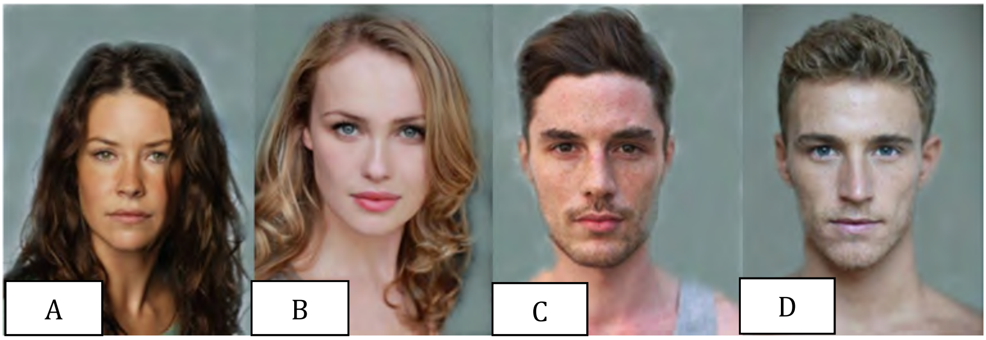

Figure 1. Survey questions and image options for men (A or B) and women (C or D)

Results

Analyzing the frequency in which these four hair shades appear in the studied population, we observe that 69 of the volunteers present brown hair, 28 black hair, 9 blonde hair and 1 person with red hair (Fig. 2). Regarding their family history, from a total number of 214 mothers and fathers, participants claimed 118 parents, with brown hair, preceded by 70 black-haired parents, 21 blondes and just 1 mother with red hair. The rest of 4 remain non-declared and statistically irrelevant (Fig. 3).

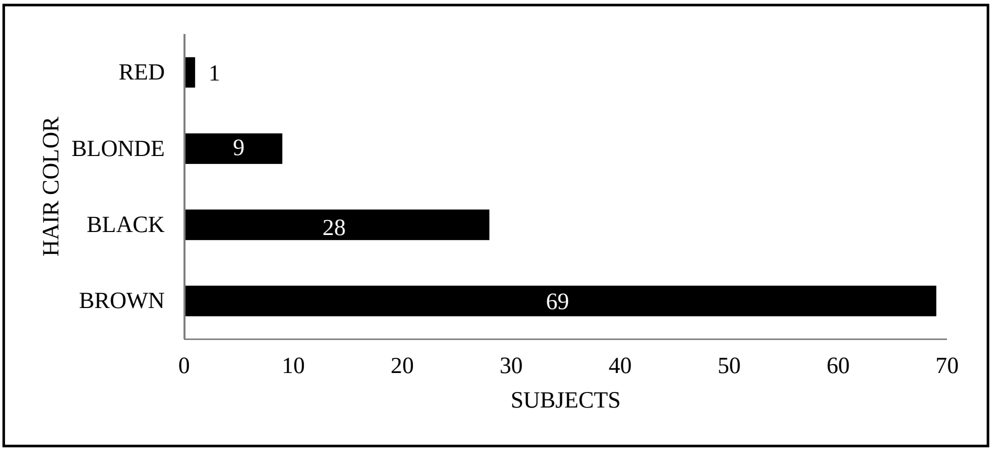

Figure 2. The total number of subjects and their hair color

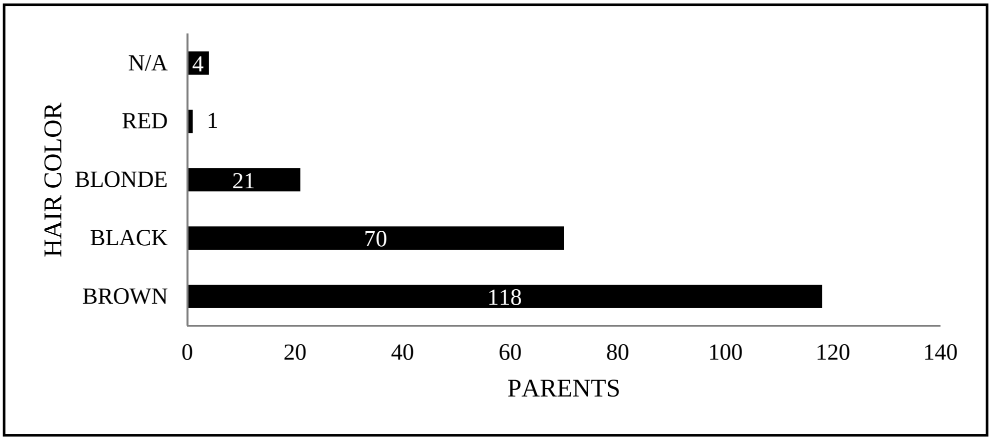

Figure 3. The total number of parents and their hair color

Regarding current romantic relationship situations, both women and men regularly declared partners who also present brown hair - 54 out of 107. The frequency of partners with black hair is also high and appears in 31 cases, followed by 10 blonde mates and another 1 red-haired (Fig. 4).

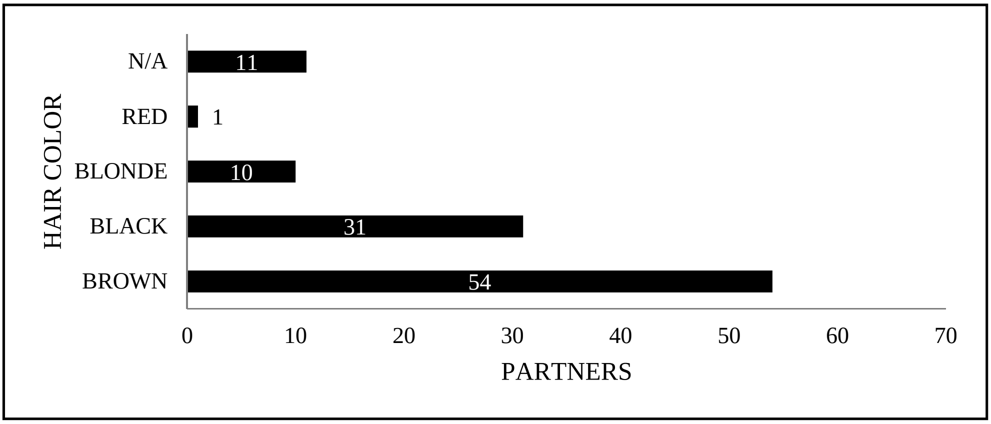

Figure 4. The total number of partners and their hair color

By comparing the results obtained from hair color analysis of parents and partners, we evaluated the chance that partners have similar hair shade as the same-sex and opposite-sex parent. Hence, for male volunteers, from a total of 50 possible cases, 45 of their partners have the same hair color as their mothers and 42 correlations between the shade of the partners and fathers have been found (Fig. 5). When it comes to female subjects, from a total of 57 situations, 44 of them present similar hair color among partners and opposite-sex parents and rest of the 43 cases display partner's hair color correspondent with the same-sex parent's hair color (Fig. 6).

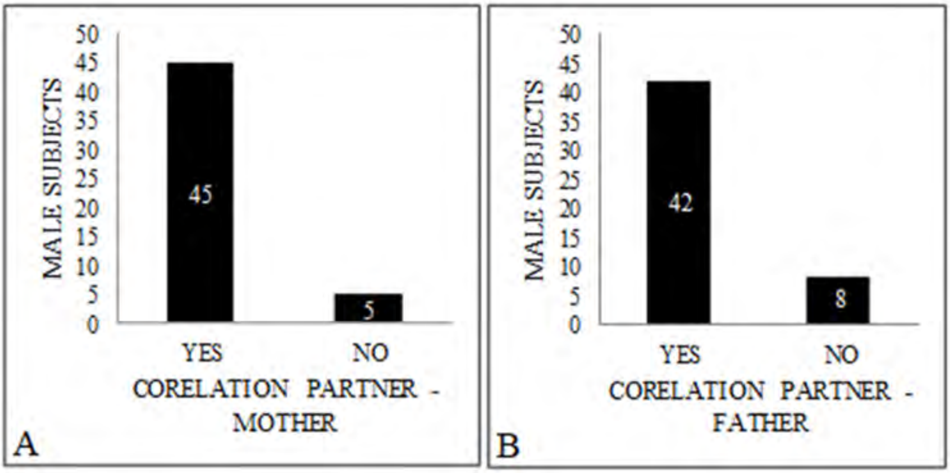

Figure 5. The number of male subjects who chose a partner with the same hair color as their opposite-sex parent (A) and as their same-sex parent (B)

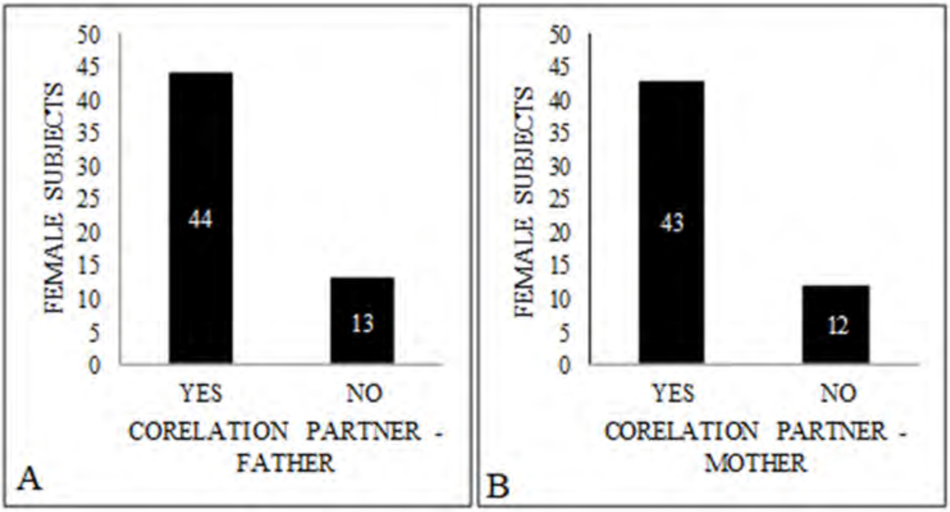

Figure 6. The number of female subjects who chose a partner with the same hair color as their opposite-sex parent (A) and as their same-sex parent (B)

In terms of choosing the favorite photograph, results indicate that 28 men preferred the one illustrating the female with dark facial features (A) and the rest of 22 chose the picture with the light facial features (B). Concerning the opposite gender, 33 females preferred the image with the brunette man (C) against the 24 who selected the blonde one (B) (Fig. 7).

When volunteers were asked about their own hair color preferences, 31 women indicate a different hair color than natural (Fig. 8). Furthermore, reports of male subjects suggest that 4 of them did not have a current partner. In the rest of the cases, 14 male volunteers declare that their partner dyed their hair in the same color as natural and 17 changed their hair color after dyeing (Fig. 9).

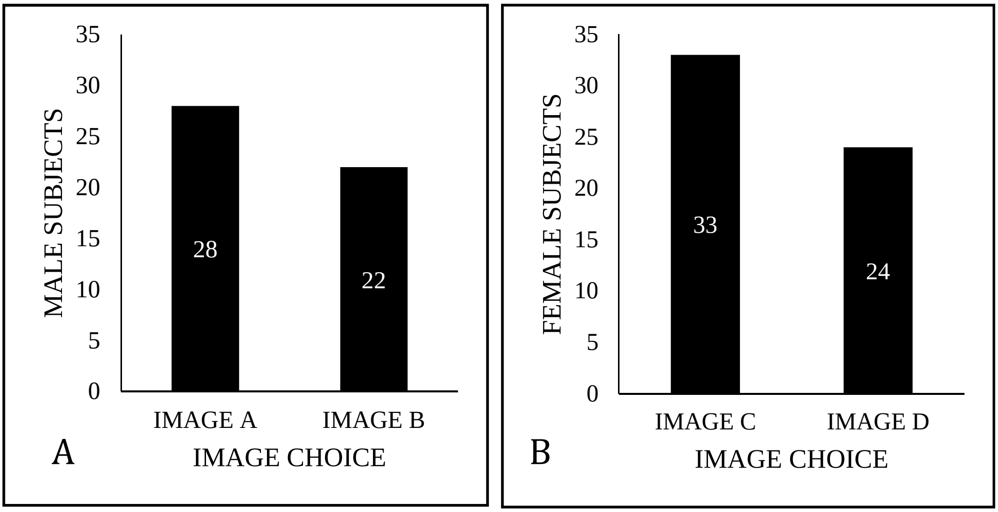

Figure 7. The number of male (A) and female (B) subjects and their image preference

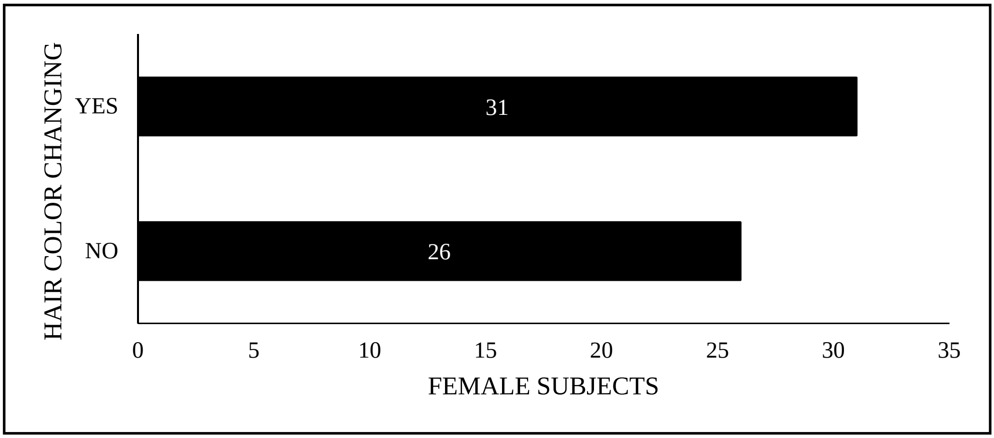

Figure 8. The number of females who would and would not prefer another hair color than natural

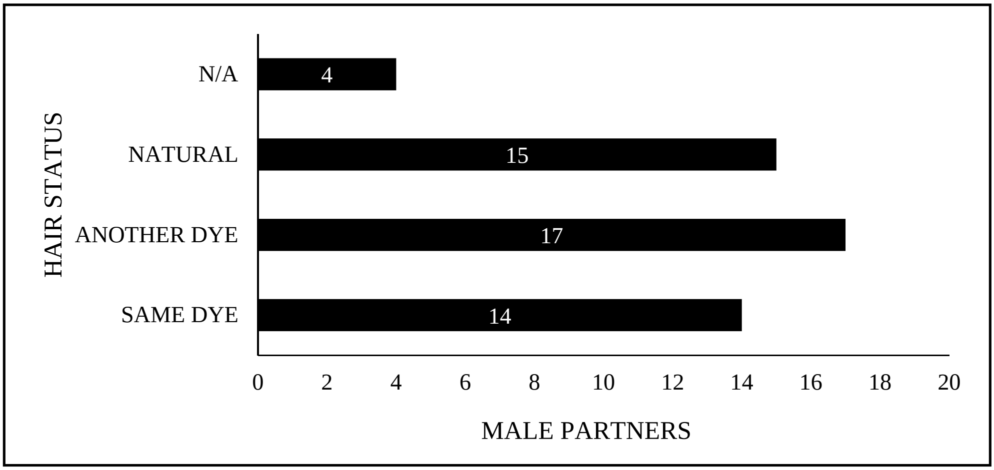

Figure 9. The number of male partners and their hair status (natural or dyed)

Discussion

Based on the survey data, it was found that most of the subjects who participated in the study had dark hair, especially brown (Fig. 2). Statements provided by male and female subjects show another high frequency of dark hair in parents and the lowest proportion of blond-haired (Fig. 3). This may be the result of the higher proportion of people with generally intermediate darker color traits such as brown eyes and hair and intermediate light skin living in this region. Also, possible influences of latitude can create an environmental diversity in human population (Janif *et al*., 2014). All these outcomes indicate that geographic positioning on the globe and cline could be determinant for eye and hair color (Darwin *et al*., 1930; Ayton, 2005; Shekar *et al*., 2008; Frost, 2014). In Romania, too, this may sustain the clinal variation process.

Some specialists have thoroughly studied the importance of hair color in particular cases like choosing a partner, and the link between it and the attractiveness of an individual. It was discovered that most people from a total of eight different cultures, considered dark hair individuals more attractive (Ayton, 2005).

The similitudes between the partner and the parent of the opposite sex have also been discussed, in terms of eye and hair color which are generally valid (Little *et al*., 2003). Recent studies consider the opposite parent's hair pigmentation a considerable predictor in choosing a partner, in humans, not excluding the positive effect of the same-sex parent's hair coloration (Westmarck, 2000). Wilson and Barret demonstrated before that eye color of the opposite-sex parent create a model of the offspring' partner. Because of the correlation between eye and hair color, we expected to see an increased similarity among the features of partners and the opposite-sex parents. Moreover, we explore trough this study that the color of the same-sex parent hair has an implication for choosing a partner, too (Wilson and Barrett, 1987). In almost all cases, regardless of gender, hair color similarities are obvious between both parents and partners (Figs. 5 and 6).

So, it can be said that parental imprinting has a significant role in mate choice and represents an adaptation strategy, in which the individual becomes marked by the general physical features of the parent with whom he grew up. So, we can agree with the statement that the opposite parent's hair pigmentation, one of the most important feature in choosing a partner (Little, *et al*., 2003), can be also considered in North-West part of Romania.

Moreover, all 107 statements and image choices affirm that both clinal variation and parental imprinting influence the favoritism for dark features in studied population due to the performed analogy between the favored image and the natural color of volunteers' partners. It can be noticed that, despite of the image choice, there is a predominance of brown-haired partners (Figs. 4 and 7).

Apart from the fact that there is a higher percentage of people with dark hair, there is also a preference for this phenotypic characteristic in Romania. This was based on the results obtained, namely the recurrence of brown hair in volunteer responses about their life partners. Considering all of this, it can be said that natural selection (existing hair, eye and skin color) overlaps the sexual selection (the preference for a certain hair, eye and skin color) and that the distinction between these two cannot be made. Both are part of one and the same type of selection, that encompasses them all.

Besides the fact that these two types of selection sum up a whole, they also influence each other. That can be the reason why even the female volunteers with blonde fathers favor a brown-haired partner. An interesting result can be observed in women who did not declare the color of their father's natural hair, but they chose exclusively black-haired partners.

The desire for transformation is more visible in women than men (Fig. 8). Alternatives proposed, in terms of hair colors, were red, violet, gray, and different shades of brown, black and blonde. The results also indicate that, most women would prefer red hair, the most rare natural hair color worldwide. Withal, a large number of the interrogated male's partners have their hair dyed (Fig. 9). Related to men's desire to have a different hair color than natural, the results are in opposition to female volunteers.

These results can demonstrate that observations made by Schweder, stating that the action of changing the natural hair color comes from the female desire to be different and intuitively attract males, behavior existing since Homo sapiens left Africa and faced the consequences of the Ice Age. This is confirmed by the hypothesis regarding the preference of novelty and rare characteristics in a population (Schweder, 1994; Frost, 2006; Frost, 2014; Zinnia *et al.*, 2015).

Conclusions

Most attractive partners for humans in Romania, studied until now, present dark features and physical appearance of the parents, which is the most important pattern for all human individuals, in terms of mate choice. This topic of major interest in biology, as well as other areas such as sociology, psychology or demography is still in a phase of ongoing debate and differing views since its original theorization given by Charles Darwin and deserves future statistical study and interventions.

REFERENCES

Ayton, P. (2005). How do men feel about women's hair colour? A survey of male and reactions to women's hair. Unpublished manuscript, City University, London.

Bateson, P. (1980). Optimal outbreeding and the development of sexual preferences in *Coturnix japonica* (Japanese quail)*, Z Tierpsychol,* 53, 231-244.

Buss, D.M. (2006). Strategies of Human Mating. *Phil. Topics, DOAJ*, 15, 239-260.

Darwin, C. (1874). The descent of man and selection in relation to sex. 2nd Ed. London, pp. 167-173.

Darwin, C., Wallace, A.R., Sarton, G., Lyell, C., & Hooker, J.D. (1930). Discovery of the Theory of Natural Selection. *UcCP,* 14, 133-154.

Frost, P. (2006). European hair and eye color. A case of frequency-dependent sexual selection?, *Evol Hum Behav, Elsevier Science*, 27, 85-103.

Frost P. (2014). The puzzle of european hair, eye, and skin color. *AA, Scientific Research,*4, 78-88.

Janif, Z.J., Brooks, R.C., & Dixson, B.J. (2014). Negative frequency-dependent preferences and variation in male facial hair. *Biology Letters , Proc. R. Soc. Lond,*10, 1-4.

Little, A.C., Penton-Voak, I.S., Burt, D.M., & Perrett, D., I. (2003). Investigating an imprinting-like phenomenon in humans. Partners and opposite-sex parents have similar hair and eye colour. *Evol Hum Behav, Elsevier Science,* 24, 43-51.

Lyon, B.E., & Montgomerie, R. (2012). Sexual selection is a form of social selection. *Philos. Trans. R. Soc. A,* 367, 2266-2273.

Parra, E.J. (2007). Human pigmentation variation: evolution, genetic basis, and implications for public health. *Yearb. Phys. Anthropol.,* 50, 85-105.

Schweder, B.I.M. (1994). The impact of the face on long-term human relationships. *Homo,* 45, 74–93.

Shekar, S.N., Duffy, D.L., Frudakis, T., Montgomery, G.W., James, M.R., Sturm, R.A., & Martin, N.G. (2008). Spectrophotometric methods for quantifying pigmentation in human hair-influence of MC1R genotype and environment. *Photochem. Photobiol.,* 84, 719-726.

Vaughn, M., Oorschot, R., & Baindur-Hudson, S. (2008). Hair color measurement and variation. *Am. J. Phys. Anthropol.,* 137, 91–96.

Westmarck, E. (2000). The history of human marriage. Ed. Adamant Media Corporation, reprint of 1903 Ed. Macmilan and Co., Limited, London, pp. 67-73.

Wilson, G.D., & Barrett, P.T. (1987). Parental characteristics and partner choice: some evidence for Oedipal imprinting. *J. Biosoc. Sci,* 19, 157–161.

Zinnia, J.J., Brooks, R.C., & Dixson, B.J. (2015). Are Preferences for Women's Hair Color Frequency-Dependent? *Adapt. Hum. Behav. Physiol*., *Springer,* 1, 54-71.

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Effects of vegetation and water seasonal variation on habitat use of herons (Aves, Ardeidae) in Tonga Lake (North-East Algeria)

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Abstract. The present study was aimed to determine the effect of some environmental factors such as water features and vegetation cover on the distribution and habitat use of herons over different seasons of the bird's life (breeding, passage and wintering) in Tonga Lake (North East Algeria) from March 2017 to February 2018. We used remote sensing techniques to study the environmental factors variations, we adopted the normalized difference vegetation index (NDVI) to give an estimate of vegetation cover and we used the normalized difference water index (NDWI) to study the water features. In our study, we determine that the abundance and richness of herons species is largely influenced by the water features and vegetation cover. The seasonal variations in the amount of these environmental factors are known to affect the availability of various food items for herons. Eight species of herons were identified during the study period. Pearson's Correlation Coefficient was used to examine the effect of NDVI and NDWI on the distribution of herons. The results showed that the NDVI was significantly positively correlated with richness and abundance of herons respectively ($r=0.728$, $p<0.05$) ($r=0.651$, $p<0.05$), wherease the NDWI was significantly negatively correlated with the richness and abundance of herons ($r=-0.65$, $p<0.05$) ($r=-0.69$, $p<0.05$) respectively.

Keywords: habitats, herons, NDVI, NDWI, Ramsar site.

Introduction

The concept of habitats has been widely documented (Santilli and Bagliacca, 2010; Hull *et al*., 2016; Ashiagbor and Danquah, 2017), although several authors have recommended of habitat in the context of ornithology (Hayes and Fox, 1991; Jones, 2001; Tommy King and Michot, 2002; Litlefeld and Johnson, 2005; Bensaci *et al.,* 2015; Swathi and Antoney, 2018).

The conspicuous nature of birds has allowed ornithologists to assemble a vast amount of information relating to the distribution and the abundance of birds to the environment's aspects (Koskimies, 1989; Paracuellos and Telleria, 2004). The characteristics of the habitat reflect the waterbird diversity, whose relative importance varies on both spatial and temporal scales (Wu *et al*., 2014). Many other authors evaluated the influence of vegetation and water as determinants of habitat use by birds (Colwellando and Tafti, 2000; Bancroft *et al*., 2002; Rajpar and Zakaria, 2014). There are frequently assumed to be the primary proximate factors determining where and how species use resources. Temporal scale is also important because birds do different things to meet varying functional needs during different times of the year and they also exhibit habitat shifts between years (Saygili *et al*., 2011; González-Gajardo *et al*., 2009).

Wetlands provide habitats for many water bird communitiesand home for large number of migratory and resident species ofbirds (Vasudeva Rao *et al*., 2015) due to habitat diversity and productivity, they are also characterized by shallow water overlying waterlogged soil, interspersed submerged and emergent vegetation (Rajpar and Zakaria, 2014; Martins *et al*., 2017).

North-East Algeria holds a wide range of wetlands, many of them designated as Important Bird Areas (IBAs) and/or Ramsar sites (Samraoui and Samraoui, 2008). Tonga Lake within North east Algeria is classified among the North Africa's top biodiversity hotspots (Samraoui *et al*., 1998). This seasonal freshwater lake has an international importance under the Ramsar Convention in 1983. In turn, Family Ardeidae is one of the best represented in the North-Eeast Algeria (Chalabi-Belhadj, 2008). Herons are wading birds of considerable importance, because they constitute indicator species in wetlands. Additionally, some species of *Ardea* have been studied as bioindicators of mercury (Goutner *et al*., 2001) and organochlorines (Albanis *et al.,* 1996).

The main objective of our study is to determine the effect of vegetation and water seasonal variation on habitat use of herons during their annual cycle of occurrence in Tonga Lake.

Throughout this contribution we tried to analyzing the relationships between changes in waterbird communities and the environmental variables across the effect of seasonal variation of vegetation and water cover on the diversity, temporal and spatial distribution of herons in the Tonga lake in the ordre to used in further management and conservation implications of these wetlands and ensure the suitable habitat requirements of waterbirds.

Materials and methods

The Tonga lake (36°52′ N, 8°31′ E), where this study was carried out is situated in the extreme north east of Algeria near the Algerian-Tunisian border, within the protected natural reserve of El-Kala National Park (PNEK)(Fig. 1). This Lake is classified as an international importance site under the Ramsar Convention in 1983 and as an Important Bird Area (IBA), which is generally, recognized as one of the four major wetland complexes in the Western Mediterranean (Bensliman *et al.,* 2015; Boucheker *et al.,* 2009). This shallow freshwater lake covers an area of 2400 ha with a maximum depth of 2.5 m. It is fed by two tributaries: Oued El Hout from the southeast and Oued El Eurg from the east. On the north part of Tonga Lake the artifcial canal of the Messida represent the connection with the Mediterranean Sea. Almost 80% of its area is covered by helophytes (Lesser Bulrush *Typha angustifolia*, Common Club-rush *Scirpus lacustris* and Common Reed *Phragmites australis*) and hydrophytes (white lotus *Nymphaea alba* and Stuckenia pectinata*Potamogeton pectinatus*) (Aissaoui *et al*., 2011; Lazli *et al*., 2011; Kadid *et al*., 2007).

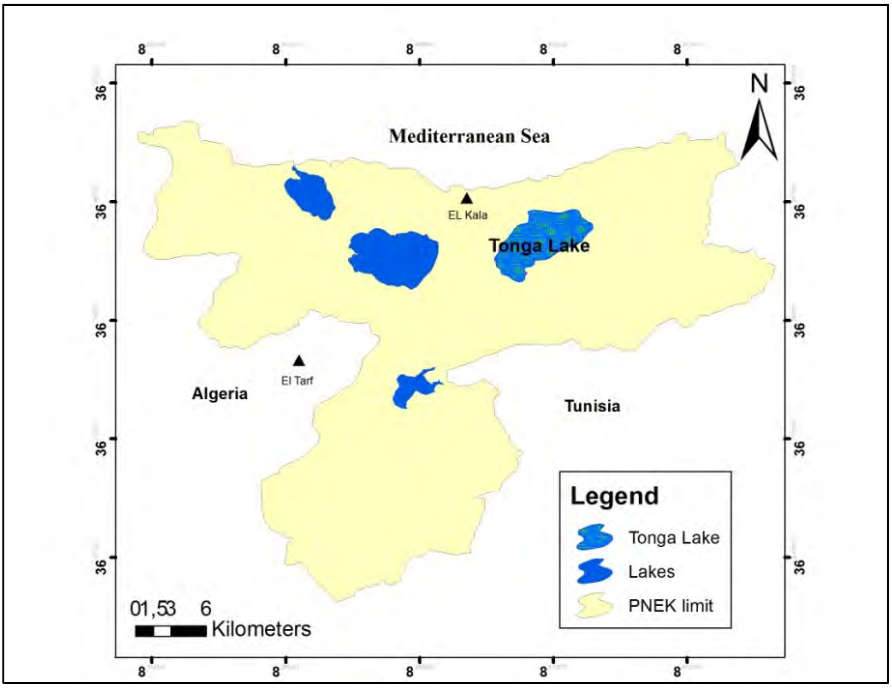

Figure 1. Location of Tonga Lake

Data collection

Heron surveys

Heron species surveys were conducted from March 2017 to February 2018, over different seasons of the bird's life (reproduction, passage and wintering). Heron censuses were conducted in breeding season(late April-late June), Passage season (late July-late November) and wintering season (late December-early March) (Yahner, 1986). Regular counts of richness and abundance of species in each habitat were undertaken by 2 observers by using a telescope 20 ×80 or 10 × 50 binoculars and birds guide of Heinzel (Heinzel *et al*., 1972). We carried out an individual count (absolute method), if thegroups of birds are less than 200 individuals and located at an inferior of 200 m, on the other hand we have made estimates of total numbers(relative method) for birds more than 200 m away, anda workforce of more than 200 individuals (Blondel, 1975). All observations and counts were made during the early hours of the day, between sunrise to 12:00 h and between 14:00 h to sunset.

Environnemental variables

In the aim to determine the effect of environment variables on the habitat use of herons over different season, two main parameters were taken in to count: spatio-temporal variation of vegetation and water cover.

To evaluate former parameters, two indexes were calculated relatively to all study period: NDVI and NDWI for vegetation and water cover respectively.

NDVI and NDWI analysis

The normalized difference vegetation index (NDVI) and the normalized difference Water index (NDWI) data were obtained from the Landsat TM images were obtained from United States Geological Survey (USGS).

The normalized difference vegetation index (NDVI) gives an estimate of the green vegetation abundance (Tucker, 1979).NDVI use band 4 (Red) come with band 5 (Near Infrared) for Landsat 8. NDVI is formulated as below:

$$\textbf{NDVI= (NIR-RED) / (NIR+RED)}$$

Where: NIR-near infrared radiation reflectance; RED-visible red radiation reflectance. The normalized difference water index (NDWI) is a method that has been developed to delineate open water features and enhance their presence in remotely-sensed digital imagery (Mcfeeters, 1996; Mcfeeters, 2013). NDVI use band 2 (Green) come with band 4(Red) for Landsat 8.The NDWI is expressed as follows:

$$\textbf{NDWI= (GREEN -RED) / (GREEN+RED)}$$

The results of NDVI and NDWI generate a value between -1 and +1.

Statistical analysis

All analyses were performed after verification of the normality test (test of Shapiro-Wilk) of the various dependent and independent variables. The one-way analysis of variance (ANOVA) and a Ttest. Those tests both are used when our data has a normal distribution. The one-way analysis of variance (ANOVA) was performed to search for a possible variation in the abundance, richness, NDVI and NDWI of herons during the deferent seasons. T test (Student's T-Test) was used to compare variation in abundance and richness between the two habitats (vegetation and water). In addition, the Pearson's correlation was used to search the relationship and association between species richness, abundance and habitat factors (NDVI and NDWI). The p-value<0.05 were *considered significant.* The species diversity in the different season was investigated by the biodiversity indices. All statistics were performed using the Software Package for Social Statistics (IBM SPSS Statistics Version 23).

Results and discussion

Heron species richness and relative abundance

During the study period, Tonga Lake recorded eight species of herons, out of which seven have breeding status. Thre especies of herons observed in Tonga Lake are threatened in Europe: the purple heron *Ardea purpurea*, little bittern *Ixobrychus minutus* (which are considered "declined") and squacco heron *Ardeola ralloides* which is considered "rare" (Leon *et al.*, 2004).

The significant difference for richness of herons among the three seasons, was compared by applying the parametric test (normal distribution), the one-way ANOVA*.* The one –way ANOVA test indicated that the richness of herons in the three seasons was significantly different (F = 3.85, p<0.05). However,there was a significant difference in the mean richness of heron species between different vegetation's habitat and water habitat's(t (18) = -5.06, p<0.001) (Tab. 1).

Table 1. Mean richness of herons in the three different seasons in Tonga Lake

	Richness			Abundance		
Season	Mean±*sd*	%	Range (*min-max*)	Mean±*sd*	%	Range (*min-max*)
Breeding	5.00±1.15	87.50	3-7	303.92±133.11	57.66	160–553
Passage	4.83±0.75	75.00	4-6	294.66±60.86	38.06	213-365
Wintering	3.50±0.57	43.75	3-4	34.25±9.06	4.27	21- 41

sd: Standard deviation

One–way ANOVA test revealed a significant difference of herons abundance between the three seasons (F= 10.17, p <0.001). Also, the variation in mean abundance between the vegetation habitat's and water habitat's was significantly different (t (18) = 2.37, p<0.05). The significant variation in species richness and abundance of herons throughout the diferent seasons could be due to seasonal movement patterns and habitat changes (Tab. 2). Yu-Seong *et al.,* 2007, recorded that the availability of feeding habitat is an important factor affecting the richness and abundance of herons species.

Table 2. Species variation of herons recorded in the three different seasons in Tonga Lake

Season	**Breeding**			**Passage**			**Wintering**		
Species name	Mean ± *sd*	%	Range (*min-max*)	Mean ± *sd*	%	Range (*min-max*)	Mean ± *sd*	%	Range (*min-max*)
Little Egret	95.63 ±38.47	26.73	38-149	133.83 ±90.21	39.24	33-250	5.5 ± 3.69	18.36	1-9
Cattle Egret	104.9 ±79.79	39.46	6-223	5.66 ±1.63	1.25	4-8	4.66 ± 0.57	10.20	4-5
Squacco Heron	118.75 ±21.26	28.14	97-159	52.23 ±52.27	17.26	7-110	-	-	-
Grey Heron	2.1 ±0.69	0.53	1-3	48.16 ±52.54	19.15	4-122	10.16 ± 4.15	32.65	4-16
Purple Heron	10.87 ± 5.02	2.83	1-16	3.33 ±4.04	1.25	1- 8	-	-	-
Night Heron	8.25 ± 5.12	2.30	1-13	-	-	-	-	-	-
Little Bittern	1.66 ± 0.57	0.35	1- 2	-	-	-	-	-	-
Great Egret	-	-	-	90 ±52.12	21.82	24-139	10.71 ± 6.60	38.77	1-19

sd: Standard deviation

Diversity pattern

The diversity of heron species in the different study seasons shows that the passage season have the highest species diversity (Shannon's index H' = 1.40; Margalef's index R = 0.86) comparatively to breeding and wintering seasons (Tab. 3).

Waterbirds diversity distribution pattern has been often explained in terms of simple environmental factors. Vegetation cover and water features have been alternatively considered as key factors in the determination of the variable distribution of waterbird diversity (Rotenberry and Wiens, 1980; Wu *et al*., 2014; Dronova *et al*., 2016; Zhang *et al*., 2017).

Table 3. Comparison of herons species diversity of breeding, passage and wintering seasons in Tonga Lake

Diversity indices	Breeding	Passage	Wintering
Shannon_H	1.34	1.40	1.32
Margalef	1.03	0.86	0.89

Normalized Difference Vegetation Index (NDVI)

The ANOVA analysis for normalized difference vegetation index (NDVI) among the three study seasons indicated that the vegetation cover was significantly different (F = 8.7; p<0.01). The results of the normalized difference vegetation index (NDVI) show that the breeding season has the highest average (0.237 ± 0.019), and the wintering season has the lowest NDVI average (0,139 ± 0.05). One-way ANOVA analysis of surface vegetation cover indicated a significant difference between the three study seasons (F = 10. 97; p<0.001), where, the breeding season has the highest surface vegetation cover (58.95%). In contrast, wintering season has the lowest surface vegetation cover (10.44%) (Tab. 4).

The examination of the relationship between seasonal NDVI variations and herons population parameters in Tonga Lake revealed a positive correlation between NDVI and species richness (Pearson's correlation r = 0.728, p<0.01) and species abundance (Pearson's correlation r = 0.651, p<0.05).

Species richness and abundance increase positively with the NDVI. Higher NDVI may indicate the presence of better vegetative food resources (Figs. 2 and 3). Lower NDVI values may indicate lower vegetation coverage, and usually determine higher water levels (Fig. 4). The species richness and abundance of many waterbirds bird species are determined by the composition of the vegetation thatforms a major element of their habitats (Bancroft *et al.*, 2002; Martins *et al.,* 2017). The breeding season has the higher NDVI average and vegetation cover; also this season has the higher abundance of herons. This might be due to the surface of vegetation cover which had created different microhabitats rich in food resources that offer suitable habitats and attracted a wide number of herons species (Cousin and Phillips, 2008; Hassen-Aboushiba, 2015). These habitats provide suitable foraging, roosting, and nesting area for the most heron's species. Additionally, vegetation may calm the water surface, thus increasing visibility and may have higher prey densities i.e. invertebrates (insects and gastropods) (Djamai *et al.*, 2019), fishes (carps and cat fishes), amphibians (frogs and salamanders). Otherreason could be that, the heterogeneity of aquatic vegetation also provides shelterfrom weather and predators. This could be too the aquatic vegetation havingshallow water level.

Table 4. Values of NDVI, surface vegetation cover (ha) and percent of vegetation cover (%) in deferent season in Tonga Lake

	Breeding	**Passage**	**Wintering**
	Mean ± *sd*	Mean ±*sd*	Mean±*sd*
Mean NDVI	0.237 ±0.019	0.221 ±0.021	0.139 ±0.05
Mean surface vegetation cover (Ha)	1415 ±227.27	1164 ±474.59	282 ±257.25
Percent of vegetation cover (%)	58.95	43.11	10.44

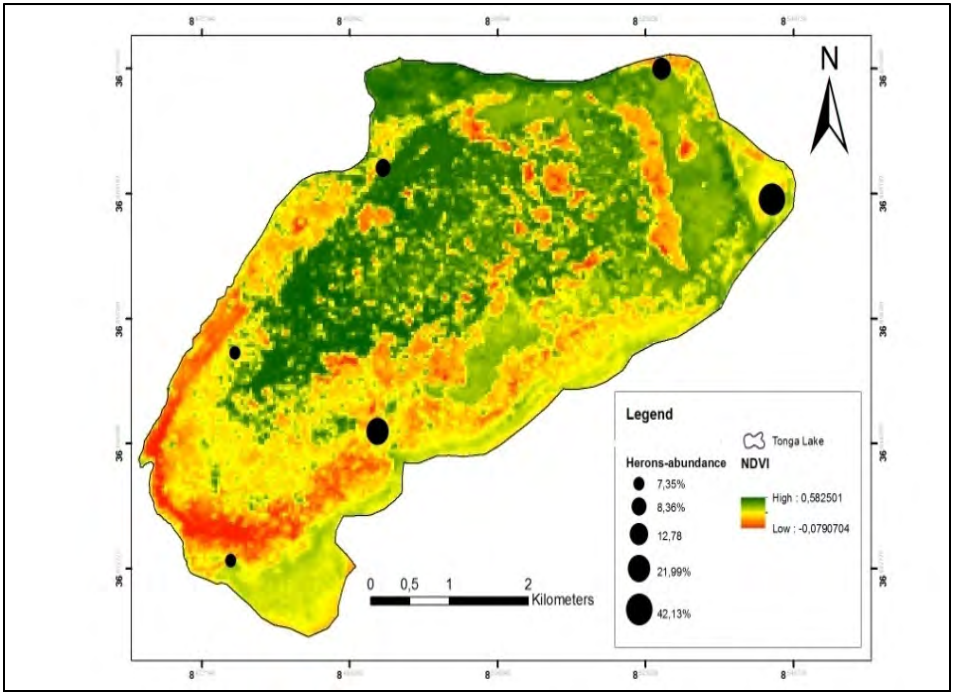

Figure 2. NDVI map of Tonga Lake in breeding season with the distribution of herons.

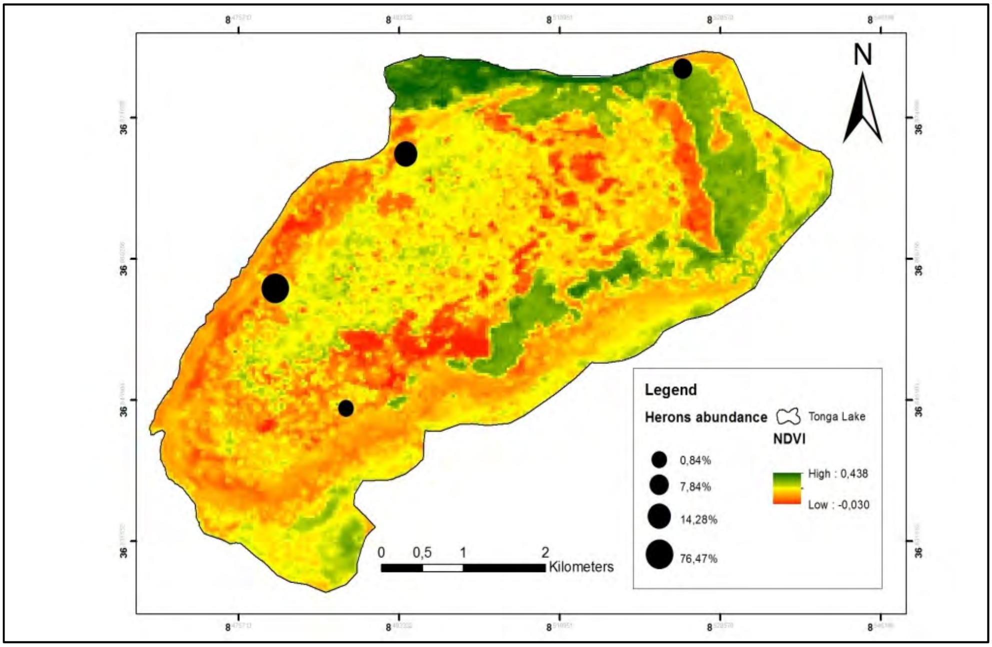

Figure 3. NDVI map of Tonga Lake in passage season with the distribution of herons.

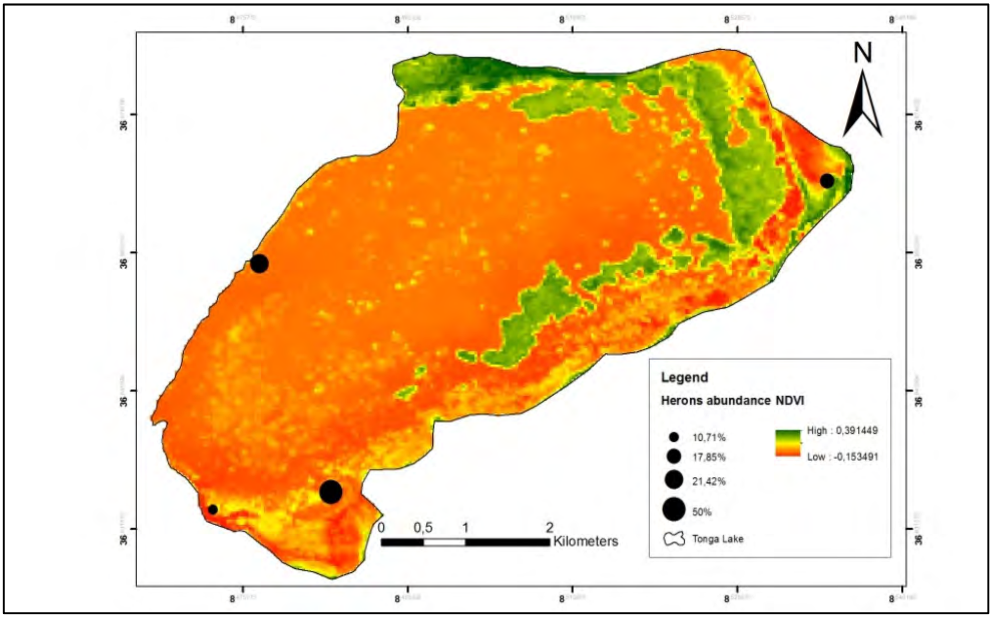

Figure 4. NDVI map of Tonga Lake in wintering season with the distribution of herons.

Normalized Difference water index (NDWI)

One-way ANOVA analysis of the normalized difference water index (NDVI) indicated that the water area of the three seasons was highly significantly different (F = 5.44, $p<0.05$). However, the wintering season has the higher NDWI average (0.081±0.021), while, the passage season has the lowest NDWI average (0.031±0.018). The one way ANOVA analysis showed that the water area of the three seasons was highly significantly different (F = 7.24, $p<0.05$).Although, the variation of water area during wintering season has the highest (41.95%), while the passage season has the lowest water area (3.79%) (Tab. 5).

The examination of relationship between seasonal variations NDWI and species abundance and richness variations revealed a negative correlation between species richness and NDWI (Pearson's correlation r = -0.65 ; $p<0.05$), and between species abundance and NDWI (Pearson's correlation r = -0.69 ; $p<0.05$).

Dubowy (1996), Colwel and Taft (2000) recorded that Herons showed a preference to shallow water because it provide suitable resources of food. Another study in Algeria of Nouidjem *et al.* (2019) recorded that herons usually seenon shallow shorelines.

Table 5. Comparison of Mean NDWI, Mean water area (Ha) and Percent of water surface (%) in deferent season of herons in Tonga Lake

	Breeding	**Passage**	**Wintering**
	Mean ± *sd*	Mean ± *sd*	Mean ± *sd*
Mean NDVI	0.047±0.016	0,031±0.018	0.081±0.021
Mean of water area (Ha)	475±264.75	91±47.07	1007±446.28
Percent of water area %	19.79	3.79	41.95

Our results showed a negative correlation between the species richness, abundance and the normalized difference water index (NDWI). While, the effect of water area on herons varied among seasons. The breeding season has an intermediate NDWI overage (Fig. 5), the passage season has a lowest NDWI average (Fig. 6) and the highest diversity index for herons species (H = 1.40). Generally, the presence of intermediate level of water decreases the species richness, abundance and diversity of herons. These results indicated that herons families showed a preference to shallow water. Dry season, recession in Tonga Lake may concentrate prey in shallow water.The water habitat includes shallow water harbored higher richness of aquatic invertebrates, fishes and amphibians which is major diet of herons species (Willard, 1977; Montesinos *et al.*, 2008; Roshnath, 2015). Wintering season showed the lowest NDVI and the highest

NDWI overage. During winter period, due to severe rainfall precipitation, the water level increase and the cover of aquatic vegetation decrease (Fig. 7). In turn, the food availability will be severely impacted; in response to this the Herons are forced to migrate in other wetlands. These results confirmed that the habitats of herons related closely with prey and food availability. Clearly, prey availability plays a key role to avian life histories (Gawlik, 2002).

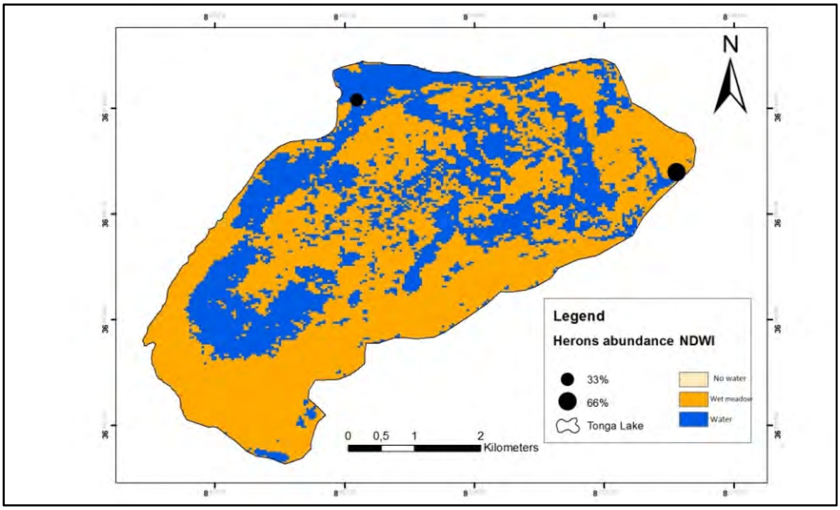

Figure 5. NDWI map of Tonga Lake in breeding season with the distribution of herons.

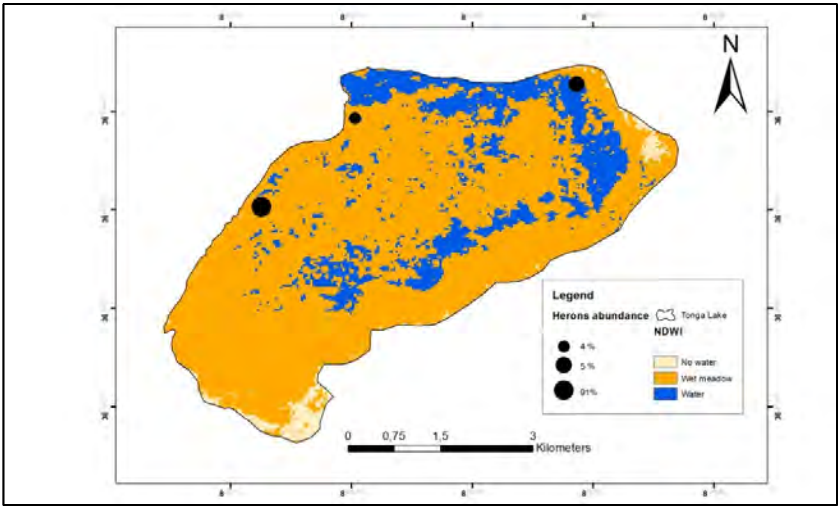

Figure 6. NDWI map of Tonga Lake in passage season with the distribution of herons.

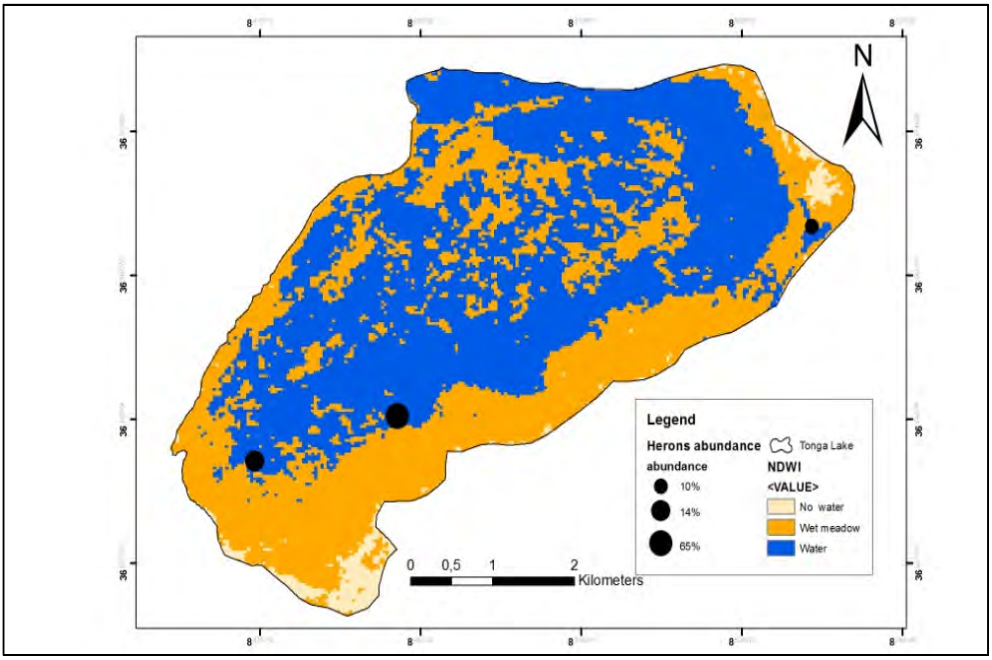

Figure 7. NDWI map of Tonga Lake in wintering season with the distribution of herons.

Conclusions

Our study concluded that herons species showed a preference for shallow waters and intermediate levels of vegetation cover. Herons family selected areas with shallow water and vegetation because these habitat features are often associated with high prey densities. Other variables, though not analysed in this study, might also influence the species richness, abundances, diversity and habitat use of herons.

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REFERENCES

Aissaoui, R., Tahar, A., Saheb, M., Guergueb, L., & Houhamdi, M. (2011). Diurnal Behaviour of Ferruginous Duck *Aythya nyroca* wintering at the El-Kala Wetlands (Northeast Algeria). *Bulletin de l'Institut Scientifique, Rabat, section Sciences de la Vie,* 33(2), 67–75.

Albanis, T.A., Hela, D., Papakostas, G., & Goutner, V. (1996). Concentration and bioaccumulation of organochlorine pesticide residues in herons and their prey in wetlands of Thermaikos Gulf, Macedonia, Greece. *Sci Total Environ,* 182,11-19.

Ashiagbor, G., & Danquah, E. (2017). Seasonal habitat use by Elephants (*Loxodonta africana*) in the Mole National Park of Ghana. *Ecologia,* 7(11), 3784-3795.

Bancroft, G.T., Gawlik, D.E., & Rutchey, K. (2002). Distribution of wading birds relative to vegetation and water depths in the Northern Everglades of Florida, USA. *Waterbirds,* 25(3), 265-277.

Bensaci, E., Saheb, M., Nouidjem, Y., Zoubiri, A., Bouzegag, A., & Houhamdi, M., (2015). Status, Habitat Use, and Behaviour of Wintering Greater Flamingos *Phoenicopterus roseus* in Semi-Arid and Saharan Wetlands of Algeria. *International Journal of Biological, Biomolecular, Agricultural, Food and Biotechnological Engineering*, 9(3), 350-355.

Benslimane, F., Labar, S., Djidel, M., Hamilton C.M.L., & Djemai R. (2015). Assessing of Tonga Lake Water Quality in the coastal basin of Northeastern Algeria. *Inter Jour Sci Engi Res,* 6(3), 202-8.

Blondel, J. (1975). L'analyse des peuplements d'Oiseaux – éléments d'un diagnostic écologique : la méthode des échantillonnages fréquentiels progressifs (E.F.P.). *Rev Ecol-Terre Vie,* 29(4), 533 – 589.

Boucheker, A., Nedjah, R., Samraoui, F., Menaï, R., & Samraoui B.(2009). Aspects of the Breeding Ecology and Conservation of the Glossy Ibis in Algeria. *Waterbirds,* 32(2), 345-351.

Chalabi-Belhadj, G. (2008). Contribution à l'étude des exigences écologiques des Ardeirdae et de l'Ibis falcinelle *Plegadis falcinellus* dans le complexe des zones humides d'El Kala (Algérie). *Thèse doctorat, Inst. Nat. Agro., El Harrach. (Alger)*, pp. 195.

Colwell, M.A., & Taft, O.W. (2000). Waterbird Communities in Managed Wetlands of Varying Water Depth. *Waterbirds,* 23(1), 45-55.

Cousin, J.A., & Phillips, R.D. (2008). Habitat complexity explains species-specific occupancy but not species richness in Western Australian woodland. *Aust J Zool,* 56(2), 95–102.

Djamai, S., Mimeche, F., Bensaci, E., & Oliva-Paterna, F.J. (2019). Diversity of macro-invertebrates in Lake Tonga (northeast Algeria), *Biharean Biol,* 13(1), 8-11.

Dronova, I., Beissinger, S.R., Burnham, J.W., & Gong, P. (2016). Landscape-Level Associations of Wintering Waterbird Diversity and Abundance from Remotely Sensed Wetland Characteristics of Poyang Lake. *Remote Sens,* 8(462), 1-22.

Dubowy, P.J. (1996). Effects of water levels and weather on wintering herons and egrets. *Southwest Nat,* 41(4), 341-347.

Gawlik, D.E. (2002). The effects of prey availability on the numerical response of wading birds. *Ecol Monogr,* 72(3), 329–346.

González–Gajardo, A., Sepúlveda, P.V., & Schlatter.P.(2009). Waterbird Assemblages and Habitat Characteristics in Wetlands: Influence of Temporal Variability on Species-Habitat Relationships. *Waterbirds,* 32(2), 225-233.

Goutner, V., Furness, R.W., & Papakostas, G. (2001). Mercury in feathers of Suacco Heron (*Ardeola ralloides*) chicks in relation to age, hatching order, growth, and sampling dates. *Environ Pollut,* 11 (1), 107- 115.

Hassen-Aboushiba, A.B. (2015). Assessing the effects of aquatic vegetation composition on waterbird distribution and richness in natural freshwater Lake of Malaysia. *Am J Life Sci,* 3(4), 316-321.

Hayes, F.E., & Fox, J.A. (1991). Seasonality, habitat use, and flock sizes of Shorebirds at the Bahia De Asuncion, Paraguay. *Wilson Bul,*103 (4), 637-649.

Heinzel, H., Fitter, R., & Parslow, J. (1972). Les oiseaux d'Europe, d'Afrique du Nord et du Moyen-Orient. *Ed. Delachaux et Niestlé, Paris,* pp.384.

Hull, V., Zhang, J., Huang J., Zhou, S., Viña, A., Shortridge, A., Li, R., Liu, D., Xu, W., Ouyang, Z., Zhang, H., & Liu, J. (2016). Habitat Use and Selection by Giant Pandas. *PLoS One,*11(9), e0162266.

Jones, J. (2001). Habitat selection studies in avian ecology.*The Auk,* 118(2), 557-562.

Kadid, Y., Thébaud, G., Pételle, G., & Abdelkrim, H. (2007). Les communautés végétales aquatiques de la classe des Potametea du lac Tonga, El-Kala, Algérie. *Acta bot. Gallica,* 154(4), 597-618.

Koskimies, P. (1989). Birds as a tool in environmental monitoring. *Ann Zool Fennici,*26, 153-166.

Lazli, A., Boumezbeur, A., Moali-Grine, N., & Moali, A. (2011). Évolution de la population nicheuse de l'Érismatureà tête blanche *Oxyura leucocephala* sur le lac Tonga (Algérie) [Evolution of the Breeding Population of the White Headed Duck *Oxyura leucocephala* on Lake Tonga (Algeria)]. *Terre Vie,* 66, 173–181.

Leon, B., Stuart, B., Jonathan, E., Michael, E., Lincoln F, Rob, P., & Alison, S. (2004). State of the world's birds 2004: indicators for our changing world. *BirdLife International*, pp.74.

Litlefeld, C.D., & Johnson, D.H. (2005). Habitat preferences of migrant and wintering Northern Harriers in Northwestern Texas. *Southwest Nat ,*50(4), 448-452 .

Martins, C.O., Rajpar, M.N., Nurhidayu, S., & Zakaria M. (2017). Habitat Selection of *Dendrocygna javanica* in Heterogeneous Lakes of Malaysia. *J Biodivers Manage Forestry,* 6,3.

McFeeters, S.K. (2013). Using the Normalized Difference Water Index (NDWI) within a Geographic Information System to Detect Swimming Pools for Mosquito Abatement: A Practical Approach. *Remote Sensing,*5,3544-3561.

McFeeters, S.K.(1996). The Use of the Normalized Difference Water Index (NDWI) in the Delineation of Open Water Features. *Int. J.* Remote *Sens,* 17,1425-1432.

Montesinos, A., Santoul, F., & Green, A. J. (2008). The diet of the night heron and purple heron in the Guadalquivir marshes. *Ardeola,* 55(2),161-167.

Nouidjem, Y., Mimeche, F., Bensaci, E., Merouani, S., Arar, A., & Saheb, M., (2019). Check list of waterbirds at Wadi Djedi in Ziban Oasis–Algeria. *Arx Misc Zool* , 17,34–43.

Paracuellos, M., &Telleria, J. L. (2004). Factors Affecting the Distribution of a Waterbird Community: The Role of Habitat Configuration and Bird Abundance. *Waterbirds*, 27(4),446-453.

Rajpar, M.N.,& Zakaria, M. (2014). Effects of habitat characteristics on waterbird distribution and richness in wetland ecosystem of Malaysia. *Journal of Wildlife and Parks,* 28, 107-122.

Roshnath, R. (2015). Preliminary study in diet composition of Indian pond Heron during breeding season. *Biotechnology,* 4 (5), 574-577.

Rotenberry, J.T., & Wiens J.A. (1980). Habitat Structure, Patchiness, and Avian Communities in North American Steppe Vegetation: A Multivariate Analysis. *Ecology,* 61(5), 1228-1250.

Samraoui, B., Segers, H., Maas, S., Baribwegure, D., & Dumont H.J. (1998). Rotifera, Cladocera, Copepoda, and Ostracoda from coastal wetlands in northeast Algeria. *Hydrobiologia,* 386, 183-193.

Samraoui, B., & Samraoui, F. (2008). An ornithological survey of the wetlands of Algeria: Important Bird Areas, Ramsar sites and threatened species. *Wildfowl,* 58, 71-96.

Santilli, F., & Bagliacca, M. (2010). Habitat use by the European wild rabbit (*Oryctolagus Cuniculus*) in a coastal sandy dune ecosystem of central Italy. *Hystrix It J Mamm (n.s.),*21(1), 57-64.

Saygili, F., Yiğit, N., & Bulut, Ş. (2011). Te spatial and temporal distributions of waterbirds in Lakes Akşehir-Eber and Lake Köyceğiz in western Anatolia, Turkey–a comparative analysis. *Turk J Zool,*35(4), 467-480.

Swathi, H.A., & Antoney, P.U. (2018). Habitat use among Shorebirds in the Lakes of Bengaluru. *Int J Zool Invest,*4(1), 46-55.

Tommy King, D., & Michot, T.C. (2002). Distribution, Abundance and Habitat Use of American White Pelicans in the Delta Region of Mississippi and Along the Western Gulf of Mexico Coast. *Waterbirds,*25(4), 410-416.

Tucker, C.J.(1979). Red and photographic infrared linear combinations monitoring vegetation. *Remote Sens Environ,* 8(2), 127-150.

Vasudeva-Rao, V., Naresh, B., Surender, G., & Swamy, K. (2015). Population Trends, Species Variations and Habitat use by Egrets, Herons and Storks at Kolleru Wetland, Andhra Pradesh, India. *Int Res J Biol Sci,* 4(2), 28-32.

Willard, D.E. (1977). The feeding ecology and behavior of five species of herons in southeastern New Jersey. *The Condor,*79,462470.

Wu, X., Lv, M., Jin, Z., Michishita, R., Chen j., Tian, H.,Tu, X., Hongmei, Z., Niu Z., Chen, X.,Yue, T., & Xu, B.(2014). Normalized difference vegetation index dynamic and spatiotemporal distribution of migratory birds in the Poyang Lake wetland, China. *Ecol Indic,*47, 219–230.

Yahner, R.H. (1986). Structure, Seasonal dynamics and habitat relationships of avian communities in Small Even-Aged forest stands. *Wilson Bull,* 98(l),61-82.

Yu-Seong, C., Kwon I. -K., & Yoo, J. C. (2007). Foraging habitat preferences of Herons and Egrets. *J Ecol Field Biol,*30 (3),237-244.

Zhang, Y., Wang, Z., Ren, C., Yu, H., Dong, Z., Lu, C., & Mao, D. (2017). Changes in habitat suitability for waterbirds of the Momoge Nature Reserve of China during 1990–2014. *J Environ Eng Landsc,* 25(4), 367–378.

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Growth and development of salinity-exposed rice (*Oryza sativa*) rhizo-inoculated with *Bacillus subtilis* under different pH levels

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Abstract. The study investigated the effects of varying pH levels on the growth and development of salt-exposed rice (*Oryza sativa* L.) after inoculation with *Bacillus subtilis.* Germinated rice seedlings (var. FARO 44) were sown in garden soils amended to 100 mM NaCl, and were thereafter inoculated with *Bacillus subtilis*. The transplants were subsequently exposed to periodic wetting with 5 mL of pH-buffered water (pH 3, 5, 7, 9 and 11) every day, and with 5 mL of 100 mM NaCl every 4 days. The set up was monitored during a 30-day period. Results showed significant reduction in chlorophyll a and b as well as lycopene and tocopherol contents of leaves due to changes in the lipid-to-protein ratio of pigment-protein complex or increased chlorophyllase activity and drought stress. There were improved morphological characteristics such as plant height, sheath and foliar dimensions due to inoculation of *B. subtilis*. Increase in salinity resulted in a decrease in plant height, leaf length and sheath. Inoculation not only promoted rice growth, but also enhanced rice tolerance towards salinity owing to the fact that FARO 44 is a salinity-tolerant rice variety. A better understanding of the interaction between microbial inoculants and soil conditions is required to harness the desired benefits towards improving crop development.

Keywords: *Bacillus subtilis*, FARO 44, *Oryza sativa*, pH, rice, salinity.

Introduction

Rice (*Oryza sativa* L.) is a staple food which is commonly consumed in African countries and other parts of the world (Ajala and Gana, 2015). This staple food is consumed by about half of the human race (Udemezue, 2018). It is classified as the most widely consumed food because larger populations depend on it. In Africa, rice has the potentials of improving nutrition, boost food security, support sustainable land care and foster rural development (Ojo and Adebayo, 2012). Rice provides more than one-fifth of the total calories consumed by human universally (Udemezue, 2018). It requires a minimum growing season of four to five months (Ajala and Gana, 2015). All stages of the growth and development of rice plant are affected by salinity, the crop responses varying with growth stages, concentration and duration of exposure to salts (Das *et al*., 2015).

Soil salinity is a serious limitation to rice cultivation under irrigated agriculture due to poor quality of water (Shrivastava and Kumar, 2015). Over 20 % of cultivated land globally is adversely affected by high salt concentration which inhibits plant growth and yield (Kapoor and Pande, 2015). Salt concentration increases in the upper soil layer in warm and dry areas due to high water losses which surpass precipitation (Prusty *et al*., 2018). The sustainability of rice to salt stress differs with growth stage. It is relatively salt tolerant at germination and becomes very sensitive at young seedling stage (Gholizadeh and Navabpour, 2011). During late reproductive stage, it is relatively less sensitive, that is, grain ripening. The detrimental effects of high salinity on plants can be very devastating, causing significant reduction in plant growth, decrease in productivity and even the loss of plants. The accumulation of sodium ion (Na^+) in leaf tissues usually results in the damage of old leaves which shortens the lifetime of individual leaves, thereby reducing the yield of the crops (Negrão *et al*., 2017). Increased NaCl levels will result to a great decrease in shoot, root, and leaf biomass (Meloni *et al*., 2001). In the majority of cultivated plants, the yields start to diminish even at relatively low salinity in soil (i.e., at electrical conductivity, EC, >1 dS m^{-1}) (Chinnusamy *et al*., 2005). Presently, 30% of global paddy fields are affected by excessive salinity (Negrão *et al*., 2017).

There has been a huge awareness in eco-friendly and sustainable agriculture which emphasis the use of beneficial microorganisms. In fact, studies have revealed that local survival of plants to their environment is determined by genetic separation in closely associated microbes (Shrivastava and Kumar, 2015). Rhizosphere microbes have been shown to improve the growth of several crops grown in a large range of root-zone salinities, and this approach is necessary to enhance improvement of germplasm collections of salt-tolerant rice species. These microorganisms living in soils affected by extreme environmental factors can reduce stresses in plants (crop), thus opening a possible and shows potential scheme in sustainable agriculture. A number of studies are now proving the suggestion that plant growth promoting rhizobacteria (PGPR) enable agricultural plants to sustain its productivity under stressing conditions by various means including enhanced accumulation of plant growth regulators like auxins, improvement of root growth for enhanced nutrient acquisition as well as improved nitrogen and phosphorus utilization by plants (Paul and Lade, 2014). Improvement in agricultural sustainability in saline soils requires managerial practices that increase soil biological activity and thereby build-up long-term soil productivity and crop health. The FARO (Federal Agriculture Research Oryza) variety 44 was selected for this study because it is an improved lowland and shallow swamp high-yielding variety which is already in cultivation by farmers in Nigeria. Furthermore, they are improved *Oryza sativa* varieties released by the National Cereals Research Institute, Badeggi, Bida, Nigeria (Dalton and Guei, 2003). They are also among the rain-fed lowland super rice cultivars in Nigeria (Akinwale *et al*., 2012). Having previously noted that soil pH affects proliferation of useful soil bacteria, the aim of this study is to investigate the effects of varying pH levels on the growth and development of salt-exposed rice after rhizo-inoculation with *Bacillus subtilis*.

Materials and methods

Sample collection

Improved high-yielding rice variety (FARO 44) was obtained from Raymos Guanah Farms Ltd, Delta State, Nigeria. Rice seeds were sown in 40 Petri dishes at the rate of 5 seeds per petri dish, until they produced seed-leaves and elaborate roots at the first week after sowing. Prior to sowing *in-vitro* in Whatman's filter paper, each filter paper was adequately moistened with 100 mM NaCl using a sterile 5 mL syringe. Thereafter, top soil (Tab. 1) was obtained from the Departmental Botanic Garden, University of Benin, Benin City, Nigeria, and placed in small nursery bags at 10 kg each. The soils were then moistened with 100 mM NaCl solution. Prior to application of NaCl solution to soil, the water-holding capacity (WHC) was previously determined following the methods of Anoliefo *et al*. (2016), to be 0.161Lkg^{-1} soil. This was necessary because the WHC in this study formed the basis for which each measured volumes of salt solution was required to moisten the soil before sowing with bacterial-inoculated rice seedlings were transplanted 2 days after. Therefore, each 10 kg sun-dried soil received 1 L of 100 mM NaCl. Control plants were never exposed to salt solution before or during experiment, neither were they inoculated with test bacterium.

Bacterial growth and inoculation

The bacterial strain used in this study (*Bacillus subtillis*) was obtained from the Graduate Research Laboratory Culture Collection Unit of the Department of Microbiology, University of Benin, Benin City, Nigeria. This had been isolated from plant rhizospheres of *Chromolaena odorata* during a previous study (Ikhajiagbe and Akendolor, 2016). Pure isolate of *Bacillus subtilis* was prepared by streaking from the previously obtained stored isolate onto agar plates and incubated at 28 ^{o}C for 24 h. Upon growth after 24 hours, the isolate was inoculated in Nutrient broth which was used to grow the isolate, and then incubated for proliferation of cells. For inoculation, the roots of the test plant were immersed completely in bacterial medium for 24 h and thereafter transplanted immediately into each previously prepared nursery bag containing salt-exposed soil.

Application of pH solutions on rice transplants

The transplants were subsequently exposed to periodic wetting with 5 mL of pH-buffered water, which were previously prepared using pH buffers. The respective selected buffer solutions for pH 3, 5, 7, 9 and 11 respectively were added to distilled water until they attained the required respective pH values. This was confirmed by using a digital pH meter Model Mk-Vi with a combination electrodes. The transplants, divided into 5 major groups, were each exposed to pH 3, 5, 7, 9 and 11 every day, and with 5 mL of 100 mM NaCl every 4 days. Whereas one part of these groups were inoculated with *B. subtilis*, the other was not. The set up was observed for 30 days.

Plant parameters considered

Rice growth measurements were taken on a weekly basis. Parameters considered (plant height, sheath, leaf length and leaf breadth of rice seedlings) were measured as described by Lutts *et al*. (1996). Total carotenoids contents was analyzed according to Lichtenthaler and Buschmann (2001). Tocopherol and lycopene were also determined following the methods of Ayodele *et al.* (2014).

Statistical analysis

Statistical analysis was performed using SPSS-20. The data represented mean calculated from 3 replicates. The analysis of variance procedure (ANOVA) was used to compare the effects of NaCl and statistical significance was set at 95% confidence interval.

Results and discussion

After exposure to saline and alkaline stress, uninoculated plants started wilting whereas inoculated plants with bacterial cells alone resisted saline stress (before the laboratory analysis took place) prior to wilting (Tabs. 2 – 4). There was significant increase in plant height over the 4-week period from 8.67 cm to 21.21 cm in the control plant (Tab. 2). Plant height of rice transplants in the salt-exposed soils moistened at pH 3 did not significantly increase ($p>0.05$) during this period; they however died on the 4th week following exposure. However, at pH 3, when rice transplants were rhizo-inoculated with *B. subtillis*, plant height was improved beyound the 4th week (13.50 – 19.67 cm), comparable with the control. Generally, plant height at 4 weeks after exposure in rice transplants exposure to pH 7 (no inoculation), pH 9 (no inoculation), pH 5 (rhizo-inoculated), and pH 7 (rhizo-inoculated) were comparable with control plant in spite of exposure to 100 mM NaCl (21.21 – 26.16 cm).

There was minimal increase in length of leaf sheath throught the 4-week exposure period ($p>0.05$) (Tab. 3). At the forth week, the shortest leaf sheath was seen in the salinity-exposed transplant constantly wetted with pH 11-buffered water (2.00 cm), compared with 9.00 cm of sheath length in salinity-exposed transplants rhizo-inoculated with *B. Subtilis*. Leaf length was impeded at pH 11 for both the bacterium-inoculated and non-inoculated transplants (6.00 – 6.50 cm^2) compared to those moistened lower pH-buffered (11.50 – 17.16 cm^2) (Tab. 4). Salt stress is an abiotic stress factor that causes various deleterious effects on the overall plant growth and development (Ghanem *et al*., 2008). The present study showed that the inoculation of *B. subtilis* strain enabled the morphological development of the test plant.

Table 1. Physical and chemical properties of garden soil used for the experiment

Parameters	**Value**
pH	5.27
Electrical conductivity (μs cm^{-1})	301.21
Total organic carbon (%)	0.49
Total Nitrogen (%)	0.18
Exchangeable acidity (meq/100 g soil)	0.22
Na (meq/100 g soil)	10.90
K (meq/100 g soil)	1.48
Ca (meq/100 g soil)	14.32
Mg (meq/100 g soil)	12.01
NO^-_2 (mg kg^{-1})	16.43
NO^-_3 (mg kg^{-1})	30.01
Clay (%)	5.13
Silt (%)	7.06
Sand (%)	87.81
Fe (mg kg^{-1})	1011.92

Table 2. Plant height of rice transplants to salt stress under varying pH regimes

	Plant height (cm) Weeks				**p-value**
	1	2	3	4	
Control	$10.33^{ab/A}$	$8.67^{a/A}$	$18.67^{b/B}$	$21.21^{bc/B}$	<0.001
No PGPR					
pH 3	$9.17^{a/A}$	$7.67^{a/A}$	$10.50^{a/A}$	$0^{a/B}$	<0.001
pH 5	$10.83^{ab/A}$	$11.67^{ab/A}$	$12.33^{a/AB}$	$15.00^{b/B}$	0.138
pH 7	$12.50^{ab/A}$	$15.83^{ab/AB}$	$19.17^{bc/BC}$	$24.67^{c/C}$	0.085
pH 9	$15.33^{ab/A}$	$15.33^{ab/A}$	$18.50^{b/AB}$	$23.83^{c/B}$	0.104
pH 11	$10.33^{ab/A}$	$15.67^{ab/B}$	$15.33^{ab/B}$	$15.00^{b/B}$	0.399
PGPR					
pH 3	$13.50^{ab/A}$	$17.33^{ab/B}$	$15.17^{ab/AB}$	$19.67^{b/B}$	0.054
pH 5	$17.17^{b/A}$	$20.00^{b/AB}$	$22.00^{bc/BC}$	$26.16^{c/C}$	0.091
pH 7	$16.00^{ab/A}$	$21.33^{b/B}$	$24.17^{c/B}$	$25.67^{c/B}$	0.138
pH 9	$8.17^{a/A}$	$14.00^{ab/B}$	$16.17^{ab/B}$	$16.16^{bc/B}$	0.331
pH 11	$9.50^{ab/A}$	$10.33^{ab/A}$	$11.17^{a/AB}$	$13.50^{b/B}$	0.411
P. value	0.137	0.08	0.181	<0.001	-

Mean values followed by the different similar lowercase alphabetic supercripts in the same column do not differ from each other ; similarly,means with uppercase alphabetic superscript on same row do not differ significantly (p>0.05)

Table 3. Leaf sheath length of rice transplants to salt stress under varying pH regimes

	Leaf sheath (cm) Weeks				**p-value**
	1	2	3	4	
Control	$4.00^{ab/A}$	$3.83^{a/A}$	$3.67^{a/A}$	$5.01^{b/A}$	0.624
Npgpr					
pH 3	$4.33^{abc/A}$	$4.16^{a/A}$	$3.50^{a/A}$	$0^{a/B}$	<0.001
pH 5	$3.3^{ab/A}$	$4.00^{a/A}$	$5.33^{a/AB}$	$7.00^{c/B}$	0.418
pH 7	$6.67^{bc/A}$	$7.00^{a/A}$	$7.00^{a/A}$	$8.83^{c/A}$	0.231
pH 9	$6.67^{bc/A}$	$6.00^{a/A}$	$7.33^{a/A}$	$8.33^{c/A}$	0.529
pH 11	$5.00^{abc/A}$	$7.00^{a/A}$	$5.67^{a/A}$	$2.00^{ab/B}$	0.138
PGPR					
pH 3	$5.16^{abc/A}$	$7.33^{a/A}$	$8.00^{a/A}$	$8.50^{c/A}$	0.329
pH 5	$7.67^{c/A}$	$6.33^{a/A}$	$7.83^{a/A}$	$9.00^{c/A}$	0.388
pH 7	$6.67^{bc/A}$	$6.83^{a/A}$	$7.83^{a/A}$	$8.67^{c/A}$	0.702
pH 9	$2.83^{a/A}$	$4.83^{a/A}$	$5.16^{a/A}$	$6.16^{bc/A}$	0.222
pH 11	$3.00^{ab/A}$	$3.50^{a/A}$	$4.67^{a/AB}$	$5.67^{bc/B}$	0.096
P. value	0.041	0.475	0.304	<0.001	

Mean values followed by the different similar lowercase alphabetic supercripts in the same column do not differ from each other ; similarly,means with uppercase alphabetic superscript on same row do not differ significantly (p>0.05)

Table 4. Leaf length of rice transplants to salt stress under varying pH regimes

	Leaf length (cm) Weeks				**p-value**
	1	2	3	4	
Control	$5.83^{a/A}$	$4.50^{a/A}$	$7.00^{a/AB}$	$9.01^{b/B}$	0.282
Npgpr					
pH 3	$5.16^{a/A}$	$5.50^{ab/A}$	$7.00^{ab/A}$	$0^{a/B}$	<0.001
pH 5	$6.00^{a/A}$	$8.16^{abc/A}$	$9.33^{ab/AB}$	$11.50^{b/B}$	<0.001
pH 7	$6.00^{a/A}$	$12.83^{bc/B}$	$14.33^{b/BC}$	$15.83^{b/C}$	<0.001
pH 9	$9.83^{a/A}$	$11.16^{abc/AB}$	$13.33^{b/B}$	$15.33^{b/B}$	0.067
pH 11	$5.33^{a/A}$	$8.67^{abc/A}$	$7.33^{ab/A}$	$6.00^{a/A}$	0.514
PGPR					
pH 3	$7.83^{a/A}$	$9.50^{abc/A}$	$10.50^{ab/AB}$	$12.50^{b/B}$	<0.001
pH 5	$7.83^{a/A}$	$13.33^{c/B}$	$14.50^{b/BC}$	$17.16^{b/C}$	0.038
pH 7	$9.00^{a/A}$	$12.00^{bc/B}$	$13.50^{b/BC}$	$16.16^{b/C}$	<0.001
pH 9	$8.00^{a/A}$	$9.00^{abc/A}$	$9.50^{ab/A}$	$10.50^{b/A}$	0.013
pH 11	$6.50^{a/A}$	$6.50^{abc/A}$	$6.67^{ab/A}$	$6.50^{a/B}$	0.725
P. value	0.69	0.121	0.057	0	

Mean values followed by the different similar lowercase alphabetic supercripts in the same column do not differ from each other ; similarly,means with uppercase alphabetic superscript on same row do not differ significantly (p>0.05)

In the rhizosphere the synergism between various bacterial genera such as *Bacillus*, *Pseudomonas* and *Rhizobium* has been demonstrated to promote plant growth and development (Figueiredo *et al*., 2010). This indicates that the rhizoinoculation not only promoted rice growth by supplying nutrient and IAA, but also enhanced rice tolerance towards salinity. This is because some plant growth promoting rhizobacteria (PGPR) stimulate plant growth and development by enhancing nitrogen acquisition and utilization, improving secretion of phytohormones, and enhancing availability and utilization of phosphate (Hayat *et al*., 2010). PGPRs also enhance plant growth and development by protecting the plant against soil-borne diseases, most of which are caused by pathogenic fungi as reported by Lugtenberg and Kamilova (2009). Saline environment inhibits rice growth, this is because rice is a saline sensitive plant (Ashraf and Harris, 2004); also because the uptake of Ca^{2+}, K^{+} and inorganic N and P are disrupted under high Na^{+} concentration (Ashraf and Harris, 2004). Rhizoinoculation enhanced morphological characteristics of test plant (*Oryza sativa*) under saline condition

The contents of photosynthetic pigments including chlorophyll a, chlorophyll b and total carotenoids contents in rice plants grown under extreme pH combined with salt stress declined significantly, being especially susceptible to salt. Yang *et al*.(2008) studies revealed that, chlorophyll a, chlorophyll b and total carotenoids contents in the salt resistant wheat grown under alkaline salt stress (pH 9.9 and 60–75 mmol L^{-1} NaCl) were 2.5–5.0 times lower than those grown under salt stress only. In the present study however, chlorophyll-a contents of salt-exposed non-inoculated rice plants were higher at pH 5 during the 4th week of exposure (0.010 mg g^{-1}) compared to the control (0.006 mg g^{-1}) (Fig. 1). Similarly, chlorophyll-b contents of salt-exposed non-inoculated rice plants were higher at pH 5 (0.017 mg g^{-1}) compared to the control (0.002 mg g^{-1}) (Fig. 2). Inoculated rice plants at similar pH 5 had a chlorophyll content of 0.015mg g^{-1}.

Chlorophyll content is often measured in plants to assess the impact of environmental stress, as changes in pigment content are linked to visual symptoms of plant illness and photosynthetic productivity. The reported decrease in chlorophyll contents of salinity-affected transplants might be due to changes in the lipid protein ratio of pigment-protein complex or increased chlorophyllase activity as reported by Dogan and Demirors (2018).

There were minimal differences in carotenoid contents of exposed plants ($p>0.05$) (Tab. 3). Lycopene, which is one of the most important bioactive compound in plants due to its benefits to human health (Aanchal *et al.*, 2019) increased under pH 5 and 9 conditions (Tab. 3), it reduced in pH 7 and 11 in the bags without PGPR, that is, the pH and NaCl (stress) could be responsible. There was a gradual decrease in the plants which were inoculated by the PGPR. Riggi *et al.* (2008) and Atkinson *et al.* (2011) found that drought stress lowered the lycopene content compared to well-watered tomato plants; however, the carotene content showed a positive increase. In contrast, Theobald *et al.* (2007) stated that the lycopene contents increased by more than 27% in water-stressed fruits. An increase in lycopene contents was also found in tomato fruits grown in Southern Italy by Favati *et al.* (2009). Moderate water stress induced an increase of the lycopene concentration of tomatoes (Sanchez-Rodriguez *et al.*, 2012).

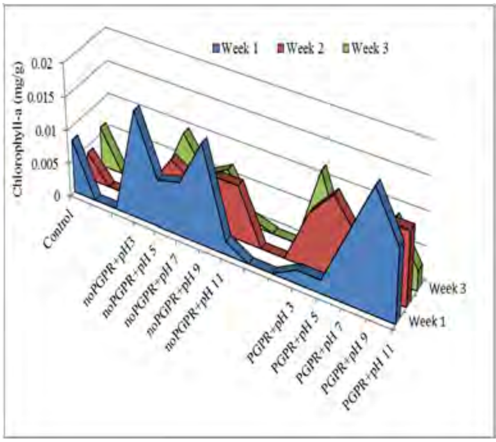

Figure 1. Cholorophyll-a content of rice transplant transplants exposed to salt stress under varying pH regimes

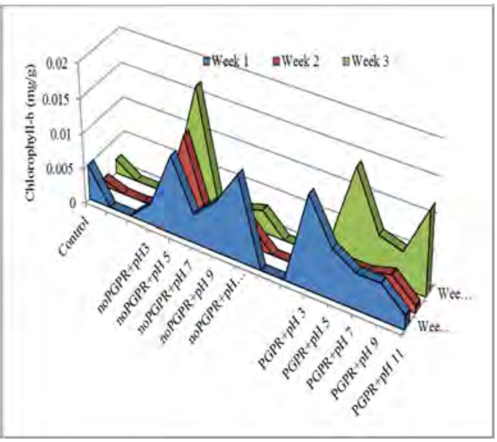

Figure 2. Cholorophyll-b content of rice transplant transplants exposed to salt stress under varying pH regimes

Table 5. Antioxidant response of the rice transplants exposed to salt stress under varying pH regimes

	Carotenoid (mg/kg)			Tocopherol (mg/kg)			Lycopene (mg/kg)		
	1	2	3	1	2	3	1	2	3
	Weeks			Weeks			Weeks		
***Control**	1137.1^{a}	1004.16^{cde}	985.61^{cd}	0.94^{bcd}	0.96^{b}	0.97^{bc}	2.35^{cd}	54.35^{b}	1.10^{e}
nPGPR									
pH 3	1100.7^{a}	985.50^{efg}	946.48^{de}	0.93^{bc}	0.99^{b}	0.98^{c}	2.78^{e}	1.91^{h}	5.65^{g}
pH 5	1108.7^{a}	983.84^{bcd}	991.70^{a}	0.96^{cd}	0.98^{b}	0.97^{bc}	2.39^{d}	91.03^{e}	91.22^{d}
pH 7	1130.7^{a}	1002.90^{bc}	1292.29^{b}	0.93^{b}	0.98^{b}	0.98^{c}	2.39^{d}	34.79^{a}	3.63^{f}
pH 9	1107.3^{a}	976.46^{g}	1004.28^{c}	0.96^{cd}	0.96^{b}	0.96^{bc}	2.01^{b}	72.73^{d}	32.32^{b}
pH 11	1109.3^{a}	951.44^{a}	1008.20^{e}	0.95^{bcd}	0.95^{b}	0.93^{b}	2.22^{c}	65.59^{c}	19.04^{b}
PGPR									
pH 3	1055.7^{a}	1024.13^{cde}	992.28^{b}	0.74^{a}	0.81^{a}	0.81^{a}	2.73^{e}	69.57^{d}	25^{ab}
pH 5	1080.7^{a}	994.52^{def}	962.09^{b}	0.94^{bcd}	0.97^{b}	0.98^{c}	2.39^{d}	1.91^{h}	60.09^{c}
pH 7	1056.4^{a}	972.44^{ab}	944.93^{cd}	0.96^{d}	0.94^{b}	0.94^{bc}	32.57^{a}	1.38^{f}	1.07^{i}
pH 9	1075.7^{a}	965.83^{fg}	996.37^{de}	0.96^{cd}	0.94^{b}	0.95^{bc}	24.99^{a}	62.43^{c}	6.56^{h}
pH 11	1116.7^{a}	935.89^{fg}	1027.99^{e}	0.96^{d}	0.95^{b}	0.95^{bc}	34.78^{a}	1.75^{g}	2.27^{j}
P. value	0.288	0.084	<0.001	<0.001	0.001	<0.001	<0.001	<0.001	<0.001

Mean values followed by the different similar alphabetic supercripts in the same column do not differ from each other (p>0.05)

Table 6. Correlation coefficient of parameters

	Plant height	**Sheath**	**Leaf length**	**Leaf breadth**	**Chlorophyll a**	**Chlorophyll b**	**Caro-tenoid**	**Toco-pherol**	**Lyco-pene**
Plant height	1								
Sheath	0.869**	1							
Leaf length	0.910**	0.820**	1						
Leaf breadth	0.828**	0.741**	0.783**	1					
Chlorophyll a	-0.28	-0.06	-0.29	-0.33	1				
Chlorophyll b	0.03	0.07	0.09	0.20	0.06	1			
Carotenoid	-0.18	-0.25	-0.25	-0.09	-0.27	-0.27	1		
Tocopherol	0.03	-0.23	0.06	0.04	-0.556**	0.11	0.14	1	
Lycopene	-0.07	-0.15	-0.14	-0.07	-0.1	0.03	0.667**	0.12	1

****. Correlation is significant at the 0.01 level (2-tailed)**

Pearson correlation matrix for all parameters of test plants indicated a significant correlation among each of chlorophyll a (r = -0.330, p<0.05) and carotenoid (r = -0.272, p<0.05) (Tab. 4). PCA, as a multivariate technique, can group individuals or objects on the basis of their characteristics. Individuals with similar descriptions are mathematically congregated within the same cluster (Ahmadikhah *et al.*, 2008). Distance, similarity and relatedness of varieties are the foundation of this method. The biplot showed that test plant parameters were not particlualry affected specific experimental conditions (Fig. 3). However, plant morphological parameters, tocopherol and chlorophyll contents were closely associated together. Tocopherol is a plant antioxidant associated with protection of the chlorophyll molecule. Chlorphyll is also assoctaed with improved plant development as this molecule is the basis for plant productivity because of its importance in cellular energetics. Therefore, the association of morphological parameters, chlorophyll contents and tocopherol underline the importance of the latter in plant survival capacities under the experimental condition. Direct exposure of the test plants to combined effects of pH and salinity would result in oxidative damage, and also may lead to impaired cellular ionic homeostasis (Yadav, 2010). It is therefore suggested that such a plant may have utilized this antioxidant as a defence strategy.

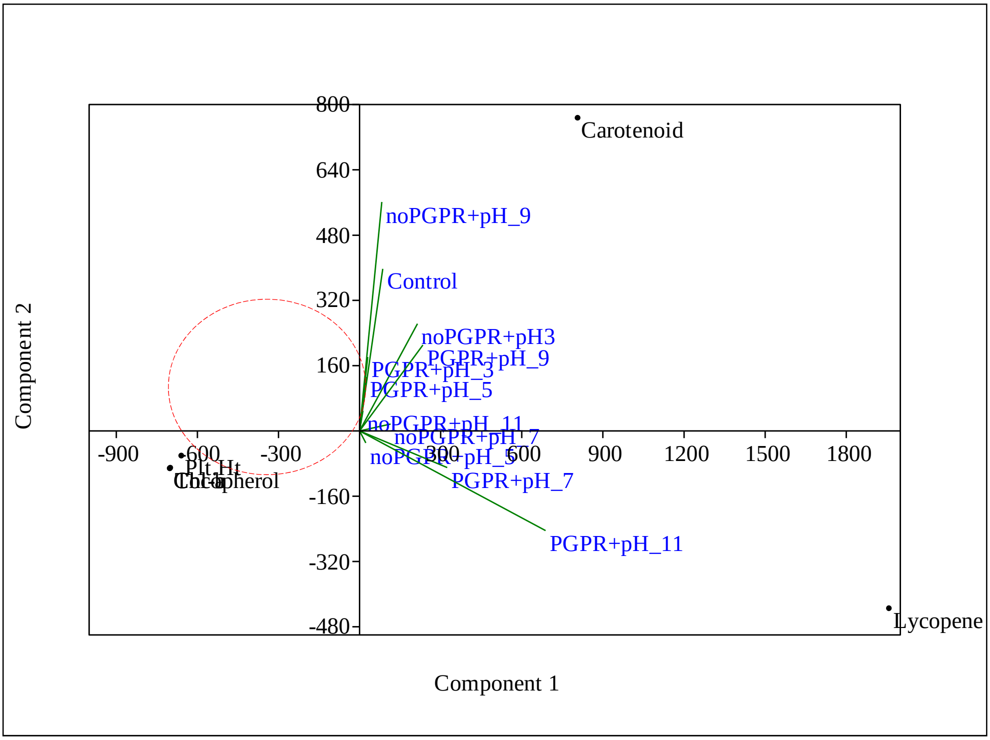

Figure 3. Pricipal component biplot showing association among selected characteristics of the test plant under experimental condition

Conclusions

The negative growth impact of salinity in plants may have significant implications for crop yield. The study investigated the effects of bacterial rhizoinoculation on growth and dvelopment of rice plants under salinity and varying pH conditions. This is predicated upon the fact that soil pH infuences microbial proliferation and hence its capability to support plant development under stress conditions. In the present study, growth enhancement of rice transplants under salinity condition was achieved rather at pH 5 and than at neutral pH.

REFERENCES

Aanchal, W., Amit, K.G., & Vatsala, S. (2019). Role of Bioactive Compounds in Human Health. *Acta Scientific Medical Sciences,* 3(9), 25-33.

Ahmadikhah, A., Nasrollanejad, S., & Alisha, O. (2008). Quantitative studies for investigating variation & its effect on heterosis of rice. *International Journal of Plant Production,* 2(4), 297 – 308

Ajala, A.S., & Gana, A. (2015). Analysis of Challenges Facing Rice Processing in Nigeria. *Journal of Food Processing,* 6, 893673.

Akinwale, M.G., Akinyele, B.O., Odiyi, A.C., Nwilene, F., Gregorio, G., & Oyetunji, O.E. (2012). Phenotypic screening of Nigerian rain-fed lowl & mega rice varieties for submergence tolerance. *Proceedings of the World Congress on Engineering*, July 4 – 6, London, pp. 640 – 648.

Anoliefo, G.O., Ikhajiagbe, B., Okoye, P.C., & Osayi, O. (2016). Utilizing local soap-derived biosurfactant for degradation of petroleum hydrocarbon polluted soils, sustainable remediation in focus. *Annals of Science & Techonology,* 1(1), 43 – 51.

Ashraf, M., & Harris, P.J.C. (2004). Potential biochemical indicators of salinity tolerance in plants. *Plant Science*, 166, 3–16.

Atkinson, N.J., Dew, T.P., Orfila, C., & Urwin, P.E. (2011). Influence of combined biotic & abiotic stress on nutritional quality parameters in tomato (*Solanum lycopersicum* L.). *Journal of Agricultural & Food Chemistry*, 59, 9673–9682.

Ayodele, O.K., Folasade, M.O., Joshua, O.A., & Chris O.A. (2014). Activity of the Antioxidant Defense System in a Typical Bioinsecticide-& Synthetic Insecticide-treated Cowpea Storage Beetle Callosobrochus maculatus F. (Coleoptera: Chrysomelidae). *International Journal of Insect Science*, 6, 99 –108.

Aziz, E.A., Al-Amier, H., & Craker, L.E. (2008a). Influence of Salt Stress on Growth & Essential Oil Production in Peppermint, Pennyroyal & Apple Mint. *Journal of Herbs, Spices & Medicinal Plants*, 14, 77 – 87.

Cha-Um, S., Supaibulwattana, K., & Kirdmanee, C. (2009). Comparative effects of salt stress & extreme pH stress combined on glycinebetaine accumulation, photosynthetic abilities & growth characters of two rice genotypes. *Rice Science*, 16, 274 – 282.

Chaves, M.M., Flexas, J., & Pinheiro, C. (2009). Photosynthesis under drought & salt stress: regulation mechanisms from whole plant to cell. *Annals of Botany*, 103, 551 – 560.

Chinnusamy, V., Jagendorf, A., & Zhu, J.K. (2005) Understanding & improving salt tolerance in plants. *Crop Science*, 45 (2), 437–448.

Dalton, T.J., & Guei, R.G. (2003) Productivity gains from rice genetic enhancements in West Africa: countries & ecologies. *World Development*, 31 (2), 359–374.

Das, P., Kamlesh, K.N., Sneh, L.S., & Pareek, A. (2015). Understanding salinty responses & adopting omics-based approaches to generate salinity tolerant cultivars of rice 6, 1 – 16.

Dogan, M., & Demirors, S.S. (2018). Pysiological effects of NaCl on *Ceratophyllum demersum* L., a submerged rootless aquatic macrophyte. *Iranian Journal of Fisheries Sciences*, 17(2), 346-356.

Favati, F., Lovelli, S., Galgano, F., Miccolis, V., Di Tommaso T., & Candido V. (2009). Processing tomato quality as affected by irrigation scheduling. *Scientia Horticulturae*, 122, 562–571.

Ghanem, M.E., Albacete, A., MartÍnez-Andújar, C., Acosta, M., Romero-Aranda, R., & Dodd, I. C. (2009). Hormonal changes during salinity-induced leaf senescence in tomato (*Solanum lycopersicum* L.). *Journal of Experimental Botany*, 59, 3039–3050.

Gholizadeh, F., & Navabpour, S. (2011). Effect of salinity on morphological and physiological characteristics in correlation to selection of salt tolerance in rice (*Oryza sativa* L.). *International Journal of Agricultural Research*, 6, 780-788.

Hayat, R., Ali, S., Amara, U., Khalid, R., & Ahmed, I. (2010). Soil beneficial bacteria & their role in plant growth promotion: a review. *Annals of Microbiology*, 60 (4), 579–598.

Ikhajiagbe, B., Anoliefo, G.O., Oshomoh, E.O., & Airhienbuwa, N. (2013). Changes in heavy metal contents of a waste engine oil polluted soil exposed to soil pH adjustments. *British Biotechnology Journal*, 3(2), 158-168.

Ikhajiagbe, B., Anoliefo, G.O., Oshomoh, E.O., Ogedegbe, U.A., & Airhienbuwa, N. (2012). Changes in polyaromatic hydrocarbon content of a waste engine oil polluted soil exposed to pH adjustments. *Annual Review & Research in Biology*, 2(3), 66-82.

Jamil, M., Lee, D.B., Jung, K.Y., Ashraf, M., Lee, S.C., & Rhal, E.S. (2006). Effects of salt (NaCl) stress on germination & early seedling growth of four vegetables species. *Journal of Central European Agriculture*, 7, 273–282.

Kapoor, N., & Pande, V. (2015). Effect of Salt Stress on Growth Parameters, Moisture Content, Relative Water Content & Photosynthetic Pigments of Fenugreek Variety RMt-1. *Journal of Plant Sciences*, 10, 210 – 221.

Lichtenthaler, H.K., & Buschmann, C. (2001). Chlorophylls and carotenoids: measurement and characterization by UV-VIS spectroscopy, In: *Current Protocols in Food Analytical Chemistry*. New York, John Wiley & Sons, pp. 1–4.

Lichtenthaler, H.K. (1987). Chlorophylls & carotenoids: Pigments of photosynthetic biomembranes. *Methods in Enzymology*, 148, 350 – 382.

Lugtenberg, B., & Kamilova, F. (2009). Plant-growth-promoting rhizobacteria. *Annual Review of Microbiology*, 63, 541–556.

Meloni, D.A., Olivia, M.A., Ruiz, H.A., & Martinez, C.A. (2001). Contribution of proline & inorganic solutes to osmotic adjustment in cotton under salt stress. *Journal of Plant Nutrition*, 24, 599 – 612.

Munns, R. (2003). Comparative Physiology of Salt & Water Stress. Plant, *Cell & Environment*, 25, 239 – 250.

Negrão, S., Schmöckel, S.M., & Tester, M. (2017). Evaluating physiological responses of plants to salinity stress. *Annals of Botany*, 119(1), 1–11.

Ojo. E.O., & Adebayo, P.F. (2012). Food security in Nigeria: an overview. *European Journal of Sustainable Development*, 1(2), 199 – 222

Paul, D., & Lade, H. (2014). Plant-growth-promoting rhizobacteria to improve crop growth in saline soils: a review. *Agronomy for Sustainable Development, Springer Verlag/EDP Sciences/INRA*, 34 (4), 737 – 752.

Prusty, M.R., Kim, S.R., & Vinarao, R. (2018). Newly Identified Wild Rice Accessions Conferring High Salt Tolerance Might Use a Tissue Tolerance Mechanism in Leaf. *Front Plant Science*, 9, 1 – 15

Riggi, E., Patanè, C., & Ruberto, G. (2008). Content of carotenoids a different ripening stage in processing tomato in relation to soil water availability. *Australian Journal of Agricultural Research*, 59, 348–353.

Sánchez-Rodríguez, E., Ruiz, J.M., Ferreres, F., & Moreno, D.A. (2012). Phenolic profiles of cherry tomatoes as influenced by hydric stress & rootstock technique. *Food Chemistry*, 134, 775–782.

Shabala, S.N., Shabala, S.I., Martynenko, A.I., Babourina, O., & Newman I.A. (1998). Salinity effect on bioelectric activity, growth, Na accumulation & chlorophyll fluorescence of maize leaves: a comparative survey & prospects for screening. *Australian Journal of Plant Physiology*, 25, 609–616

Shrivastava, P., & Kumar, R. (2015). Soil salinity: A serious environmental issue & plant growth promoting bacteria as one of the tools for its alleviation. *Saudi Journal of Biological Sciences*, 22, 123–131.

Udemezue, J.C. (2018). Analysis of Rice Production & Consumption Trends in Nigeria. *Journal of Plant Sciences & Crop Protection*, 1(3), 1 – 6.

Yadav, S.K. (2010). Heavy metal toxicity on plants: an overview on the note of gluthathione & phytochelatins in heavy metal the tolerance of plants. *South African Journal of Botany*, 76, 167 – 179.

Yang, F., Liang, Z.W., Wang, Z.E., & Chen, Y. (2008).Relationship between diurnal changes of net photosynthetic rate & influencing factors in rice under saline sodic stress. *Rice Science*, 15, 119 – 124.

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Microbial activity in soils from the Făgăraș Mountains

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Abstract. The aim of this paper was to assess the microbial activity and detection of some enzymes from soils of mountain zones in order to understand the complex processes that occur in these habitats. The sampling sites are located in three zones from the Făgăraș Mountains (woodside, forest and grassland). Six soil samples from these zones were subjected to microbiological and enzymological studies. The physical-chemical analyses were carried out using a portable multiparameter (pH, Eh, electrical conductivity and salinity). The following four ecophysiological bacterial groups have been studied: aerobic mesophilic heterotrophs, ammonifiers, nitrifiers and denitrifiers. The presence of all ecophysiological bacterial groups was registered in all the soil samples. The studied bacterial groups present fluctuations according with the sampling zones. The descending ranking of their abundance in the soil samples was: aerobic mesophilic heterotrophs, ammonifiers, nitrifiers, denitrifiers. Based on the bacteria number of each ecophysiological group, the bacterial indicators of soil quality (BISQ) were calculated. All of the four enzymatic activities analysed in soils (phosphatase, catalase, actual and potential dehydrogenase) were registered at every sampling site. Based on the absolute values of enzymatic activities, the enzymatic indicator of soil quality (EISQ) was obtained. The EISQ and BISQ values indicate a good intensity of these activities in all the analysed zones.

Keywords: enzymatic activity, enzymatic and bacterial indicator of soil quality, the Făgăraș Mountains microbial activity.

Introduction

Forest soils are in a continuous dynamic with the plant communities due to the different substrate inputs (leaves litter, rhizodeposition), forming a natural pedodiversity, caused by this development (Zhang *et al.*, 2019). These are in a tight correlation with the forest growth and the variation of natural conditions (relief, rock, climate, vegetation) (Samec *et al*., 2018). The soil fauna represents an important component of the soil biota, including three branches: macrofauna, mezofauna and microbiota. These exhibit important roles in the nutrient cycles, which finally reflect in the total energy flow of ecosystems (Wu and Wang, 2019). Beneath each spot of earth in the forest there is a living network, interconnected, considered by some researchers as "the hidden intelligence" by which underground symbiotic relations (mycorrhiza) with the fungi are established, sending messages of alarm and resources share (Ågren and Weih, 2012). The ecosystem processes are shaped according to the plant layer composition, the tree species, the successive stages and the environmental factors which influence the redistribution of nutrients (C, N and P) between plants and soils (Ågren and Weih, 2012). The soil composition determined by the C:N:P nutrients ratio shows the soil fertility and the regulation of the growth/development of plants, indicating the nutrients composition at plants level (Bui and Henderson, 2013; Fan *et al*., 2015).

Being an integrant part of the forest ecosystems, the soil microorganisms fulfil important roles in regulating the carbon and nitrogen cycles and in determining the responses of the terrestrial ecosystems subjected to environmental changes (Ren *et al.,* 2018; Yao *et al*., 2018). Thus, small changes at microbiota level can lead to significant changes of the nutrients transformation in the soil-plant system (Bragazza *et al*., 2015; Deng *et al*., 2016; Ren *et al*., 2018). The soil microorganisms can modify the diversity and productivity of plants and affect the sustentation of soil fertility. Also, the plant and soil properties significantly affect the microbial communities in soil (Yao *et al*., 2018). The microbial communities of soil can quickly respond to the changes in the plant-soil interactions (Yao *et al*., 2018).

The soil nutrients are correlated to the enzyme activities, considered one of the best representations of the soil activity and health (Lucas-Borja *et al*., 2016; Yao *et al*., 2019). The activity of soil enzymes is often used as bioindicator to assess the soil quality. These are involved in microbial decay, in the basic biochemical processes (C, N and P cycles, decomposition of organic matter hydrolysis of esters etc.) and react quickly to internal and external changes of the local environmental conditions (Ananbeh *et al*., 2019; Guo *et al*., 2019; Yao *et al*., 2018). The soil enzymes participate to the biochemical reactions and thus can regulate the nitrogen cycle in soil level. The biogeochemical cycle of nitrogen (N cycle) is one of the most important functions of soil performed by microorganisms, from soil (Pereg *et al*., 2018). Nitrogen (N) is an essential element for plant growth (Pu *et al*., 2019), but it is also responsible for the existence of life in the biosphere, because it is included in the structure of all proteins and nucleic acids and in many other biomolecules (Cabello *et al.*, 2019). It is widely present in nature, but it is in a great measure found in forms directly inaccessible to plants or animals. The microorganisms use reactions from the N cycle in assimilating, respiratory or dissimulating purpose. Ammonium assimilation consists in its incorporation in the carbon skeletons, usually by glutamine synthesis (Cabello *et al.*, 2019). The bacteria which fix the molecular nitrogen, reduce N_2 to NH_4^+ (ammonium), while other bacteria (ammonificators) decompose the organic compounds with nitrate to NH_3. The nitrifying bacteria oxidize $NH_4^+$to NO_2^- (nitrites) and then NO_3^- (nitrates), the preferred form of N for plants. The denitrification process occurs under anaerobic conditions, mediated by the denitrifying bacteria which perform the reduction of nitrates (NO_3^-) to nitrites, NO, N_2O and N_2, turning back as N in the atmosphere and completing the N cycle (Pereg *et al.*, 2018; Abbas *et al.*, 2019).

The human impact affect the N cycle at global scale, with implications on environment and health. Actually, over half of the fixed nitrogen which enters the ecosystems has anthropogenic origin, while the human activities modify, also, the composition and dynamic of the microbial populations in the terrestrial environment (Cabello *et al.*, 2019).

Materials and methods

Soil sampling. In the summer of 2018, in the Făgăraș Mountains (Viștișoara Valley, Natura 2000 site ROSCI0122) 6 soil samples were sterile taken from forest, woodside and meadow zones. These were transported on ice to the lab, where they were subjected to microbiological and enzymological analyses.

Physical and chemical analyses. Using a portable multiparameter, pH, redox potential (Eh), electrical conductivity and salinity in aqueous solutions were measured (1:50).

Microbial analyses in soil samples. The following ecophysiological bacterial groups have been studied: aerobic mesophilic heterotrophs, ammonifiers, nitrifiers and denitrifiers. The number of aerobic mesophilic heterotrophs was determined on plates nutrient agar medium (Atlas, 2010). After incubation the number of colonies in each Petri dish was counted, the average of the parallel samples values was calculated from the most significant dilution and it was multiplied with the reverse value of the respective dilution. The probable number of three groups of studied bacteria (ammonifying, nitrifying and denitrifying) was determined through MTM (multiple tubes method) according to Cușa, 1996. For ammonifiers a peptone medium was used, for nitrifying bacteria (nitrate and nitrite bacteria) in aerobe condition, the Barjac culture medium was used. For the determination of most probable number of bacteria that produce denitrification the Allen culture media was used. The most probable number was calculated according to the statistical table of Alexander (1965). Based on the bacteria number of each ecophysiological groups, the bacterial indicators of soil quality (BIQS) were calculated.

Enzymatic activities in soil samples. Activities of the following four enzymes in soil were measured: actual and potential dehydrogenase, phosphatase, catalase (Alef and Nannipieri, 1995; Carpa *et al*., 2014). Dehydrogenase activity (actual and potential) was determined after 24h incubation of the soil samples at 37ºC with TTC solution, and expressed by the amount of the formed 2,3,5-triphenylformazan (mg formazan / g soil). Enzymatic activity of dehydrogenases was determined by spectrophotometry using an Able Jasco V530 spectrophotometer at 440 nm wavelength. Phosphatase activity was determined after 24h incubation of the soil samples at 37ºC with phenyl phosphate disodic solution, and it is expressed in mg phenol/ 2.5 g soil. Phosphatase activity was determined by using an Able Jasco V530 spectrophotometer measuring the absorbance at 620 nm wavelength. Catalase activity was determined after 1h incubation of the soil samples at room temperature with H_2O_2 solution. The residual H_2O_2 is determined by titration with $KMnO_4$ in the presence of H_2SO_4. Catalase activity was expressed in mg split H_2O_2/ 1.5 g soil. The analytical data serves as the base for calculating the enzymatic indicator of soil quality (EISQ) (Muntean *et al*., 1995-1996).

Result and Discussions

The function of the terrestrial ecosystems depends on the interaction between above-ground and below-ground communities (Bardgett and Wardle, 2010). Below-ground community components, such as bacteria, provide necessary nutrients for the plants and, in turn, plants supply the necessary resources for them. The soil sampling zones belong to the Natura 2000 Site the Făgăraș Mountains, ROSCI0122 (198 618 ha in the central part of the country) (Fig. 1).

In this protected area are found a series of plant and animal species which are rare, vulnerable or even endemic. The habitats are very diverse, from floodplains and meadows to forests, subalpine shrubs and alpine meadows. The flora is well represented, being recorded over 900 plant species. Of the dominant species we can mention: *Campanula persicifolia, Corydalis cava, Geranium robertianum, Agrostis tenuis, Festuca rubra, Fagus sylvatica, Picea abies, Sphagnum* sp. and *Polytrichum* sp.

The samples were prelevated from three zones in the Făgăraș Mountains: two samples from the woodside (1 and 2), two samples from forest (3 and 4) and two samples from the meadow (5 and 6) (Fig. 1).

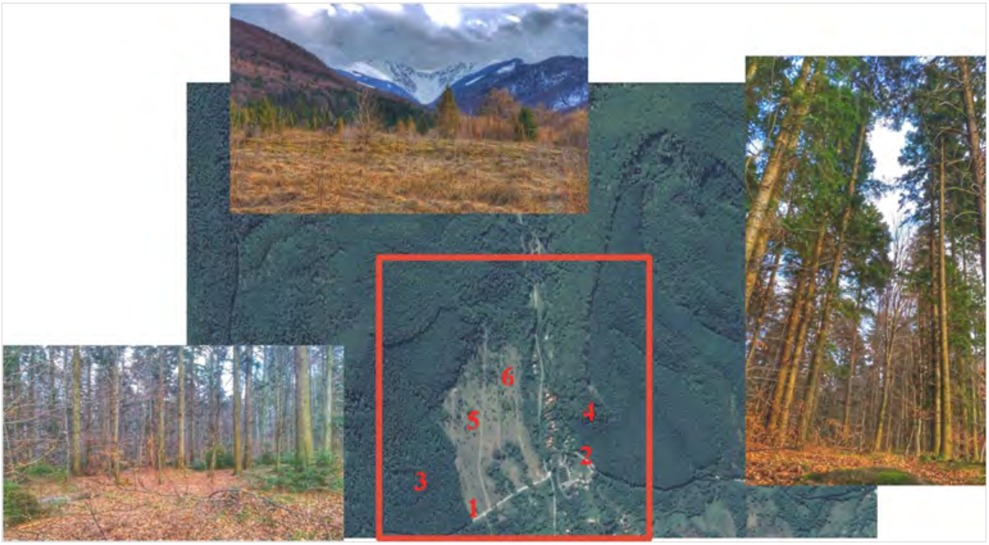

Figure 1. Soil sampling location (1, 2- woodside; 3,4- forest; 5, 6- meadow)

Physical and chemical analyses. All the analysed soils presented a strongly acid character (Blaga *et al.*, 2005), exhibiting pH values between 4.29 in the woodside samples and 4.9 in the meadow samples (Fig. 2).

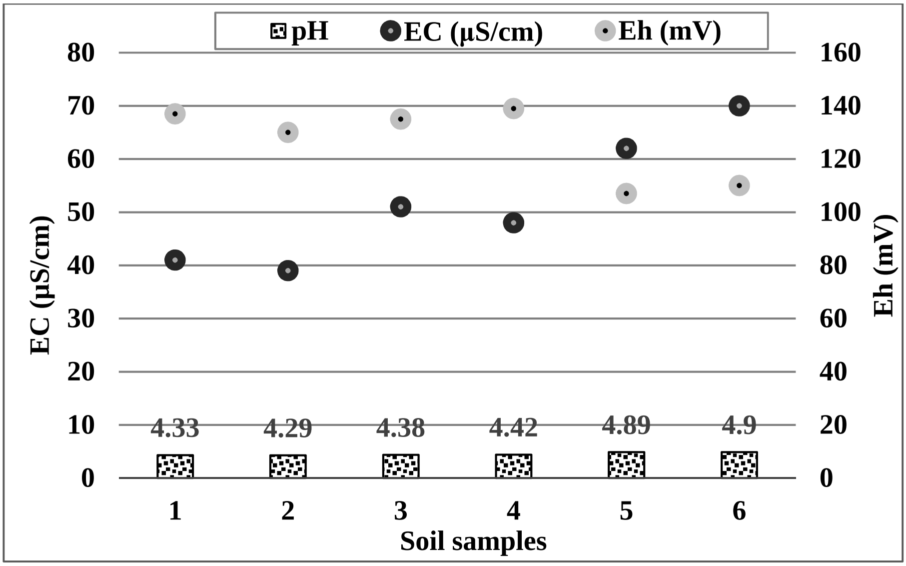

Figure 2. Physical and chemical parameters

The redox potential (Eh) presented values inversely proportional to pH, reaching 137 mV in the woodside zone and 110 mV at the meadow samples. The electrical conductivity (μS cm^{-1}) followed slightly rising trend, following the pH one (Fig. 2). No salinity was detected in these soil samples.

Microbial analyses. Soils are highly dynamic systems that are influenced by the interaction between organic and inorganic components and biota. Bacteria represent the main soil organisms in establishing the soil micro-food web (Wardle, 2002). Heterotrophic bacteria are microorganism that require organic carbon supply for their metabolism. Heterotrophic microorganisms in terrestrial systems have an important role in organic matter decomposition.

In the soils from the Făgăraș Mountains aerobic heterotrophic bacteria were the most numerous of all the ecophysiological groups studied. The highest number was detected in the meadow soils, reaching an average of 4565683 bacteria/ g soil /grassland zone (Tab. 1).

Ammonification consists in mineralization of protein nitrogen to NH_3 (ammonia) by decomposing organic matter or nitrite reduction; stage preceded by fixation of molecular nitrogen under the action of over 100 genera of different bacteria. Ammonifying bacteria were significantly higher in woodside compared to other samling sites. The highest level of ammonifying bacteria was founded in forest (sample 4)(8500 bacteria/g soil). In the other zones the presence of these bacteria was lower, specially in the woodside zone, where values of 2800 and 6800 bacteria/g soil (Tab. 1) were recorded. This fact may be due to the composition of the organic matter which require different time spans for decomposion, this process being one slower and with continuous activity.

Table 1. Probable number of bacteria in soil samples from the Făgăraș Mountain

Soil samples	Heterotrophs	Ammonifiers	Nitrate bacteria	Nitrite bacteria	Denitrifiers	BISQ
1	197667	6800	6800	400	1700	3.76
2	677533	2800	3600	1800	1200	3.83
3	825000	7200	6100	3600	3200	4.12
4	2340333	8500	6000	4500	2900	4.02
5	5158333	7200	1400	200	2400	3.88
6	3973033	8100	9100	2100	2000	4.22

The next stage of the nitrogen biological cycle is the nitrification, composed of two stages: first the oxidation of NH_3 to nitrites (NO_2^-) and than to nitrates (NO_3^-). In the analyzed soil samples the presence of nitrifying bacteria was much more reduced than the presence of ammonifying bacteria. The highest presence of nitrate bacteria was noticed at meadow (sample 6) (9100 bacteria/g soil). In the forest samples the nitrate bacteria activity was comparable to the one in the meadow. The smallest value of nitrate bacteria was obtained at the forest zone (600 bacteria/g soil) (Fig. 3).

The presence of nitrite bacteria in the soils from the Făgăraș reached almost half of the values for nitrate bacteria. Detection was minimal in the samples of meadow soil (2000 bacteria/g soil) (Fig. 3).

Denitrification is the last stage of nitrogen cycle. This, if very intense, can be harmful to soil, because leads to loosing the nitrogenous compounds assimilable by plants and, finally, to reducing the soil fertility (Muntean, 2009). The presence of denitrifying bacteria was the lowest of all the studied groups. The highest value was 3200 bacteria/g soil, in the forest samples. The lowest values were recorded in the woodside samples (Fig. 3).

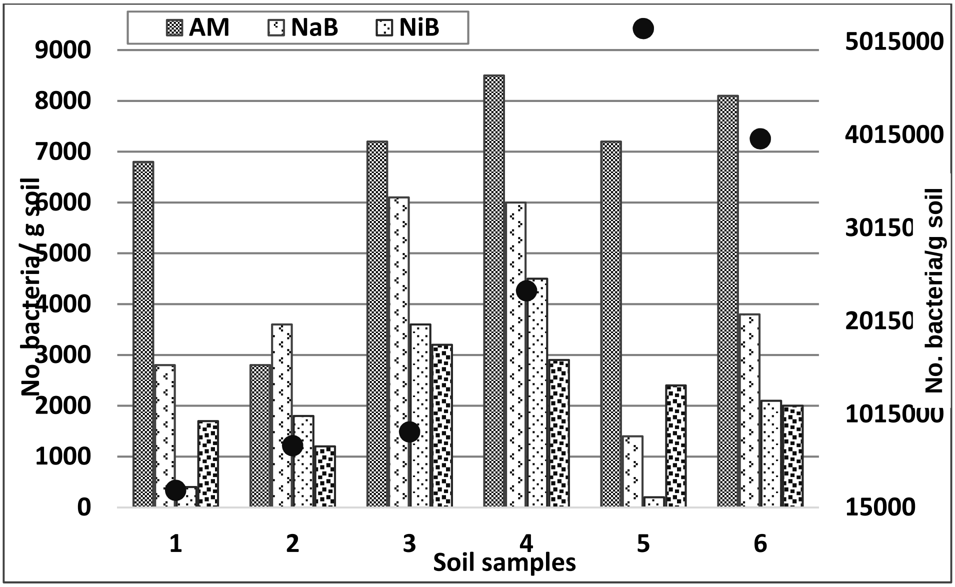

Figure 3. Bacterial determination in soil samples from the Făgăraș Mountain

Enzymatic analyses. The soil enzymes participate in important ecosystem processes, as decomposition of organic matter, formation of humus in soil and, naturally, in the nutrients cycle. These enzymes are known as sensitive indicators of natural and anthropogenic changes in ecosystems, used to evaluate the impact of different pollutants (Wang *et al.*, 2018). The following enzyme activities were detected in all the soil samples: actual and potential dehydrogenase, phosphatase activity and catalase activity (Tab. 2).

Table 2. Enzymatic activities in soil samples from the Făgăraș Mountain

Soil samples	**Dehydrogenases activity (mg formazan/g soil)**		**Phosphatase activity** (mg phenol/ 2.5 g soil)	**Catalase activity** (split H_2O_2 / 1.5 g soil)	**EISQ**
	Actual	Potential			
1	0.525	0.873	1.109	48.52	0.593
2	0.464	0.961	1.071	49.11	0.600
3	0.818	1.274	1.221	4976	0.621
4	0.771	1.361	1.167	49.91	0.632
5	1.196	1.813	1.150	43.34	0.563
6	1.074	1.749	1.100	43.60	0.563

Dehydrogenase activity is an indicator of the variability of live microbiota. Actual dehydrogenases activity (ADA) and potential dehydrogenases activity (PDA) were detected in all the analysed soil samples, with a few variations according to the prelevation zone. PDA presents values almost double compared to the actual one for the simple reason that a nutritive supplement rich in glucose was added, before incubation. The highest dehydrogenase activity was recorded for the meadow (samples 5 and 6), where the vegetation was more abundant (Fig. 3). Here ADA reached values of 1.196 mg formazan/g soil while PDA reached values of 1.813 mg formazan/g soil.

Phosphatases are the expression of organic phosphorus mineralization in all the ecosystems. The phosphorus cycle is one of the most important biological processes in the soil environment. The highest phosphatase activity was noticed in the forest samples, where a value above 1.18 mg phenol/ 2.5 g soil was recorded. In general, it was noticed an average activity on the studied zones; the lowest activity was found in woodside (sample 2), from woodside zone, 1.07 mg phenol/ 2.5 g soil (Fig. 4).

Catalase is the enzyme which accumulates in soils, keeping its activity for a long time. Through the activity of catalase the decomposition of H_2O_2 to H_2O and O_2 occurs.The enzyme is present in all the aerobic microorganisms. Regarding the areas studied an increased activity was noticed, slightly uniform, with a small difference regarding meadow samples (5 and 6), from the mountain meadow, where 43.6 and 43.34 split H_2O_2/1.5 g soil were recorded.

The enzymatic activity of microorganisms is used as a biological indicator of soil fertility and also has an important role in understanding the functioning of ecosystems.

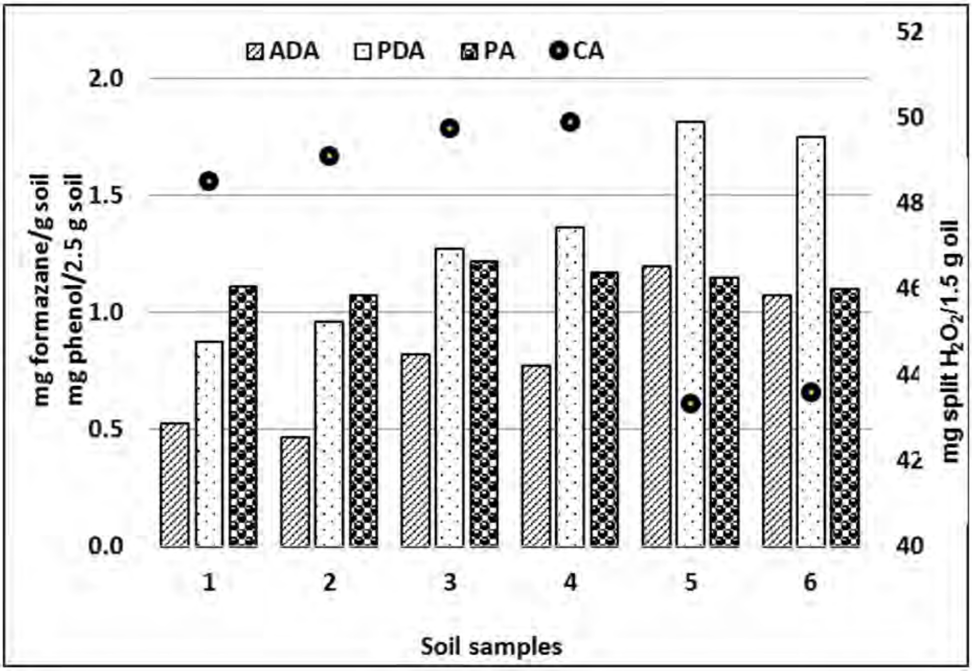

Figure 4. Enzymological activities in soil samples from the Făgăraș Mountains

Based on the microbiological and enzymological activities detected in soil samples, the bacterial and enzymatic indicators of soil quality (BISQ and EISQ) were calculated (Fig. 5). For this study the EISQ values varied between 0.59 and 0.63. Normally this indicator has values between 0 and 1 (Muntean, 1995-1996). We can affirm that a medium activity is noticeable at soil level, as well as a good ecosystem function.

The bacterial indicator of soil quality presented quite close values, ranging between 3.7 and 4.2. This indicator also evidences a good activity in the analysed mountain soils (Fig. 5).

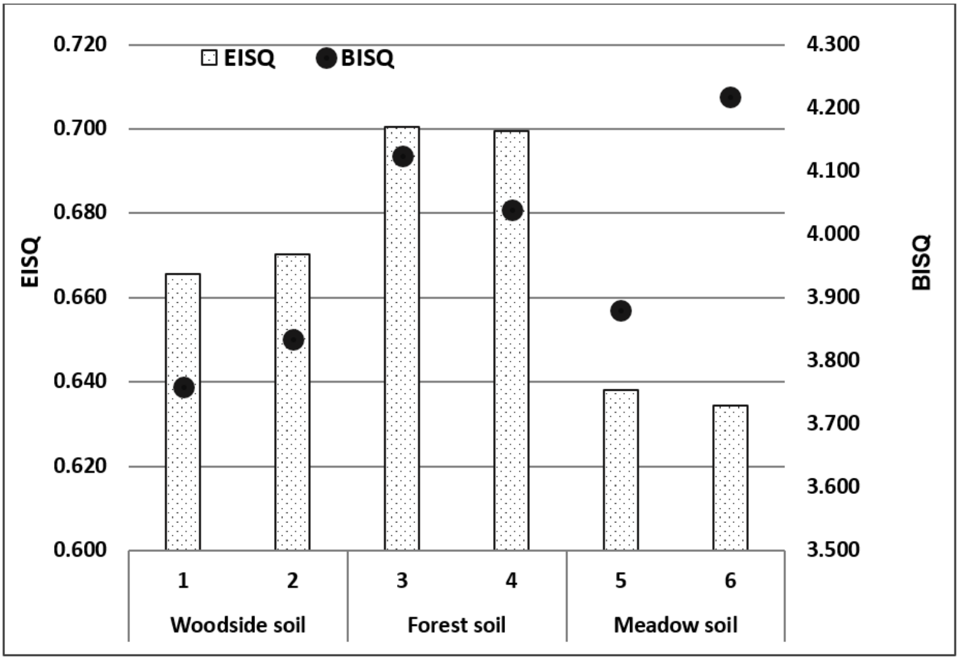

Figure 5. Bacterial and Enzymatic indicator of soil quality

Conclusions

Results of physico-chemical parameters analyses of soil samples revealed: a pH close to strong acidity; the redox potential ranging between 107-139 mV; conductivity detected in all sampling sites (values between 39 and 70 µS cm^{-1}).

A correlation between the microbial activity to each sampling sites and specific vegetation was observed (with differences between woodside, forest and grassland zones). The number of different bacteria (heterotrophic, ammonifying, nitrifying, denitrifying bacteria) is influenced by the amount of nutrients in the soil, season and zone. The highest values were registered in the sampling sites form forest zone. The bacterial indicator of soil quality (BISQ) presents variations according to the sampling sites.

An appreciable enzymatic potential of soils was registered in all the studied soils. The highest values of the enzymatic indicator of soil quality (EISQ) were registered in the forest soil (EISQ = 0.632).

The high enzymatic and bacterial potential registered in the forest soil, could be correlated with putative organic materials originating from the end of the vegetation period.

REFERENCES

Abbas, T., Zhou, H., Zhang, Q., Li, Y., Liang, Y., Di, H., & Zhao, Y. (2019). Anammox co-fungi accompanying denitrifying bacteria are the thieves of the nitrogen cycle in paddy-wheat crop rotated soils. *Environment International*, 130, 104913.

Alexander, M. (1965). Most-probable number method for microbial populations. In Black, C.A., Evans, D.D., White, J.L., Ensminger, L.E., Clark, F.E. (eds.), Methods os Soil Analysis, Ed. Am. Soc. Agron., Madison.

Ågren, G.I., & Weih, M. (2012). Plant stoichiometry at different scales: element concentration patterns reflect environment more than genotype. *New Phytol*, 194, 944–952.

Ananbeh, H., Stojanović, M., Pompeiano, A., Voběrková, S., & Trasar-Cepeda, C. (2019). Use of soil enzyme activities to assess the recovery of soil functions in abandoned coppice forest systems. *Science of The Total Environment*, 694, 133692.

Bardgett, R.D., & Wardle, D.A. (2010). Aboveground-belowground Linkages, Biotic Interactions, Ecosystem Processes, and Global Change (Oxford University Press, New York, 2010), pp. 1–59.

Blaga, G., Filipov. F., Rusu, I., Udrescu, S., & Vasile, D. (2005). *Pedologie*, Ed. Academic Press, Cluj Napoca.

Bragazza, L., Bardgett, R.D., Mitchell, E.A.D., & Buttler, A. (2015). Linking soil microbial communities to vascular plant abundance along a climate gradient, *New Phytol.* 205, 1175–1182.

Bui, E.N., & Henderson, B.L. (2013). C:N:P stoichiometry in Australian soils with respect to vegetation and environmental factors. *Plant Soil,* 373, 553–568.

Cabello, P., Luque-Almagro, V.M., Roldán, M.D., & Moreno-Vivián, C. (2019). Nitrogen Cycle, Encyclopedia of Microbiology (Fourth Edition), 301-310.

Carpa, R., Drăgan-Bularda, M., & Muntean, V. (2014). Microbiologie Generală, Lucrări Practice, Ed. Presa Univ. Clujeană, Cluj-Napoca [*in Romanian*].

Cuşa, V. (1996). The methodological instruction for microbiological analysis of the aquatic sediments, vol. 4, Bucharest Research and Environmental Engineering Institute, 2-7.

Deng, Q., Cheng, X., Hui, D., Zhang, Q., Li, M., & Zhang, Q. (2016). Soil microbial community and its interaction with soil carbon and nitrogen dynamics following afforestation in central China. *Science of the Total Environment*, 541, 230–237.

Fan, H., Wu, J., Liu, W., Yuan, Y., Hu, L., & Cai, Q. (2015). Linkages of plant and soil C:N:P stoichiometry and their relationships to forest growth in subtropical plantations. *Plant Soil*, 392, 127–138.

Guo, D., Ren, C., Ali, A., Li, R., Du, J., Liu, X., Guan, W., & Zhang, Z. (2019). *Streptomyces pactum* combined with manure compost alters soil fertility and enzymatic activities, enhancing phytoextraction of potentially toxic metals (PTMs) in a smelter-contaminated soil. *Ecotoxicology and Environmental Safety,* 181, 312-320.

Lucas-Borja, M.E., Hedo, J., Cerdá, A., Candel-Pérez, D., & Vinegla, B. (2016). Unravelling the importance of forest age stand and forest structure driving microbiological soil properties, enzymatic activities and soil nutrients content in Mediterranean Spanish black pine (*Pinus nigra Ar. ssp. salzmannii*) Forest. *Science of The Total Environment*, 562, 145-154.

Muntean, V. (1995–1996). Bacterial indicator of mud quality, *Contrib. Bot*., 73–76.

Pereg, L., Mataix-Solera, J., McMillan, M., & García-Orenes, F. (2018). The impact of post-fire salvage logging on microbial nitrogen cyclers in Mediterranean forest soil. *Science of The Total Environment,* 619-620, 1079-1087.

Pu, Y., Zhu, B., Dong, Z., Liu, Y., Wang, C., & Ye, C. (2019). Soil N_2O and NO_x emissions are directly linked with N-cycling enzymatic activities. *Applied Soil Ecology*, 139, 15-24.

Ren, C., Zhang, W., Zhong, Z., Han, X., Yang, G., Feng, Y., & Ren, G. (2018). Differential responses of soil microbial biomass, diversity, and compositions to altitudinal gradients depend on plant and soil characteristics. *Science of The Total Environment*, 610-611, 750-758.

Samec, P., Vozenilek, V., Vondrakova, A., & Macku, J. (2018). Diversity of forest soils and bedrock in soil regions of the Central-European highlands (Czech Republic). *Catena,* 160, 95-102.

Wu, P., & Wang, C. (2019). Differences in spatiotemporal dynamics between soil macrofauna and mesofauna communities in forest ecosystems: The significance for soil fauna diversity monitoring. *Geoderma,* 337, 266-272.

Yao, X., Zhang, N., Zeng, H., & Wang, W. (2018). Effects of soil depth and plant–soil interaction on microbial community in temperate grasslands of northern China. *Science of The Total Environment,* 630, 96-102.

Yao, Y., Shao, M., Fu, X., Wang, X., & Wei, X. (2019). Effects of shrubs on soil nutrients and enzymatic activities over a 0–100 cm soil profile in the desert-loess transition zone. *Catena,* 174, 362-370.

Zhang, W., Liu, W., Xu, M., Deng, J., Han, X., Yang, G., Feng, Y., & Ren, G. (2019). Response of forest growth to C:N:P stoichiometry in plants and soils during *Robinia pseudoacacia* afforestation on the Loess Plateau, China. *Geoderma*, 337, 280-289.

Wardle, D.A. (2002). Communities and Ecosystems: Linking the Aboveground and Belowground Components. (Princeton University Press, Princeton, 2002), pp. 7-55.

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Ultrastructural aspects of *Eriogaster catax* and *Eriogaster lanestris* (Lepidoptera: Lasiocampidae)

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Abstract. *Eriogaster catax* (Linnaeus, 1758) and *E. lanestris* (Linnaeus, 1758) are two related species in the family Lasiocampidae with decreasing distribution areas and extremely isolated populations, thus being vulnerable to numerous threats, particularly human impact. Until presently, the biological and ecological studies regarding the species in question are scarce, particularly for *E. catax*, a species protected through Annexes II and IV of the Council Directive 92/43/EEC, Annex II of the Bern Convention and Law no. 49/2011 in Romania. Taking into account the importance of the *E. catax* species and the insufficient information reported on the ecological need of the species, a highly detailed analysis at an ultrastructural level could provide valuable information. The primitive aspect of the wings explained the weak distribution of adults. The different flight period of adults and egg laying season were reflected on the structure, adherence and size of eggs. Also, the structure of the fibers was correlated with the different ways in which *E. catax* and *E. lanestris* use the nest.

Keywords: *Eriogaster catax*, *E. lanestris*, oviposition strategy, ecological niche.

Introduction

Eriogaster catax (Linnaeus, 1758) and *E. lanestris* (Linnaeus, 1758), (Lepidoptera: Lasiocampidae) are two cohabitant species (Sáfián, 2006) which are mainly found in the semi-natural habitats that have a predominant shrubbery vegetation (Kadej *et al.,* 2018).

E.catax is one of the least studied species due to living in very localized areas (Drews and Wachlin, 2003) and it is also considered an endangered species in several European countries. Currently it is protected by law at European level by the *Directive 92/43/EEC*, Annexes II and IV and the *Berne Convention*, Annex II

(Höttinger, 2005), while also being categorized as data deficient (DD) in the IUCN red list of threatened species (Farkač *et al.,* 2005). The species spreads throughout entire Europe, starting from the north of Spain up to the Balkans, and to the south down to the Ural Mountains and Anatolian peninsula (Baillet, 2013; Borges, 2012; Bury, 2015; Freina, 1996; Freina and Witt, 1987; Karsholt and Razowski, 1996; Konvička *et al*., 2005; Leraut, 2006; Ruf *et al*., 2003).

E. lanestris has a much larger distribution and can be found in almost all Europe except for the Tundra habitats, the Mediterranean region and in Asia with reports up to north of Caucasus, Kazakhstan, Siberian southern region, Central Yakutia and the Amur basin (Ruf *et al*., 2003; Ebert, 1994; Freina and Witt, 1987; Dubatolov and Zolotuhin, 1992; Pro Natura, 2005).

In their larval stage, both species feed primarily on the *Prunus spinosa* and *Crataegus monogyna* shrubs. The female moths of both species lay their eggs only once, on the branches of the host plant (Baillet, 2011; Caron, 2009; Höttinger, 2005; Oleksa, 2010). Due to the absence of a "mouth", the adult moths do not feed in their life span and rely solely on their larval nutrition (Malkiewicz, 2015).

A distinguishing feature of the two species is their highly social behavior during the larval stage, categorized as gregarious (Baillet, 2013; Bury, 2015; Caron, 2009; Chrzanowski *et al.,* 2013; Freina, 1996; Ruf *et al.,* 2003).

In the literature published so far, the growth stages of *E. catax* and *E. lanestris* have been described from a morphological point of view. By using a scanning electron microscopy technique, Fitzgerald (1995) highlights a series of ultrastructural characteristics such as the texture of the silk nest woven by the caterpillars or different types of glands from the genus *Malacosoma*, which is related to the genus *Eriogaster.*

Taking into account the importance of the *E. catax* species and the scarce information reported on the ecological need of the species, a highly detailed morphological analysis at an ultrastructural level could provide valuable information. Due to the fact that *E. catax* and *E. lanestris* share the same habitat and sometimes even the same shrub during their larval development stage, the investigation of both species was considered necessary.

Materials and methods

Electron microscopy analysis

Samples (adults, eggs, larva and nest silk) were prepared for scanning electron microscopy (SEM) analysis using the turbomolecular pumped coater Quorum Q150T ES, from the Integrated Electron Microscopy Laboratory (LIME) of National Institute for Research and Development of Isotopic and Molecular Technologies (INCDTIM), Cluj-Napoca, Romania, and examined at SEM Hitachi SU8230 (LIME-INCDTIM, Cluj-Napoca, Romania).

Statistics

Statistical analyses were conducted using online *t* test calculator.

Results and discussions

Adults

Both species had palmate scales (Fig. 1 A, B, D), fixed on the surface of the wings through a peduncle (Fig. 1 C), and were randomly dispersed on the surface.

The fact that the scales were fixed through a peduncle indicates that they originate from trichomes. Some studies showed the relation between Lepidoptera scales and sensory trichomes (Galant *et al.*, 1998; Zhou *et al.*, 2009). The scares distribution of the scales is correlated with the underdeveloped flight ability, explaining also the low dissemination of females. Peduncle-like scales and no mouthpieces are primitive characters of *Eriogaster.*

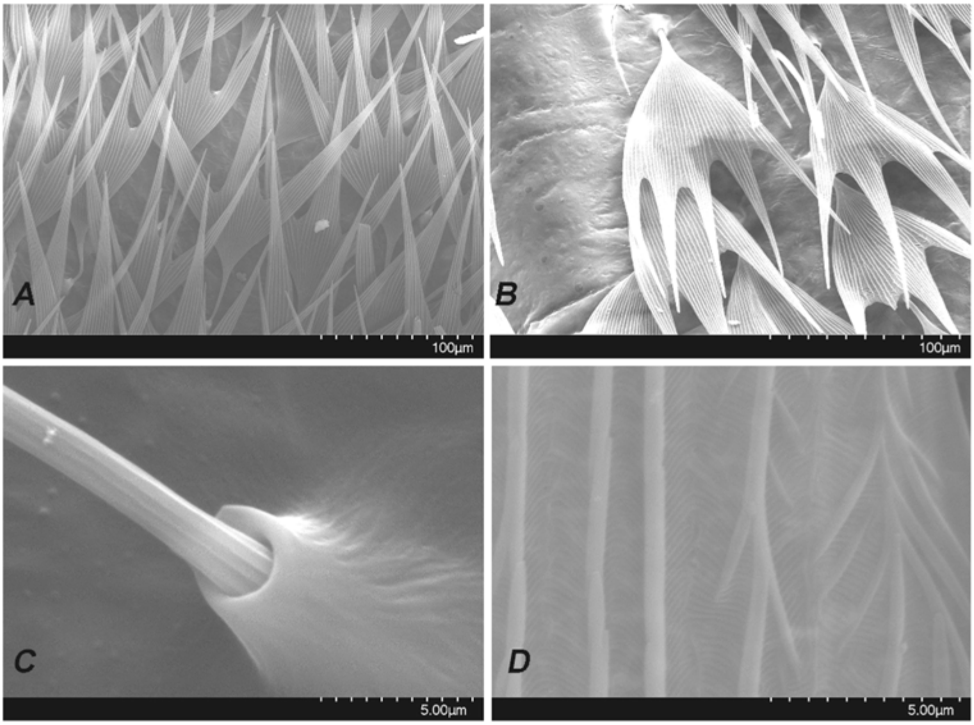

Figure 1. A. Palmate scales of *E. catax*; B. Palmate scales of *E. lanestris*; C. The insertion point of a peduncle to the scale; D. The surface of a scale in detail.

The antennae of both sexes were consisted of three segments: scape, pedicel and a long (Ma *et al.,* 2017; Mark *et al.,* 2017) bipectinate flagellum (Fig. 2 A). The bipectinate flagellum is composed of approximately same number of flagellomeres in males and females. Each flagellomere bears two lateral branches (Fig. 2 C, 4 A). The females of both species had shorter and thicker branches than the males. There are also differences in shape and thickness between the flagellomere branches of the two species i.e., those of *E. lanestris* were significantly thickened towards the base giving them a pear-like shape (Fig. 2 C, D).

Branches of flagellomeres in males of the two species increased progressively in length from the proximal end to the middle, and then decreased towards the apex. Branch length was sexually dimorphic, with males possessing significantly longer branches throughout.

The dorsal surface of the flagellum (antennal spindle) and the entire scape and pedicel were covered in overlapping lamellar scales (Fig. 2 B) (Mark *et al.,* 2017; Yuvaraj *et al.*, 2018).

Based on the external morphology of the sensilla observed under SEM, many sensilla with role in olfaction which plays a critical role for insects were identified (Yuvaraj *et al.*, 2016).

Sensilla trichodea were predominantly distributed on the ventral side of the lateral branches (Fig. 3A, B; 4A) (Fernandes *et al.*, 2017; Li *et al.,* 2018; Mark *et al.*, 2017). In males they were grouped in clusters of 5–6 sensilla (Fig. 4 B, C, D). Sensilla trichodea are the most abundant sensory receptors on the antennae of both sexes and species (Fig. 3 A, B and 4 A).

On the dorsal side of the lateral branches (Fig. 2 C, D; 4 A) we identified 8-9 (n=100) sensilla chaetica (Fig. 3 E, F and 4 E, F) (Mark *et al.,* 2017) at antennae of both species.

On the apex of of the lateral branches of both sexes we identified 1-3 sensilla basiconica (Fig. 3 C, D) (Mark *et al.*, 2017).

Eggs

For *E. catax* the eggs had an elongated, cylindrical form, nicely organized in rows and very well welded together and to the substrate. On the apex, the eggs had the micropyllar region formed like a small dent, with numerous pores (Fig. 5 A, C, E).

E. lanestris had less organized eggs, with weak connections in between and to the surface. The eggs were elongated and with a wider apex compared with the basal part. The micropyllar region is slightly curved (Fig. 5 B, D, F). Because the eggs are not so close to each other, the pores developed on the lateral of the egg, and not on top, like *E. catax* (Fig. 6 A).

Both species hatch by penetrating the micropyllar region and the missing part indicated that the caterpillars consumed this region which contributed to their nutrition (Fig. 6 B).

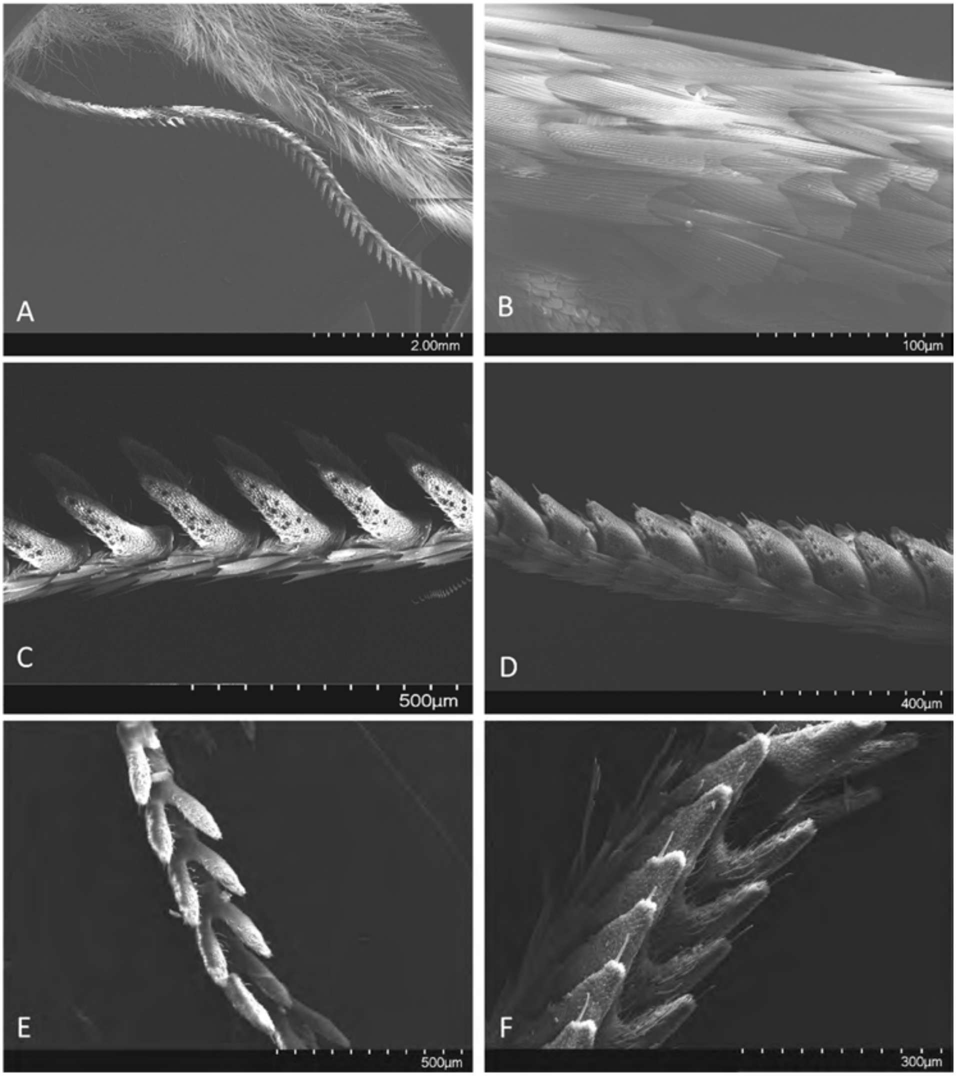

Figure 2. SEM micrographs showing the external morphology of the antennae of *E. catax* and *E. lanestris:* A. The bipectinate antennae of the *E. catax* female; B. The dorsal surface of the flagellum is covered with overlapping lamellar scales at both species; C. Female antenna of *E. catax* - lateral view. D. Female antenna of *E. lanestris* - lateral view; E. Female antenna of *E. catax* - ventral view. F. Female antenna of *E. lanestris* - ventral view;

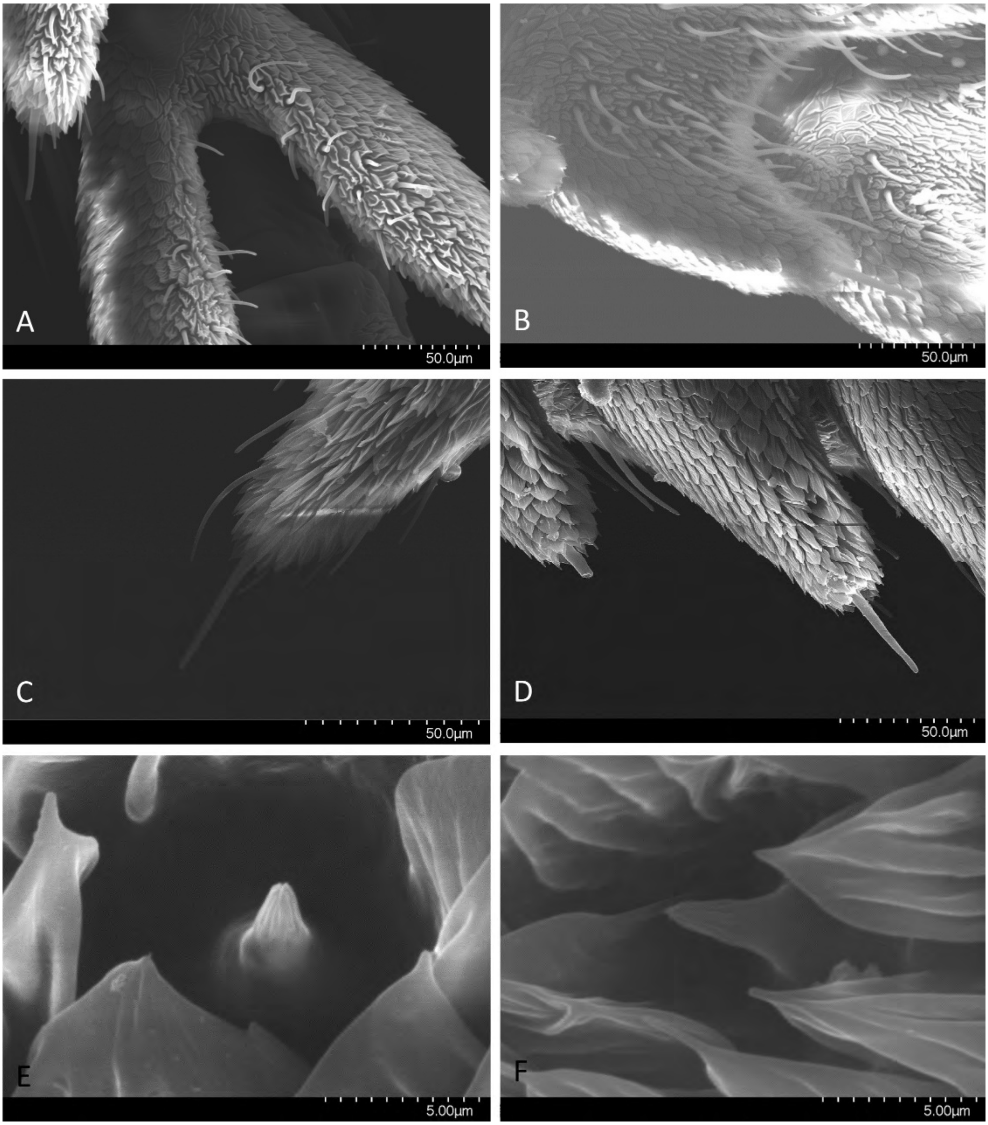

Figure 3. The external morphology of the sensilla observed using SEM on the female antennae of *E. catax* (A., C., E.) and *E. lanestris* (B., D., F.). A., B. Sensilla trichodea on the ventral side of the lateral branches; C., D. Sensilla basiconica on the apex of the lateral branches; E., F. Sensilla chaetica on the dorsal side of the lateral branches.

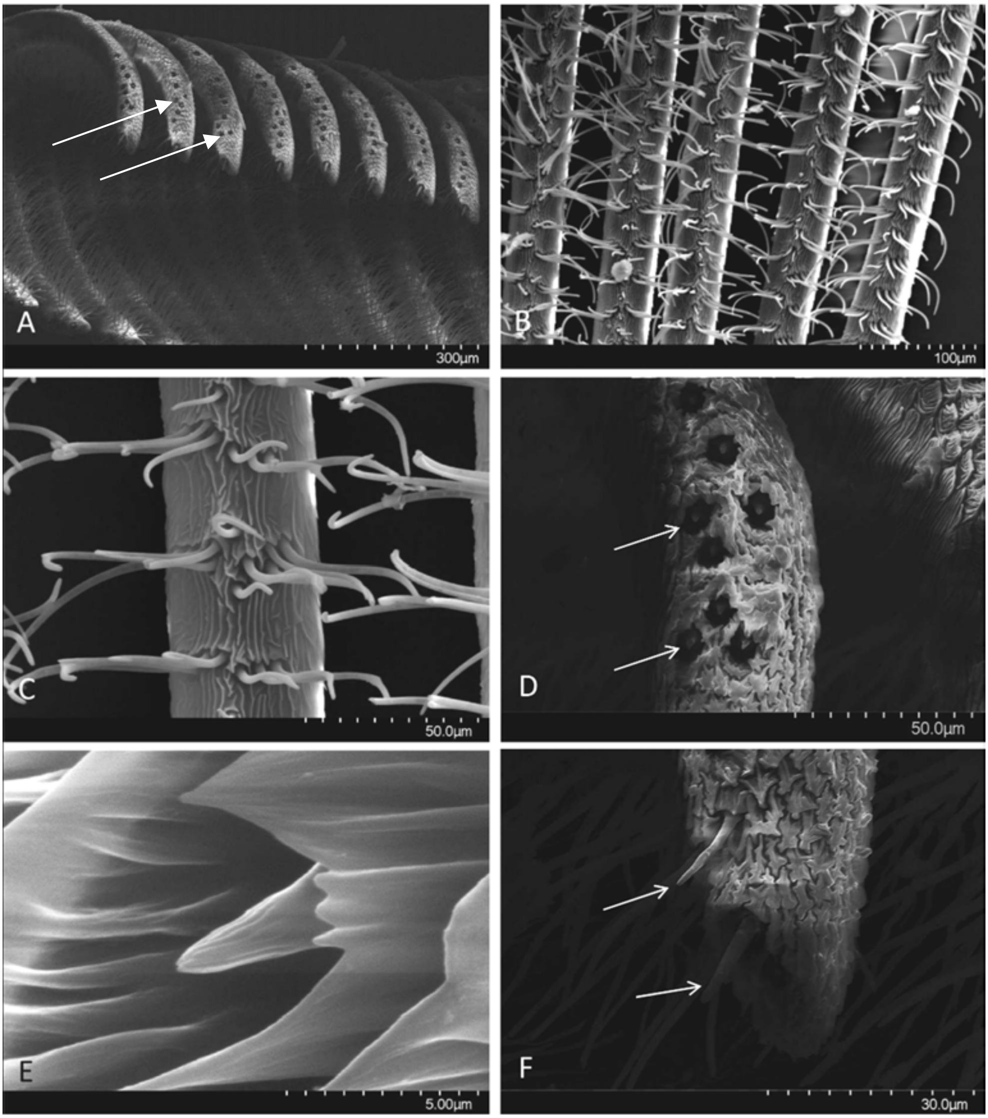

Figure 4. The external morphology of the antennae and the sensilla observed on SEM on the male's antennae of *E. catax.* A. Male antenna of *E. catax* - ventral view. Arrows indicate the location of the chaetica sensilla. B., C. Sensilla trichodea on the ventral side of the lateral branches of both species; They were grouped in clusters of 5–6 sensilla; E. Sensilla chaetica on the dorsal side of the lateral branches. F. Sensilla basiconica on the apex of of the lateral branches.

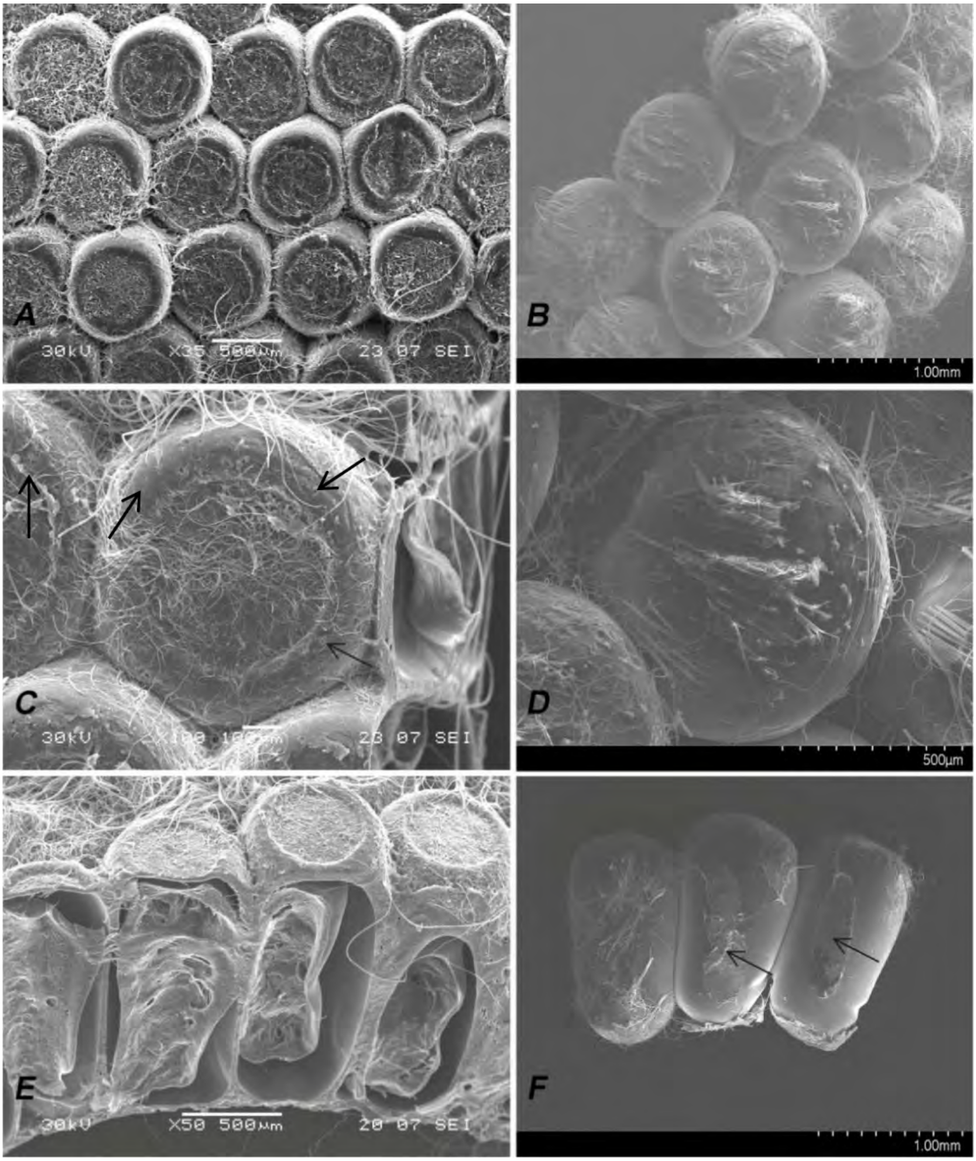

Figure 5. A. Eggs of *E. catax* arranged in ordered.rows The eggs are tightly bound together and adheres very well to the substrate; B. Eggs of *Eriogaster lanestris* **-** the rows are more slightly ordered and the adhesion to the substrate is weak; C. Around the micropillary region of the *E. catax* egg, numerous pores indicated by arrows can be observed; D. The micropillary region of the E. *lanestris* egg is slightly bulged like a dome. E. Lateral view of *E. catax* eggs; F. Lateral view of *E. lanestris* eggs. Arrows indicate the contact area with adjacent eggs.

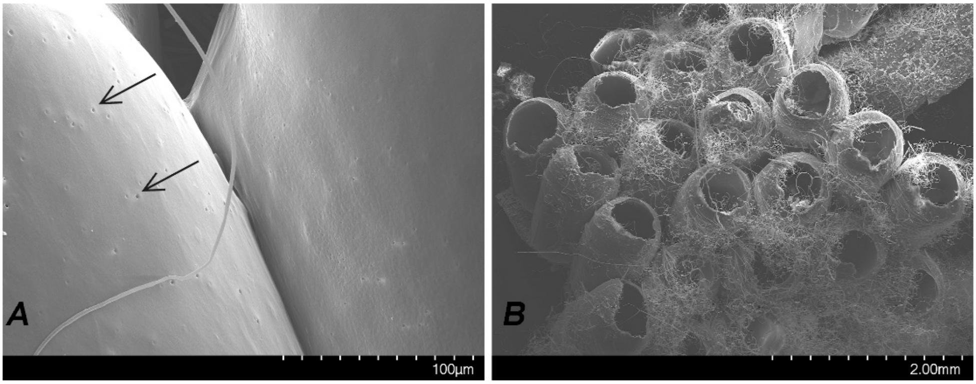

Figure 6. A. Respiratory pores on the egg surface of *E. lanestris*;
B. Hatch orifices of *E. lanestris* caterpillars.

E. lanestris has smaller eggs compared to *E. catax* and this could be explained by the different time periods when the eggs are laid. *E. catax* lays its eggs in September-October (Bury, 2015; Chrzanowski et al., 2013; Freina, 1996), and *E. lanestris*, during March-April (Ruf *et al.,* 2003).

Compact and well adhered eggs on the surface of the leaves, along with a small number of large eggs are adaptations of *E. catax* to overcome unfavorable conditions during winter period.

Larva

The polypod caterpillar of *E. catax* had five stemmata (the visual organs of the larva) on each side of the cephalic capsule. Also, numerous pores were observed on the cephalic capsule most probably act like atmospheric pressure sensors (Fig. 7 A, B, C).

Both species had protective tubular trichomes on the surface of the body and on the cephalic capsule. To evaluate the differences, trichomes' width on the cephalic capsule were measured and very significant differences were observed ($p<0.05$). *E. catax* had an average width of trichomes of 8.96 µm ± 0.2 µm (N=30, ± SEM) and for *E. lanestris* the trichomes measured 4.51 µm ± 0.25 µm (N=30, ± SEM) (Fig. 4 D, E, F).

On the sides of the body, eight pairs of spiracles were observed. Spiracles contain numerous atrial structures that close or open the airway. The atrial structures of the spiracles are finger-shaped and they have many cilia (Fig. 8. A, B).

On the ventral side the larva had three pairs of thoracic legs and four pairs of prolegs provided with numerous crochets (Fig. 9).

E. catax hatch in early spring, along with bud break of *Prunus spp.*and *Crataegus spp.* (Baillet, 2013; Caron, 2009; Höttinger, 2005).

Access to food is still limited during this period and cold weather in the morning and at night lead to high consumption of energy for thermoregulation. *E. lanestris* hatches 3 to 4 weeks later (Konvička *et al*., 2006), when both *Prunus* spp. and *Crataegus* spp. are blooming, the temperatures slightly increased and food sources are plenty.

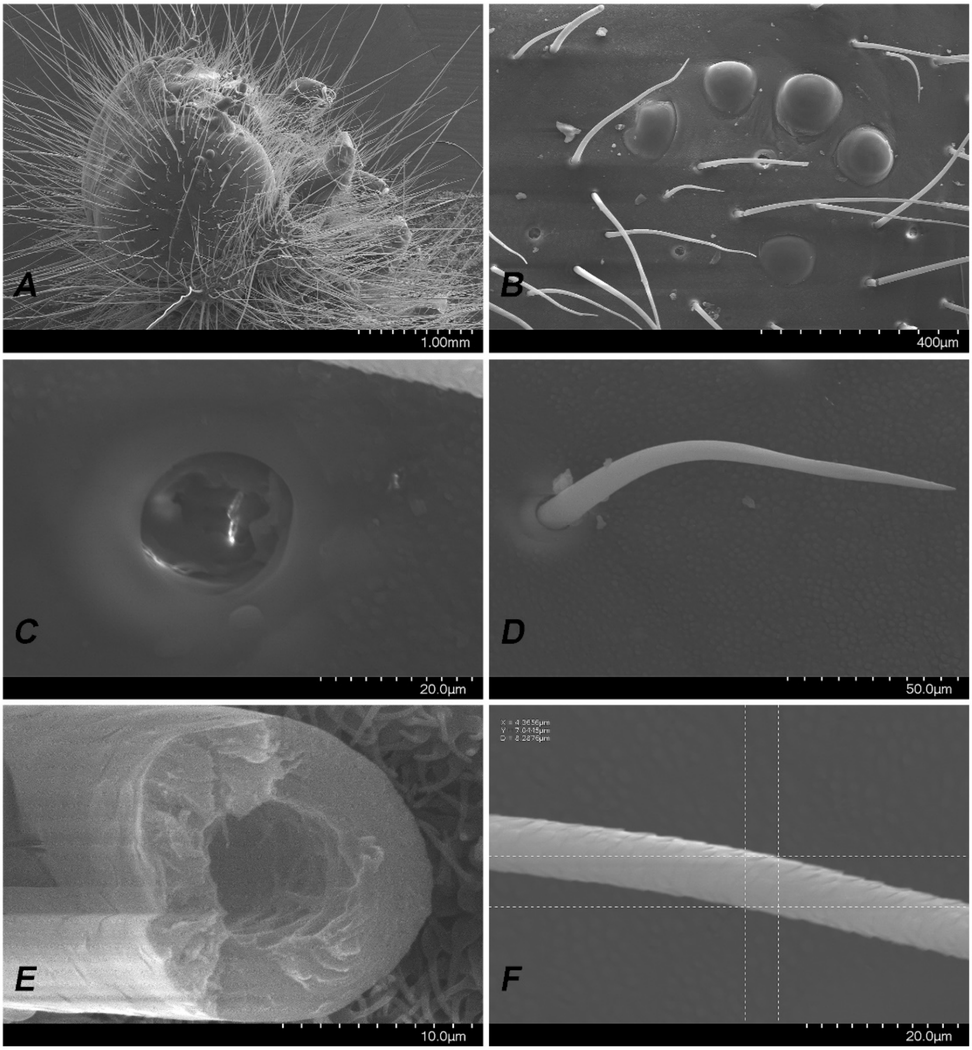

Figure 7. A. Cephalic capsule of the caterpillar; B. Five stemmata - the visual organs of the larva; C. Cephalic capsule of the caterpillar has many pores - atmospheric pressure sensors; D. Protective trichomes present on the body and on the cephalic capsule; E. Protective trichome - cross section; F. *E. catax* average width of trichomes - 8.96 µm.

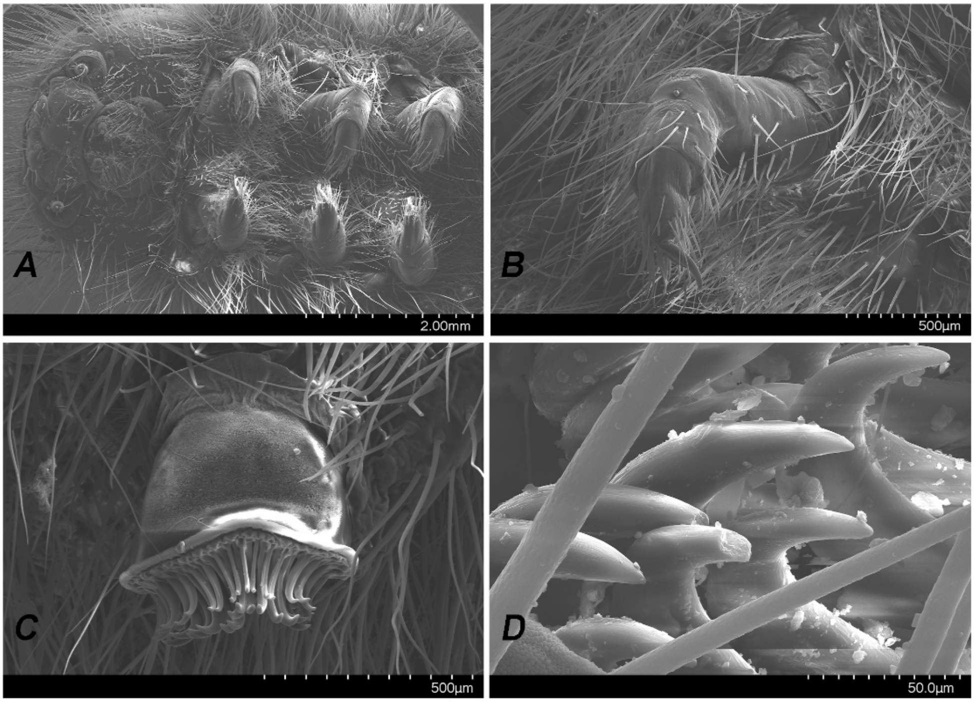

Figure 8. Two different types of caterpillars legs. A., B. Three pairs of articulated abdominal legs C., D. Proleg provided with numerous crochets.

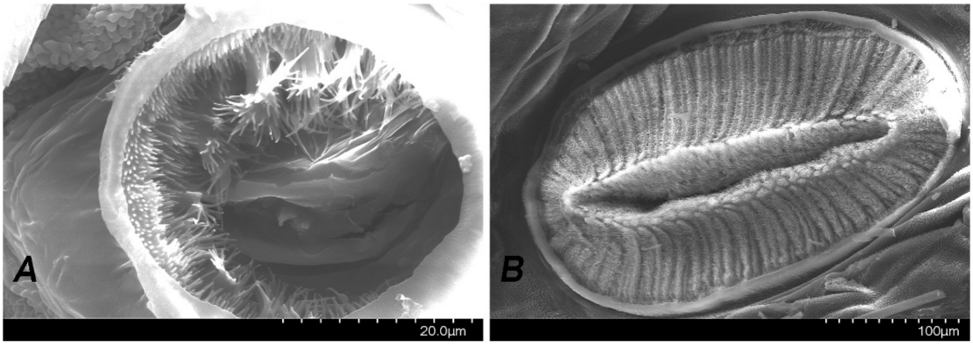

Figure 9. Spiracle with atrial structure. A. Caterpillar in first instar *L1*; B. Caterpillar in last instar *L5*.

Nest silk

E. catax had curvy-like fibers, welded together in different spots, and *E. lanestris* had straight fibers which did not form agglomerations, and with calcium oxalate crystals for increased strength. The width of the fibers was measured and highly significant differences were observed ($p<0.001$). *E. catax* had 0.89 µm ± 0.04 µm thick fibers, and *E. lanestris* had 1.78 µm ± 0.07 µm ($N = 100$, ±SEM), making it double in width (Fig. 10).

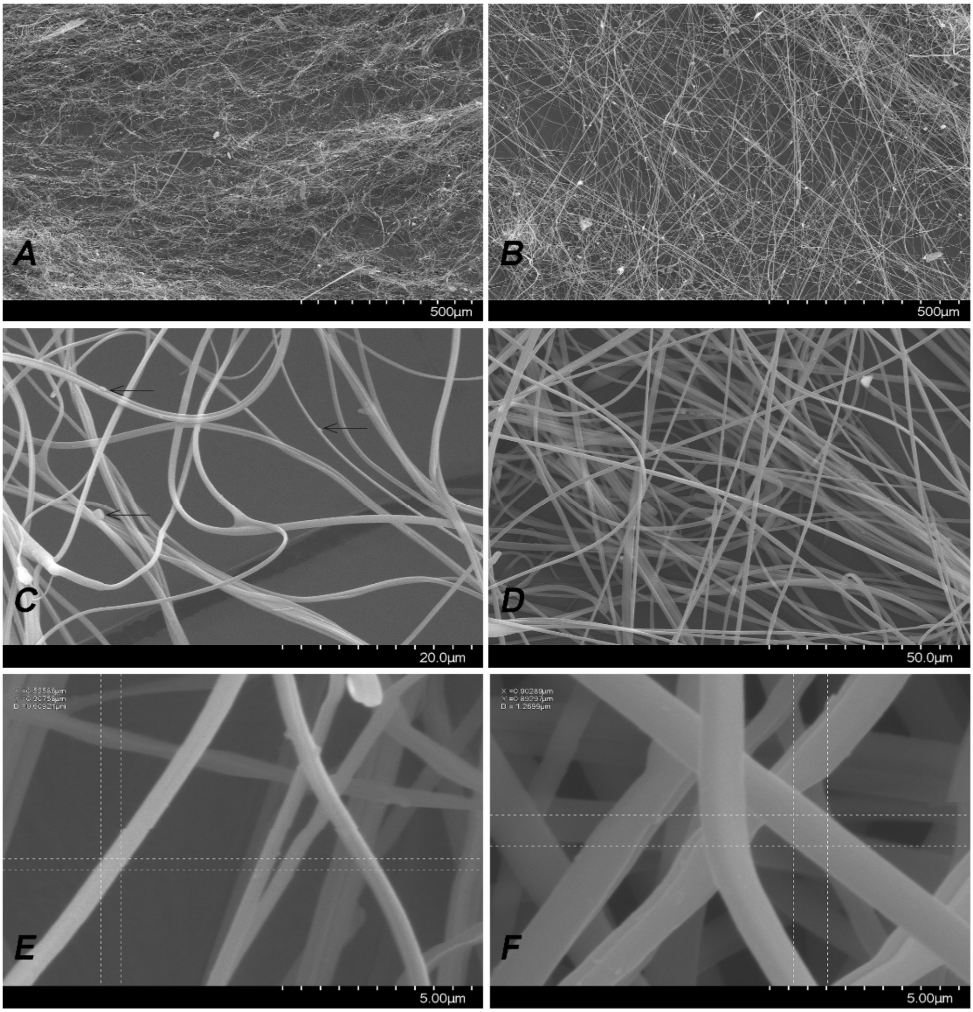

Figure 10. The ultrastructural aspects of *E. catax* (A, C, E) and *E. lanestris* (B, D, F) silk

The small width of the fibers of *E. catax* is compensated by their capacity to weld and form a felt-like structure, which confers rigidity to the nest. For *E. lanestris* the time required for the fibers to stiffen is shorter compared to *E. catax.* This explains why for *E. catax* the fibers adhere to each other.

The nest made by *E. catax* is used as a platform to rest on, gain warmth and shelter. The nest grows until the larva reach L3 stage, but they do not have enough space to stay inside it, compared to *E. lanestris*, where the larva can get inside the nest.

The nest serves as an activity center and plays an important role in the thermoregulation of the colony (Fitzgerald and Costa, 1999; Costa, 1997).

Conclusions

SEM analysis revealed precious information about the ecology and biology of the species. The primitive aspect of the wings explained the weak spatial distribution of the adults. The different flight period of adults and egg laying season were reflected in the structure, adherence and size of eggs. Also, the structure of the fibers was influenced by the ways in which *E. catax* and *E. lanestris* use the nest.

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REFERENCES

Baillet, Y. (2011). Inventaire de Eriogaster catax (Laineuse du Prunellier) sur l'ENS des communaux de Trept. *Rapport d'étude rendu au format Excel de Flavia, Trept.*

Baillet, Y. (2013). Inventaire de Eriogaster catax (Laineuse du Prunellier) sur l'ENS des communaux de Trept. *Rapport d'étude rendu au format Excel de Flavia, Trept.*

Borges, A. (2012). La Laineuse du prunellier – 2.localisation des chenilles (dans les nids communautaires) : c'est maintenant ! I NSECTES n°164 - 2012 (1)

Bury J. (2015). Nowe dane o występowaniu Eriogaster catax (Linnaeus, 1758) (Lepidoptera: Lasiocampidae) w południowo-wschodniej *Polsce. Acta Ent. Siles*. 23 (online 012)1–10.

Caron, G. (2009). La laineuse du prunellier Eriogaster catax (Linnaeus, 1758) (Lepidoptera, Lasiocampidae) victime des changements climatiques ? Ecologie de l'espèce et hypothèses sur son déclin dans la région genevoise. *Entomo Helvetica, 2:* 49-60.

Chrzanowski, A., Kuźmiński, R., Łabędzki, A., Mazur, A., & Rutkowski, P. (2013). Occurrence of *Eriogaster catax* (Linnaeus, 1758) (Lasiocampidae, Lepidoptera) and the proposed protective actions on the Polish territory. *Nauka Przyroda' Technologia* 7, 2, (24): 1–7.

Costa, J.T. (1997). Caterpillars as social insects. *American Scientist:* 85, 150–159.

Drews, M., & Wachlin, V. (2003). *Eriogaster catax* (Linnaeus, 1758). W: Petersen B., Ellwanger G., Biewald G., Hauke U., Ludwig G., Pretscher P., Schröder E., Ssymank A. (red.). Das europäische Schutzgebietssystem Natura 2000. Ökologie und Verbreitung von Arten der FFH-Richtlinie in Deutschland. Band 1: Pflanzen und Wirbellose. Schr.R. Landschaftspflege Naturschutz 69/1: 459.464.

Dubatolov, V.V., & Zolotuhin, V.V. (1992). A list of the Lasiocampidae from the territory of the former USSR. Atalanta 23 (3/4): 531-548.

Ebert, G. (1994). Die Schmetterlinge Baden-Württembergs. Band 4, Nachtfalter II. Ulmer Verlag, Stuttgart.

Farkač, J., Král D., & Škorpík, J. (2005). Červený seznam ohrožených druhů České republiky Bezobratlí. Agentura ochrany přírody a krajiny ČR. 760pp

Fernandes, F.F., & Pimenta, P.F.P. (2018). Typology and distribution of antennal sensory organs of adults Haematobia irritans of the Midwest Region of Brazil. *Pesquisa Veterinária Brasileira*, 38(1), 183-188. doi:10.1590/1678-5150-pvb-5499

Fitzgerald, T.D. (1995). The tent caterpillars. Cornell University Press. ISBN 0-8014-2456-9

Fitzgerald, T.D., & Costa J.T. (1999). Collective behavior in social caterpillars. pp. 379–400 in Detrain C, Deneubourg JL and Pasteels JM (eds.) Information processing in social insects. Birkhäuser Verlag, Basel.

Freina, J.J. de, (1996). *Eriogaster catax* (Linnaeus, 1758). W: Helsdingen P.J., van Willemse L., Speight M.C.D. (red.): Background information on invertebrates of the Habitat Directive and the Bern Convention. Part I: Crustaceae, Coleoptera and Lepidoptera. *Nature and Environment* No. 79: 117.120.

Freina, J.J. de, & Witt, T.J. (1987). Die Bombyces und Sphinges der Westpaläarktis (Insecta, Lepidoptera). Edition Forschung und Wissenschaft, München.

Galant, R., Skeath, J.B., Paddock, S., Lewis, D.L., & Carroll, S.B. (1998). Expression pattern of a butterfly achaete-scute homolog reveals the homology of butterfly wing scales and insect sensory bristles. *Current Biology* 1998, 8:807–813.

Höttinger, H. (2005). Der Hecken-Wollafter (*Eriogaster catax* L.) in Wien (Lepidoptera: Lasiocampidae). Endbericht einer Studie im Auftrag der Wiener Magistratsabteilung MA 22 (Umweltschutz).

Kadej, M., Zając, K., & Tarnawski, D. (2018). Oviposition site selection of a threatened moth *Eriogaster catax* (Lepidoptera: Lasiocampidae) in agricultural landscape - implicationsfor its conservation. *Journal of Insect Conservation*, 22, 29–39.

Karsholt, O. & Razowski J. (eds.). (1996) The Lepidoptera of Europe. A distributional checklist. Apollo Books, Stenstrup.

Konvička, M., Beneš, J., & Čížek, L. (2005). Ohrožený hmyz nelesních stanovišť: ochrana a management. Sagittaria 2005. 127 pp.

Konvička, M., Hula, V., & Beneš, J. (2006). Metodika monitoringu evropsky významného druhu bourovec trnkový (*Eriogaster catax*). Nepublikovaný rukopis, deponován na UP AOPK ČR Praha. 7pp.

Leraut, P. (2006). Moths of Europe. Volume 1: Saturnids, Lasiocampids, Hawkmoths, Tiger Moths. N.A.P. Editions, Verrières le Buisson. 395 pp.

Li, Y., Liu, F., Du, X., Li, Z., & Wu, J. (2018). Ultrastructure of antennal sensilla of three fruit borers (Lepidoptera: Crambidae or Tortricidae). *PLoS ONE* 13(10): e0205604. doi:10.1371/journal.pone.0205604

Ma, M., Chang, M., Lu, Y., Ley, C., & Yang, F. (2017). Ultrastructure of sensilla of antennae and ovipositor of *Sitotroga cerealella* (Lepidoptera: Gelechiidae), and location of female sex pheromone gland. *Scientific Reports* 7, 40637. doi:10.1038/srep40637

Malkiewicz, A. (2015). Barczatka kataks w Krainie Łęgów Odrzańskich – występowanie, zagrożenia, zalecenia ochronne. In: Pietkiewicz M. and Sztwiertnia H. 2015 Kronika Łęgów Odrzańskich nr 1. Rzadkie i chronione zwierzęta krainy Łęgów Odrzańskich - zagrożenia i perspektywy rozwoju: Zimowanie ptaków w Krainie Łęgów Odrzańskich.Prochowice 2015. ISBN: 978-83-942200-0-6

Mark, C., Parsons, S., & Holwell, G. (2017). Antennal morphology and micro-sensory architecture of the New Zealand magpie moth, Nyctemera annulata (Lepidoptera: Erebidae): diversity, distribution and dimorphism. *Austral Entomology*. doi: 10.1111/aen.12279

Oleksa, A. (2010). 1075 Barczatka kataks *Eriogaster catax* (Linnaeus, 1758) [Eastern eggar *Eriogaster catax* (Linnaeus, 1758)]. In: Monitoring gatunków i siedlisk przyrodniczych ze szczególnym uwzględnieniem specjalnych obszarów ochrony siedlisk Natura 2000. Wyniki monitoringu. GIOŚ Warszawa [in Polish].

Pro Natura (2005). Lepidopterologen-Arbeitsgruppe (2000): Schmetterlinge und ihre Lebensräume, Band 3. Pro Natura - Schweizerischer Bund für Naturschutz, ISBN 3855870322, 928 Seiten.

Ruf, C., & Fiedler, K. (2002). Plasticity in foraging patterns of colonies of the small eggar moth, *Eriogaster lanestris* (Lepidoptera: Lasiocampidae). *Oecologia* 131: 626–634.

Ruf, C., Freese, A., & Fiedler, K. (2003). Larval sociality in three species of central-place foraging lappet moths (Lepidoptera: Lasiocampidae): a comparative survey. Zoologischer Anzeiger - *A Journal of Comparative Zoology* 242 (3): 209.222.

Sáfián, S. (2006). The occurrences of *Eriogaster catax* (Linnaeus, 1758) and *Eriogaster lanestris* (Linnaeus, 1758) (Lepidoptera: Lasiocampidae) in the Körös Valley (South-Eastern Hungary) *Folia Entomologica Hungarica*, 67, 137-143.

Yuvaraj, J.K., Andersson, M.., Anderbrant, O., & Löfstedt, C. (2018). Diversity of olfactory structures: A comparative study of antennal sensilla in Trichoptera and Lepidoptera. *Micron*, vol. 111, pp. 9-18. doi:10.1016/j.micron.2018.05.006

Zhou, Q., Yu, L., Shen, X., Li, Y., Xu, W., Yi, Y., & Zhang, Z. (2009). Homology of Dipteran Bristles and Lepidopteran Scales: Requirement for the Bombyx mori achaete-scute Homologue ASH2. *Genetics* 183: 619–627. doi: 10.1534/genetics.109.102848

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Effect of Fluocinolone-N treatment on fetal liver development in White Wistar Rats

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Abstract. Exposure to synthetic glucocorticoids during development can result in later cardiovascular and renal disease in sheep and rats. Although prenatal glucocorticoid exposure is associated with impaired renal development, less is known about effects on the developing liver. The main objective of this study is to analyze the side effects of glucocorticoid excess when treatment is done with Fluocinolone-N ointment, to see if it has any effect on rat newborns liver which have an important role in development of fetuses. Our results demonstrate that Fluocinolone-N treatment has negative impact upon the embryologic development of liver.

Keywords: glucocorticoid excess, fetal development, liver.

Introduction

Glucocorticoids are hormones that play a major role in energy homeostasis and stress response of the body (Vegiopoulus and Herzig 2007). As drugs they are most frequently used for immunosuppressive and anti-inflammatory purposes (Cosío *et al*., 2005; Newton *et al.,* 2010; Coutinho and Chapman, 2011; Spies *et al.,* 2011; Laugesen *et al.,* 2017). Glucocorticoids are used successfully in the treatment of a wide variety of diseases; however, some patients develop side effects (Crăciun *et al*., 1997a,b, 1998 a,b, 1999a,b,c, 2001; Rose *et al.,* 2010; Rebeyrol *et al.,* 2012; Woods and Weeks, 2005), while others fail to respond to this form of therapy (Schäcke *et al.,* 2002; Oppl *et al*., 2013). Fetal exposure to stress and its glucocorticoids hormone mediators exerts influences on organ growth, development and subsequent offspring physiology (Drake *et al*., 2007). In clinical situations, sources of maternal exposure to glucocorticoids includes; maternal stress, treatment with synthetic glucocorticoids in threatening preterm delivery and treatment of medical condition such as asthma (Singh *et al*., 2012).

Prenatal glucocorticoid exposure has been associated with a reduction in birth weight and postnatal alterations in glucose homeostasis and hypothalamic–pituitary–adrenal (HPA) axis function (Kapoor *et al.,* 2006; Kis *et al*., 1999, 2000; Sloboda *et al*., 2002; Jeje and Raji 2015; Paragliola *et al*., 2017). The mechanisms underlying these responses are unknown, although changes in fetal hepatic development may play an important role. The fetal liver produces key regulators of fuel metabolism and of the developing HPA axis, which are altered by hyperglucocorticoidemia (Fu *et al.*, 2016)

Animal studies have shown that prenatal exposure to synthetic GCs can have deleterious effects on the development of organs (such as the kidney, brain and the heart) which may in the longer term contribute to adult onset disease, including hypertension (Moritz *et al*., 2011; Gallo *et al*., 2012; Singh *et al*., 2012; Sullivan *et al.,* 2013). The role of synthetic glucocorticoids such as dexamethasone (DEX) in the programming of adult-onset diseases has been well studied (Jeje and Raji 2015). These studies have consistently demonstrated a reduction in nephron endowment following DEX treatment in sheep, spiny mice (Oppl *et al*., 2013). After glucocorticoid treatment similar changes can be observed in other mammals such as rats, rabbits and mice (Ortiz *et al*., 2003; O' Sullivan *et al*., 2013). Maternal administration of DEX for 48 h early in rat kidney development results in offsprings with a reduced nephron endowment. The authors hypothesized that DEX may indirectly inhibit nephrogenesis by inhibiting ureteric branching morphogenesis (Shingh *et al*., 2007). Damage to the kidney tissues may explain the hematological effect of DEX treatment. Chronic elevations in glucocorticoids induced by DEX are associated with increased liver fat (Harvey *et al*., 2017). Increased concentrations of glucocorticoids in humans and animals are associated with enhanced hepatic rates of lipogenesis and increased hepatic and plasma lipids (Amatruda *et al*., 1983; Beaudry *et al*., 2013; Drake *et al*., 2010; Yang *et al*., 2018). A number of facts studies suggest that some adult diseases may be the result of biochemical changes before birth produced by the elevation of glucocrticoid level (Romero and Butler 2007). Fetal organ systems are highly sensitive to changes in the intrauterine environment, including overexposure to glucocorticoids. Structural and functional alterations resulting from such changes may persist throughout life and have been associated with diverse diseases (Tegethoff *et al*., 2009). Antenatal corticosteroid therapy may increase the risk of cardiovascular disease in adulthood. Mice with global GR knockout have small and immature hearts that function poorly (Agnew *et al*., 2018).

Starting from the above observations, we can hypothesise that the abnormal hormone levels, can modify the program of the fetus development, causing such changes, which lead to dysfunction later in life. The mechanism of glucocorticoid excess induced tissue damage has not yet been fully elucidated. In literature there are very few data about glucocorticoid excess and its relationship with embryological development. Therefore, the main objective of this study is to determine correlations in pregnant animals treated with Fluocinolon-N ointment, between glucocorticoid excess, viability of rat newborns and liver development.

Materials and methods

The experiments were carried out in pregnant (60-day-old) and newborn Wistar rats. The animals were kept under standardized bioclimatic conditions and fed on common rat chow, with water *ad libitum*.Commercial Fluocinolone-N ointment containing 25 mg Fluocinolone-acetonid-N/100 g excipient, was applied topically to the skin at 2 cm^2, for five consecutive days, by smearing 50 mg ointment/100 g b.w on the inguinal region. The daily dose of Fluocinolone-acetonid-N being equal to 12,5 g/100 g b.w. The newborn animals were divided into the following groups:

C-control group, newborns of untreated dams
FC1 – newborns of pregnant dams, treated with Fluocinolone-N in the 9th- 13th days of pregnancy
FC1- newborns of pregnant dams, treated with Fluocinolone-N in the 16th- 20th days of pregnancy
FC3 - newborns of pregnant dams, treated with Fluocinolone-N in the 9th- 20th days of pregnancy. These newborns did not survive 24 hours.

After 16 hours of fasting and 24 hours following the cessation of the treatment, the treated animals together with controls, were sacrificed by exsanguinations. The body and liver weights were measured with an accuracy of 0,00001 g immediately after excision. The significance levels were determined by parametric t-test. A $p<0,05$ was considered statistically significant. We measured parameters only from newborns which survived the first 24 hours after parturition, therefore only three groups of neonates were studied. We have no data from FC_3 newborns because none survived 24 hours after parturition.

For structural analysis the organs of the slaughtered animals were fixed in Bouin liquid and afterwards processed in view of being embedded in paraffin. The fragments were sectioned at the microtome with a thickness of 7 μ. The staining of liver was made by means of hematoxilin-eosin method (Muresan *et al.,* 1974)

Results and discussions

Viability and body weight of newborn rats

The parturition occurred on the twenty–first day of pregnancy. The autopsy was performed 24 hours after birth but not all infants survived the critical first 24 hours. At necropsy we found in mother's (FC2 and FC3) womb dead fetuses. Therefore we considered necessary to distinguish between fetuses, neonates and viable infants (Fig. 1).

The numbers of fetuses and newborns in the control and in the early stage of pregnancy treated FC1 group are equal, the viability of fetuses in these groups are approximately 100 %. The numbers of newborns in the FC2 and FC3 groups decreased considerable in comparison to control group. The viability of fetuses in the late stage of pregnancy treated group FC2 is approximately 66%, and in the FC3 is 0%.

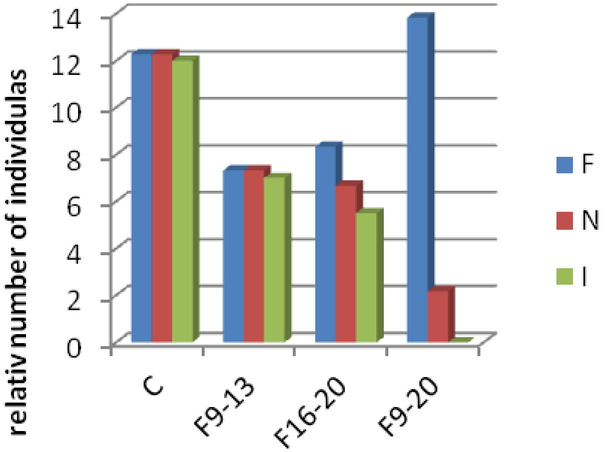
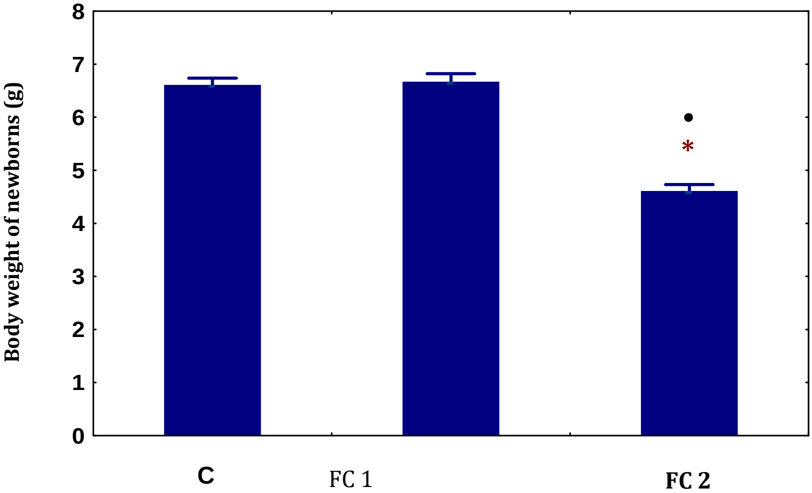


Figure 1. Relative number of foetuses (F), newborns(N) and viable infants (I)

The body weight of offspring FC1 was statistically equal to the control value (Fig. 2). Fluocinolone treatment did not cause long-term weight loss in newborns if the treatment was applied in the first stage og pregnancy. Nine days after treatment end the newborn's weight returned to the control value. In the late stages of pregnancy treated group (FC2) offspring body weight was significantly reduced compared with the control group. This means that glucocorticoid overdose of mothers in the late stage of pregnancy affected embryological development more than in FC1.



***significant alteration compared with the control group (p<0.000001),**
• significant alteration compared with the FC^{2} group(p<0.0022)

Figure 2. Newborns body weight after 24 h to parturition

The body weight of offspring FC1 statistically was equal to the control value. We could interpreted this observation in two ways: firstly the fluocinolone treatment did not cause long-term weight loss in newborns, if the treatment was applied in the first stage of pregnancy. Nine days after treatment end the newborn's weight returned to the control value. Secondly overdose of pregnant females with glucocorticoids did not affect the fetus development. In the late stages of pregnancy treated group FC2 offspring body weight was significantly reduced compared with the control group. This means that glucocorticoid overdose of mothers in the late stage of pregnancy affected embriological development more than in FC1. We could interpreted this observation also in two ways: firstly the glucocorticoid treatment caused weight loss but there was no time to returned to the control value because they were sacrificed, in the second way the glucocorticoid treatment in the late stage of pregnancy determined irreversible modification in fetus development. In these stage neurons develop more sensitive GR receptors, which cause stimulation of adrenergic neurons from brainstem, dopaminergic neurons from hypothalamus down regulated the secretory function of hipotalamo-hipophyseal axis.

The magnitude and nature of long-term effects of glucocorticoids might depend on tissue specific differences in ontogenetic expression of the GR (Bakker *et al.*, 2001). Prenatal glucocorticoid treatment, is often GR specific, prenatal stress involves both MR and GR signalling and catecholamine release. And there is evidence that endogenous maternal and foetal glucocorticoids (and possibly other stress-related hormones) reduce birth weight and have implications for the developing foetal hypothalamic - pituitary – adrenal axis (Teghetoff *et al.,* 2009; Harris and Seckl, 2011).

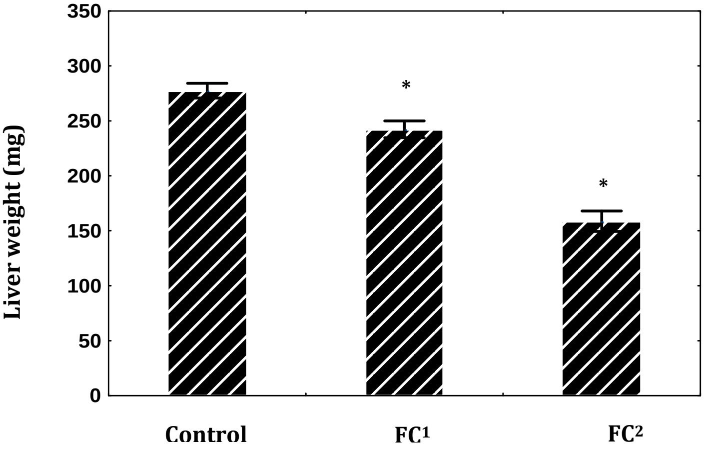
The weight loss of newborns in conditions of glucocortioid excess were in consensus with our previous experimental findings that glucocorticoid treatment overregulated the somatotrope, tireotrope cells morphology and function (Kis and Crăciun, 2005).

Earlier studies with bethametasone have also observed reductions in birth weight of children exposed to prenatal corticosteroid treatment (Blomm *et al.*, 2001, Pesonen *at al.*, 2009). The hypothalamic- pituitary-adrenocortical axis plays an important role in human behavior regulation and, thus, may have a role in explaining the associations of longer duration of betamethasone exposure with impulsivity and slower fetal growth with lower effortful control and higher negative affectivity (Seckl, 2008). There is evidence that endogenous maternal and fetal glucocorticoids reduce birth weight and have implications for the developing fetal hypothalamic-pituitary axis (Main *et al.*, 2006; Harris and Steckl, 2011). The weight loss of newborns in conditions of glucocorticoid excess was in consensus with our previous experimental findings that glucocorticoid treatment overregulated the thyroid gland structure and function (Kis and András, 2017).

After fluocinolone treatment, the effect of glucocorticoids excess significantly reduced the liver weight in FC1 and FC2 groups compared with the control group (Fig. 3). In the early stages of embryonic development (FC1), the liver is less vulnerable to Fluocinolone-N ointment treatment, than in a later stages (FC2). FC^2 group presents a significantly alteration of liver weight compared with the liver weight in FC1 group.

In advanced stages of pregnancy, treated females offspring's are more sensitive to glucocorticoid excess. The placenta of dams in the later stage of pregnancy is more permeable to glucocorticoids. The excess of this hormone passes from the dam's body more easily and has a negative impact on the fetus liver development.

Histological study of the liver in controls and treated groups. Liver sections from control animals (Fig. 4a) displayed normal histological structure, the hepatic lobules have a visible connective tissue around and a relaxed structure. The lobules are roughly hexagonal, and consist of plates of hepatocytes radiating from a central vein. A distinctive component of a lobule is the portal triad (Fig. 4b), which can be found running along each corner of the lobule.



* significant alteration compared with the control group (p<0.004)

Figure 3. The newborn's liver weight

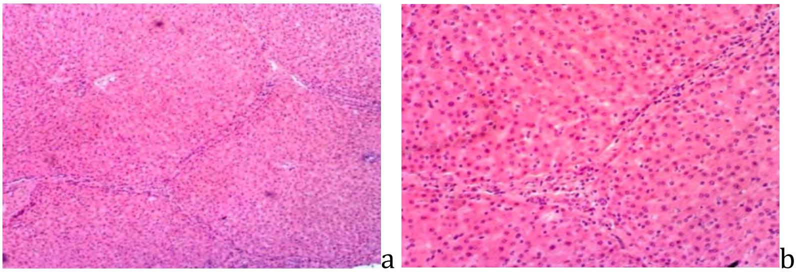


Figure 4. Control newborn's liver, a) Normal histological structure, 4x b) Relaxed structure of hepatic lobules, 20x

Between the hepatocyte plates are liver sinusoids, which are enlarged capillaries through which blood from the hepatic portal vein and hepatic artery enters via the portal triads, then drains to the central vein. In FC1 animals, liver tissue (Fig. 5a) is very similar to the liver tissue of control animals, the hepatic lobules can be well distinguished, but visible lipidosis can be observed in liver cells (Fig. 5b).

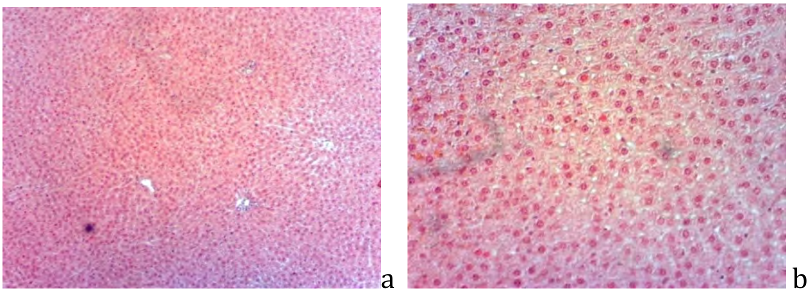


Figure 5. FC1 newborn's liver a) More condensate lobules 4x, b) Lipid droplets in the hepatic cells, 20x

Elevated glucocorticoids promote very-low-density lipoprotein production and secretion as well as triglyceride synthesis via fatty acid synthase and acetyl-CoA carboxylase. Along with the inhibition of free fatty acid oxidation via interference with the activity of Acyl-CoA dehydrogenase, glucocorticoids thereby, trigger hepatic fat accumulation (steatosis) as well as systemically elevated blood lipid levels (Crăciun *et al.,* 1998a, Vegiopoulos and Herzig, 2007, Woods *et al*., 2015). Clinical administration of exogenous glucocorticoids to patients, subjected to organ transplantation or suffering from severe inflammation-related diseases, is known to cause side effects, including deregulation of lipid metabolism and hepatic steatosis (Liu *et al.,* 2016). In the FC2 group, the liver sections of newborns are majorly different from the control animals (Fig. 6a), we can distinguish hepatic lobules, but they are more condensate than the control ones, the connective tissue underlying the lobules disappear definitively. We can see a hyperemia between the liver cells. In the zone of hyperemia the hepatic cells have granulo-vacuolar structure (Fig. 6b).

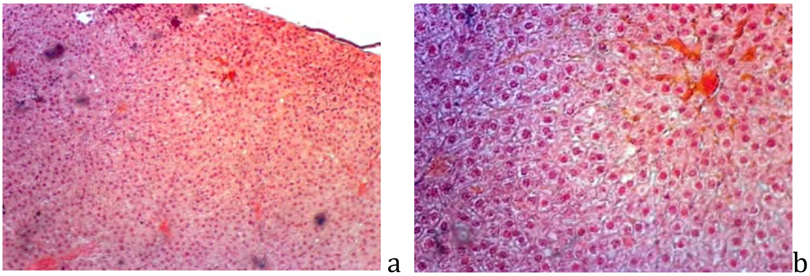


Figure 6. FC2 newborn's liver, 4x a) Condensate hepatic lobules with hyperaemia, b) Granulo-vacuolar structure of the hepatic cells, 20x

The presence of pyknotic cells in this liver zone indicates massive necrosis of liver cells, caused by the treatment with fluocinolone.

These histological changes of the liver tissues in the FC2 group are in consensus of alteration in liver weight. Literature data also show that excessive glucocorticoid level caused by glucocorticoid treatment affects the liver function. A significant proportion of patient with Cushing's syndrome will develop hepatic steatosis (Koliwad *et al*., 2009, Tarantino and Finelli, 2013). Glucocorticoid excess increases the circulating FFA and induce ectopic lipid accumulation in skeletal muscle and liver, all are associated with insulin resistance (Wang *et al.,* 2012).

Conclusions

The dose of Fluocinolone-N ointment used in the present study induced alteration of the rat fetus liver development.

The glucocorticoid excess induced by Fluocinolone-N treatment decrease the body and liver weight in fetuses of dam's treated in different stage of pregnancy.

The more affected animal group is the FC3, these did not survive 24 hours after the cessation.

REFERENCES

Agnew, E.J., Ivy, J.R., Stock, S.J., & Chapman, K.E. (2018). Glucocorticoids, antenatal corticosteroid therapy and fetal heart maturation. *Journal of molecular endocrinology*, *61*(1), R61-R73.

Amatruda, J.M., Danahy, S.A., & Chang, C.L. (1983). The effects of glucocorticoids on insulin-stimulated lipogenesis in primary cultures of rat hepatocytes. *Journal of biochemistry*, *212*(1), 135–41.

Beaudry J.L., Anna M.D., Teich T., Tsushima, R., & Riddell, M.C. (2013). Exogenous glucocorticoids and a high-fat diet cause severe hyperglycemia and hyperinsulinemia and limit islet glucose responsiveness in young male Sprague-Dawley rats. *Endocrinology*, *154*(9), 3197-208.

Bloom, S. L., Sheffield, J. S., Mcintire, D., & Leveno, K. (2001). Antenatal dexamethasone and decreased birth weight. *Obstetric and gynecol*ogy, *97*, 485–490.

Cosío, B.G., Torrego, V., & Adcock, I.M. (2005). Molecular mechanisms of glucocorticoids. *Archivos de bronconeumologia*, *41*(1), 34-41.

Coutinho, A.E., & Chapman, K.E., (2011). The anti-inflammatory and immunosuppressive effects of glucocorticoids, recent developments and mechanistic insights. *Molecular and cellular endocrinology,* 335, 2–13.

Crăciun, C., Ardelean, A., Madar, J., Tarba, C. Şildan, N., Crăciun, V., & Farcaş, T., (1997a). Ultrastructural studies of the secondary effects induced at the level of thymus by topic application of Fluocinolone-acetonid N in prepuberal rats, In: *Current Problems and Technique in Cellular and Molecular Biology,* (eds) Ardelean, A., Crăciun, C., 176-186, Mirton, Timişoara.

Crăciun, C., Frăţilă, S., Ardelean, A., Madar, J., & Crăciun, V. (1999a). Ultrastructural and biochemical studies concerning the action of some topical dermocorticosteroids on prepubertal rat thymuses, before and simultaneously with the adrenoreceptors blocking, In: *Current Problems in Cellular and Molecular Biology* (eds) Ardelean, A., Crăciun, C., 209-248, Risoprint, Cluj-Napoca.

Crăciun, C., Frăţilă, S., Madar, J., Ardelean, A., Crăciun, V., & Ilie, A. (1999b). Effects of some topical dermocorticoids on pubertal rat thymuses after beta-adrenoreceptros blocking, In: *Current Problems in Cellular and Molecular Biology,* (eds) Ardelean, A., Crăciun, C., 249-283, Risoprint, Cluj-Napoca.

Crăciun, C., Kis, E., Sandu, V.D., Paşca, Crăciun, V., & Madar, J. (2001). Comparative studies of the adrenal cortex structure and ultrastructure in mature rats treated with topical dermocorticosteroids, *Studia Universitatis Babeş-Bolyai, Biologia*, *46*(1), 91-98.

Crăciun, C., Kis, E., Sandu, V.D., Paşca, C., Puică, C., Crăciun, V., & Madar, J. (1999c). Comparative studies of the adrenal cortex structure and ultrastructure in prepubertal rats treated with topical dermocorticosteroids, In: Ardelean, A., Crăciun, C., *Current Problems in Cellular and Molecular Biology,* 209-248, Risoprint, Cluj-Napoca.

Crăciun, C., Madar, J., Frăţilă, S., Crăciun, V., Miclăuş, V., Ardelean, A., & Ilyes, I. (1998a). Comparative study of thymus ultrastructure, thymolysis and thymus and blood-serum lipids contents in prepubertal rats treated with topical corticosteroids, In: *Current Problems in Cellular and Molecular Biology* (eds) Crăciun, C., Ardelean, A., 200-217, Risoprint, Cluj-Napoca.

Crăciun, C., Madar, J., Tarba, C., Frăţilă, S., Ardelean, A., Crăciun, V., & Ilyes, I. (1998b). Correlation between ultrastructural thymus modifications, thymolisis, thymus and blood-serum lipids contents in response to epicutaneously applied dermocorticoids in pubertal rats, In: *Current Problems in Cellular and Molecular Biology* (eds) Crăciun, C., Ardelean, A., 218-235, Risoprint, Cluj-Napoca.

Crăciun, C., Tarba, C., Madar, J., Ardelean, A., Şildan, N., Fărcaş, T., & Crăciun, V. (1997b). Study of the secondary effects induced by the topic application of Fluocinolone-acetonid N in the thymus ultrastructure of pubertal rats, In: (eds) Ardelean, A., Crăciun, C. *Current Problems and Technique in Cellular and Molecular Biology,* 187-199, Mirton*,* Timişoara.

Drake A.J., Raubenheimer, P.J., Kerrigan, D., McInnes, K.J., Seckl, J.R., & Walker, B.R. (2010). Prenatal dexamethasone programs expression of genes in liver and adipose tissue and increased hepatic lipid accumulation but not obesity on a high-fat diet. *Endocrinology*, *151***,** 1581-7.

Drake A.J, Tang J.I, & Nyirenda M.J. (2007). Mechanisms underlying the role of glucocorticoids in early life programming of adult disease. *Clinical science*, *113*, 219-232.

Fu, J., Ma, S., Xin Li, An, Sh., Li, T., Guo, K., Lin, M., Qu, W., Wang, Sh., Dong, X., Han, X., Fu, T., Huang, X., Wang, T., & He, S. (2016). Long-term stress with hyperglucocorticoidemia induced hepatic steatosis with VLDL overproduction is dependent on both 5-HT2 receptor and 5-HT synthesis in liver. *International journal of biologica sciences, 12*(2), 219-234.

Gallo L.A., Tran M., Moritz K.M., Mazzuca M.Q., Parry L.J., Westcott, K.T., Jefferies, A.J., McEwen, L.A. C., & Wlodek, M.V. (2012). Cardiorenal and metabolic adaptations during pregnancy in female rats born small: implications for maternal health and second generation fetal growth. *Journal of physiology*, *590*, 617–630.

Harris A., & Seckl, J. (2011). Glucocorticoids, prenatal stress and the programming of disease. *Hormones and behavior*, *59*(3), 279-289.

Harvey, I., Stephenson, E.J., Redd, R.J., Tran, Q.T., Hochberg, I., Qi, N., & Bridges, D. (2017). Glucocorticoid-induced metabolic disturbances are exacerbated in obesity. *Endocrinology*, *159*(6), 2275-2287.

Jeje, S.O., & Raji, Y. (2015). Effects of maternal dexamethasone exposure on hematological indices in the male offspring. *International journal of biological chemical science*. *9*(1), 48-55.

Jeje, S.O., & Raji, Y. (2015). Effects of maternal Dexamethasone exposure during lactation on metabolic imbalance and oxidative stress in the liver of male offsprings of Wistar rats. *Nigerian journal of physiology science*. *30*, 131-137.

Kapoor, A, Dunn, E, Kostak, A, Andrews, M.H, & Mathews, S.G. (2006). Fetal programming of hypothalamo - pituitary adrenal function: prenatal stress and glucocorticoids. *Journal of physiology***,** *572*, 31–44.

Kis, E., & András, P. (2017). Has the Fluocinolon-acetonid N ointment any effect on the kidneys and the thyroid gland structure and function? *Studia Universitatis Babeș-Bolyai Biologia*, *62*(2), 41-52.

Kis, E., Puică, C., Paşca, C., Sandu, V.D., & Madar, I. (1999). Attenuation of the Fluocinolone-acetonid N induced histological alterations of hypothalamic-pituitary-adrenal axis by Propranolol in white Wistar rats. *Studia Universitatis Babeş-Bolyai Biologia*, *44*(1-2), 127-134.

Kis, E., Puică, C., Sandu, V.D., Paşca, C., Crăciun, C., & Madar, I. (2000). Study on the histological alterations of hypothalamic-hypophyseal-adrenal axis induced by Dermovate-cream in Wistar prepubertal rats. *Evolution and adaptation*, *6*, 199-206.

Koliwad, S.K., Kuo, T., Shipp, L.E., Gray, N.E., Backhed, F., So, A.Y.-L., Farese Jr, R.V., & Wang, I.C. (2009). Angiopoietin-like 4 (ANGPTL4, fasting-induced adipose factor) is a direct glucocorticoid receptor target and participates in glucocorticoid-regulated Triglyceride Metabolism. *The journal of biological chemistry*, *284*(38), 25593– 25601.

Laugesen, K., Otto, J., Jrgensen, L., Srensen, H.T., & Petersen, I. (2017). Systemic glucocorticoid use in Denmark: a population-based prevalence study. *British medical journal*, 7:e015237. doi:10.1136/bmjopen-2016-015237.

Liu, Y-F., Wei, J-Y., Shi, M-H., Jiang, H., & Zhou, J. (2016). Glucocorticoid induces hepatic steatosis by inhibiting activating transcription Factor 3 (ATF3)/S100A9 protein signaling in granulocytic myeloid-derived suppressor cells. *The journal of biological chemistry*, *291*(41), 21771–21785.

Main, K.M., Jensen, R.B., Asklund, C., HoiHansen, C.E., & Skakkebaek, N.E. (2006). Low birth weight and male reproductive function. *Hormon researche in pediatrics*, *65*, 116–122.

Moritz K.M., De Matteo, R., Dodic, M., Jefferies, AJ., & Arena, D. (2011). Prenatal glucocorticoid exposure in the sheep alters renal development in utero: implications for adult renal function and blood pressure control. *American Journal of physiology. Regular integrative comparativ physiology*, *301*, R500–509.

Mureşan, E., Gaboreanu, M., Bogdan, A.T., & Baba, A.I. (1974). Tehnici de histologie normală şi patologică. *Ed. Ceres,* Bucureşti.

Newton, R., Leigh, R., & Giembycz, M.A. (2010). Pharmacological strategies for improving the efficacy and therapeutic ratio of glucocorticoids in inflammatory lung diseases. *Pharmacology & Therapeutics 125*, 286–327.

O'Sullivan, L., Cuffe, J.S.M., Paravicini, T.M., Campbell, S., Dickinson, H., Singh, R.R., Gezmish, O., Black, J.M., & Moritz, K.M. (2013). Prenatal exposure to dexamethasone in the mouse alters cardiac growth patterns and increases pulse pressure in aged male offspring. *Public Library of science*, *8*(7), 1-10.

Paragliola, R.M., Papi, G., Pontecorvi, A., & Corsello, S.M. (2017). Treatment with synthetic glucocorticoids and the hypothalamus-pituitary-adrenal axis. *International journal of molecular science*, *18*(10), 2201; doi:10.3390/ijms18102201.

Pesonen, A.K., , Raikkonen, K., Lano, Au., Peltoniemi, Ou., Hallman, M., & Kari, M. (2009). Antenatal betamethasone and fetal growth in prematurely born children: Implications for temperament traits at the age of 2 years. *Pediatrics*, 123(1), 31-37.

Rebeyrol, C., Saint-Criq, V., Guillot, Lo., Riffault, L., Harriet Corvol, H., Chadelat, K., Ray, D., Clement, A., Tabary, Ol., & Le Rouzic, P. (2012). Glucocorticoids reduce inflammation in cystic fibrosis bronchial epithelial cells. *Cellular signaling*, *24*, 1093–1099.

Rose, A.J, Vegiopoulos, A, & Herzig, S. (2010). Role of Glucocorticoids and the glucocorticoid receptor in metabolism: insights from genetic manipulations. *The journal of steroid biochemistry and molecular biology*, *122*, 10-20.

Schäcke, H., Döcke, W-D., & Asadullah, K. (2002). Mechanisms involved in the side effects of glucocorticoids. *Pharmacology & Therapeutics*, *96*, 23–43.

Seckl J. (2008) Glucocorticoids, developmental programming and the risk of affective dysfunction. *Progress in brain researce, 167*, 17–34.

Singh, R.R, Moritz, K.M, Bertram, J.F, & Cullen-McEwen, L.A. (2007). Effects of dexamethasone exposure on rat metanephric development: in vitro and in vivo studies. *The American journal of physiology-Renal physiology*, *293*, F548–F554.

Singh, R.R, Cuffe, J.S.M., & Moritz, K.M. (2012). Short and long term exposure to natural and synthetic glucocorticoids during development. *Proceeding of Australian physiological society*, *43*, 57-69.

Spies, C.M., Strehl, C., van der Goes, M.C., Bijlsma, J.W.J., & Buttgereit, F. (2011). Glucocorticoids. *Best practice & Research clinical rheumatology* 25, 891–900.

Tarantino, G., & Finelli, C. (2013). Pathogenesis of hepatic steatosis: The link between hypercortisolism and non-alcoholic fatty liver disease. *World journal of gastroenterology*, *19*(40), 6735-6743.

Tegethoff, M., Pryse, C., & Meinlschmidt, G. (2009). Effects of intrauterine exposure to synthetic glucocorticoids on fetal, newborn and infant hypothalamic-pituitary-adrenal axis function in human. *Endocrine Reviews*, *30*(7), 753-789.

Vegiopoulos, A., & Herzig, S. (2007). Glucocorticoids, metabolism and metabolic diseases. *Molecular and cellular endocrinology, 275*, 43–61.

Wang, J.C., Gray, N.E., Kuo, T., & Harris, C.A. (2012). Regulation of triglyceride metabolism by glucocorticoid receptor. *Cell&Bioscience* 2(1):19. doi: 10.1186/2045-3701-2-19.

Woods, C.P., Hazlehurst, J.M., Jeremy, W., & Tomlinson, J.W. (2015). Glucocorticoids and non-alcoholic fatty liver disease. *Journal of steroid biochemistry & Molecular biology 154*, 94–103.

Woods, L.L., & Weeks, D.A. (2005). Prenatal programming of adult blood pressure: role of maternal corticosteroids*. American Journal of physiology. Regulatory intergrative and comparative physiology, 289*, R955–R962.

Yang, F., Dai, Y., Min, C., & Li, X. (2018). Neonatal overfeeding induced glucocorticoid overexposure accelerates hepatic lipogenesis in male rats. *Nutrition and metabolism*, https://doi.org/10.1186/s12986-018-0272-0.

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Chlorophyll *a* measurement as an index of phytoplankton bloom and primary production in the Gulf of Guinea, Nigeria, West Africa

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Abstract. Chlorophyll *a* levels of oceanic waters in Nigeria located in the Gulf of Guinea and adjoining water bodies were investigated in this study. Sample collections were done at quarterly intervals from March 2014 to January 2015 from fifty-five sites and spanned over eight coastal states. Integrated water sample for chlorophyll analysis was taken from a depth of 10m to the surface using a silicone hose. Determination of chlorophyll was done by a combination of filtration, extraction, homogenization, centrifugation and spectrophotometry using the trichromatic method. The range of chlorophyll *a* concentration was 27.5 – 1419.35 μgL^{-1} in oceanic waters and 32.79 – 1649.64 μgL^{-1} in adjoining water bodies. Chlorophyll a concentrations varied spatially and temporally, with a general observation of higher concentrations in the south-west oceanic locations in the Bight of Benin axis of the Gulf of Guinea, compared to the south-south oceanic locations in the Bight of Bonny axis of the Gulf of Guinea In the adjoining coastal waters, the Gbaji River in Badagry, Lagos, located in the South-West, had the highest overall total chlorophyll a concentration while the Brass River in Bayelsa, located in the South-South had the lowest overall total chlorophyll *a* concentration throughout the study period. The mean primary production, ranging from 439.85 μgL^{-1} to 1051.89 μgL^{-1}, were included in six categories of <500 μgL^{-1}; 600-699 μgL^{-1}; 700-799 μgL^{-1}; 800-899 μgL^{-1}; 900-999 μgL^{-1} and >1000 μgL^{-1}. Principal component analysis showed strong positive correlations of chlorophyll *a* with total biomass of major phytoplankton groups (Bacillariophyta, Dinophyta and Cyanophyta) and dissolved oxygen levels; negative correlation of chlorophyll *a* with Fe, NO_3, PO_4, TDS, SiO_3 and turbidity.

Keywords: algal bloom, chlorophyll *a*, Gulf of Guinea, Nigeria, primary production.

Introduction

Chlorophyll *a* is a universal photosynthetic pigment found in plants, cyanobacteria and algae. It occurs as the primary photosynthetic pigment in all autotrophic and mixotrophic phytoplankton and its concentration can be used as a proxy for total phytoplankton biomass. Chlorophyll *a* concentration is one of the key indices in the study of the health status of any aquatic ecosystem (Boyer *et al.*, 2009; Jamshidi *et al.*, 2010; Jamshidi and Abu Bakar, 2011). Therefore, the investigation on variations of chlorophyll *a* is very important in the study of water quality and pollution in the aquatic environments. Additionally, chlorophyll is an indicator of productivity, an indicator of algal abundance in aquatic environment, and an effective measure of trophic status of waterbodies (Kadiri 1993). The application of chlorophyll *a* as an index of the productivity and trophic condition of estuaries, coastal and oceanic waters is age long (Boyer *et al.,* 2009). Chlorophyll *a* connotes the instantaneous (standing stock) or net matter of both growth and loss processes of autotrophic organisms in the pelagic ecosystems.

Variability of chlorophyll *a* concentrations determines the ecological conditions of marine systems such as the changes in the physical and chemical characteristics of the environment, aquatic systems health, composition, and ecological status (Rakocevic-Nedovic and Hollert, 2005). Phytoplankton requires an array of macro and micronutrients for growth; the most important being nitrogen and phosphorus. However excess nutrients inputs into coastal waters which mainly arise from anthropogenic sources can cause algae to bloom, resulting in a myriad of effects including toxicity, hypnoxia, increased turbidity, low light intensity, impairment of aquaculture production, degradation of recreational amenities, ecological problems like fish kill~~,~~ and community structure alteration (Anderson *et al.*, 2002).

In recent decades, coastal areas have undergone considerable development and increased utilization and this trend is expected to continue (Neumann *et al.,* 2015). As consequence of this development, there is increased population growth and development which are important drivers of changes in coastal areas through high increased utilization and pollution (Hardy and Patterson 2008; Crossland *et al.,* 2005).

In Nigeria, about 20 million people live along the coastal zone and depend directly on the coastal waters for their nutritional and economic sustenance. Because phytoplankton is the first link in food chain of most aquatic ecosystems, its productivity affects organisms that feed on them directly or indirectly and thus can impart fishery resources which many coastal communities in Nigeria rely on.

Research on chlorophyll *a* in Nigerian waterbodies are not only generally very few, such information on coastal waters is exceptionally very rare. The few studies that exist are mainly restricted to small inland waterbodies and they include reports of (Nwadiaro and Oji, 1985; Kadiri, 1993; Nwankwo *et al.,* 2013; Onyema and Akingbulugbe, 2017; Ayeni and Adesalu, 2018). Additionally, no information on chlorophyll *a* exists in the Atlantic Ocean of Nigeria, coupled with the lack of comprehensive study in the entire coast of Nigeria. Therefore this study represents the first comprehensive study of this nature in the entire coast, spanning the Bight of Benin to the Bight of Bonny, Gulf of Guinea.

The objectives of the study was to investigate the concentrations, seasonal and spatial profile of chlorophyll *a* in the Atlantic oceanic, Gulf of Guinea and adjoining waters of coastal waters of Nigeria and establish relationship between environmental variables and chlorophyll *a*.

Materials and methods

The study was carried out in the Nigerian Coast-Atlantic Ocean, from the Bight of Bonny to Bight of Benin with 55 sampling sites located in Cross River, Akwa-Ibom, Rivers, Bayelsa, Delta, Ondo, Ogun, Lekki, Bar Beach, and Badagry (Lagos) States (Fig. 1). Geographically, the Nigerian costal states are divided into two regions namely: South-South region and the South-West region. The South-South consists of five states namely: Cross River, Akwa Ibom, Rivers, Bayelsa and Delta. Three stations were sampled in each of these locations and the mean value was taken.

There are two main seasons in the area, namely the rainy (wet) season spanning from May to October and dry season which extends from November to April. The coastal area is humid with a mean average temperature of 24°C to 32°C and an average annual rainfall ranging between 1,500 m to 4,000 m (Kuruk, 2004).

Sampling was done in March, 2014, July 2014, October, 2014 and January, 2015, corresponding to dry-wet, wet, wet-dry and dry seasons respectively.

Integrated water sample of the water column was taken from a depth of 10 m to the surface using a silicone hose with an inner diameter of 2.5 mm and provided with a heavy-weight, into clean 1litre sample containers. The samples were immediately stored in ice-chests and transported to the Limnology and Algology laboratory of the University of Benin, Nigeria within 6 hours of collection. Determination of chlorophyll a concentration was done according to the trichromatic method of Vollenweider (1974), using the combined procedures of filtration, extraction, homogenization, centrifugation and spectrophotometry.

Two-Way Analysis of Variance (ANOVA) and Principal Component Analysis (PCA) was done using Paleontological Statistics *Software* Package (PAST), Version 3.26 (Ogbeibu, 2005). Principal Component Analysis was done to determine environmental and biological relationships with chlorophyll *a* concentrations while ANOVA was employed to test differences between means of chlorophyll concentrations in the study area.

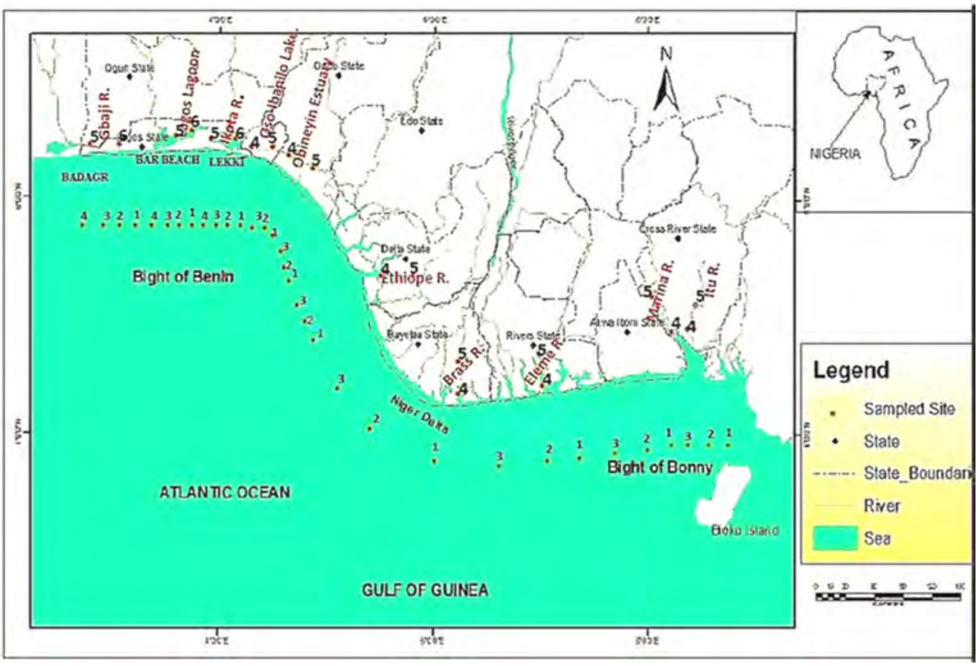

Figure 1. Coastal map of Nigeria showing the study area

Results and discussion

The total chlorophyll *a* concentrations of oceanic waters of Nigeria is presented in figure 2. The concentration values ranged from 27.5 – 1419.35 µgL^{-1}, with the minimum recorded in Ogun in July and maximum obtained in Lekki also in July. Chlorophyll *a* exhibited significant spatial and temporal variations. Generally, the chlorophyll *a* levels of July were the lowest and those of January were the highest. A deviation from this trend was noted where minimum and maximum values were recorded respectively in March and July (Lekki, Bar Beach and Badagry areas), in March and October (Ondo); a maximum in July (Lekki, Lagos) and minimum value obtained in October in Bayelsa oceanic water. Spatial comparison revealed overall that Lekki had the highest and Bayelsa had the lowest annual total chlorophyll *a* concentration throughout the entire period.

A horizontal gradient was observed. Generally, the South-West oceanic areas were higher in chlorophyll *a* than the South-Southern ones.

Statistically, the results for the two-way ANOVA indicated a significant main effect for season, F (3, 132) = 52.341, $p < 0.001$ and a significant main effect for sampling locations, F (9, 132) = 7.202, $p < 0.001$. Additionally, the results show a significant interaction between season and location, F (27,132) = 9.873, $p < 0.001$, an indication that seasonal variations in chlorophyll *a* were dependent upon the spatial variations in chlorophyll *a* in the oceanic waters of the Nigerian coast. Also, 83.8 % (R^2= 0.838) of the total variance of chlorophyll *a* was attributed to the interaction of season and location of sampling. Post hoc analyses using *DMR* test for seasonal variation of chlorophyll *a* showed that the mean value for January was highest and differed significantly from October, July and March seasons. Post hoc analyses using *DMR* test for determination of spatial variation in chlorophyll *a* concentrations showed that mean chlorophyll *a* values were in a descending order of Lekki >Badagry> Ondo> Bar Beach >Ogun> Akwa Ibom> Rivers > Cross River >Delta > Bayelsa.

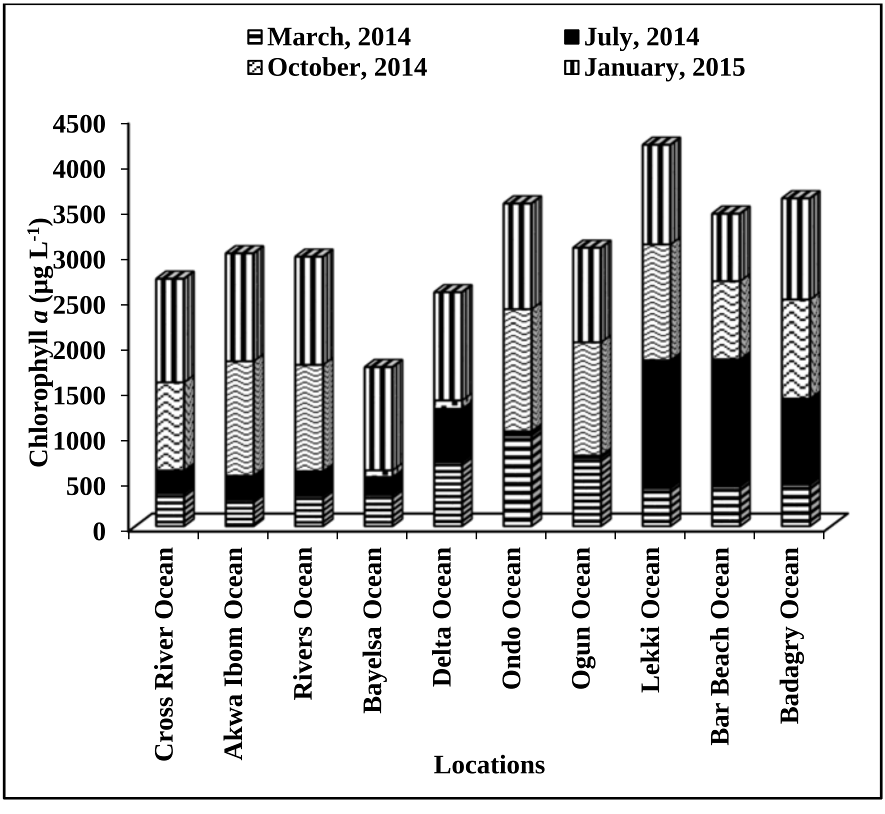

Figure 2. Total seasonal chlorophyll *a* concentration of Atlantic oceanic waters of Nigeria

The total chlorophyll *a* values of adjoining water bodies of oceanic waters of Nigeria is represented in figure 3. The range was 32.79 – 1647.64 μgL^{-1}, with minimum recorded in July in Oso-Ibanilo Lake and maximum obtained in the Gbaji River in July. The trend in adjoining rivers was more variable than in the oceans. The minimum and maximum chlorophyll values were recorded in only 50% of the locations in July and October, respectively. Divergence from this trend was observed in the Itu, Ethiope and Brass Rivers where maximum chlorphyll *a* concentration was recorded in January while Lagos Lagoon and the Gbaji River had maximum chlorophyll *a* values in July. In contrast with the general trend, minimum chlorophyll *a* values were recorded in March for the Ikota River, Lagos Lagoon and the Gbaji River while the Brass and Ethiope Rivers had minimum chlorophyll *a* cocentrations in October.

Spatial comparison revealed that the Gbaji River in Badagry, Lagos State had the highest overall (total) chlorophyll *a* while the Brass River in Bayelsa recorded the lowest overall (total) chlorophyll *a* throughout the entire period. Generally, chlorophyll levels in the South-West Rivers were higher than the South-Southern ones.

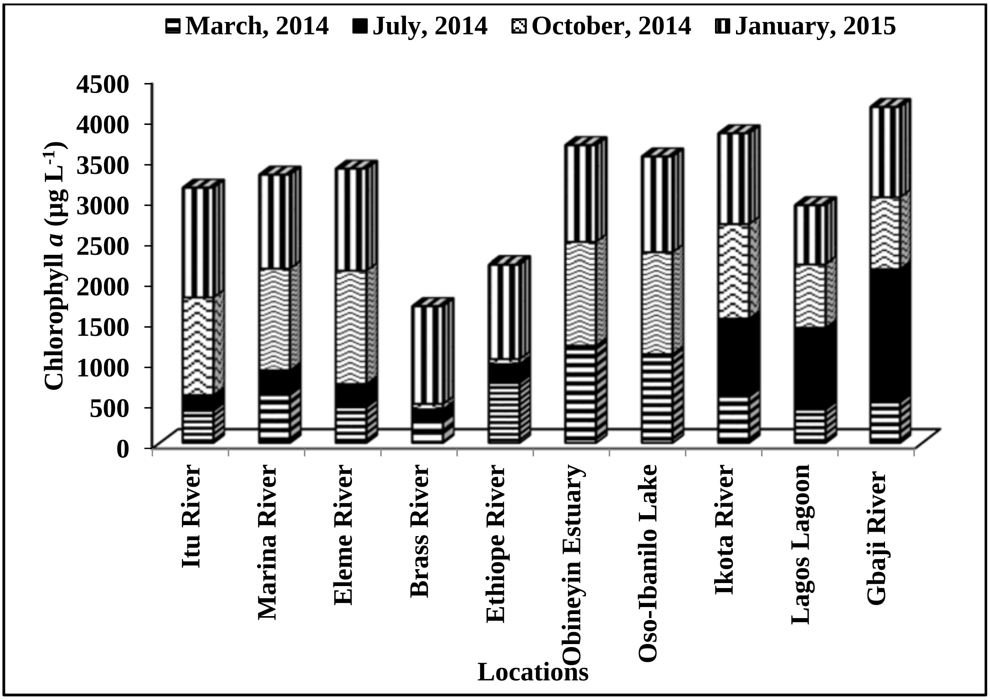

Figure 3. Total Seasonal Chlorophyll *a* concentration of adjoining coastal waterbodies of Nigeria

Similarly, for the adjoining water bodies, the results for the two-way ANOVA indicated a significant main season effect, $F\ (3, 80) = 36.685$, $p > 0.001$ and a significant main effect for location, $F\ (9, 80) = 60.724$, $p > 0.001$. Furthermore, the results showed a significant interaction between season and location, $F(27, 80) = 9.177$, $p > 0.008$. It could be an indication that seasonal variations in chlorophyll *a* of the water samples were dependent upon the spatial variations in chlorophyll *a* in the various adjoining rivers from South-West to South-South locations. Also, 95.8 % of the total variance in chlorophyll *a* was attributed to the interaction of season and location. Post hoc analyses using *DMR* test for seasonal variation of chlorophyll *a* showed that the differences in mean values were in the following order January >March>July and October.

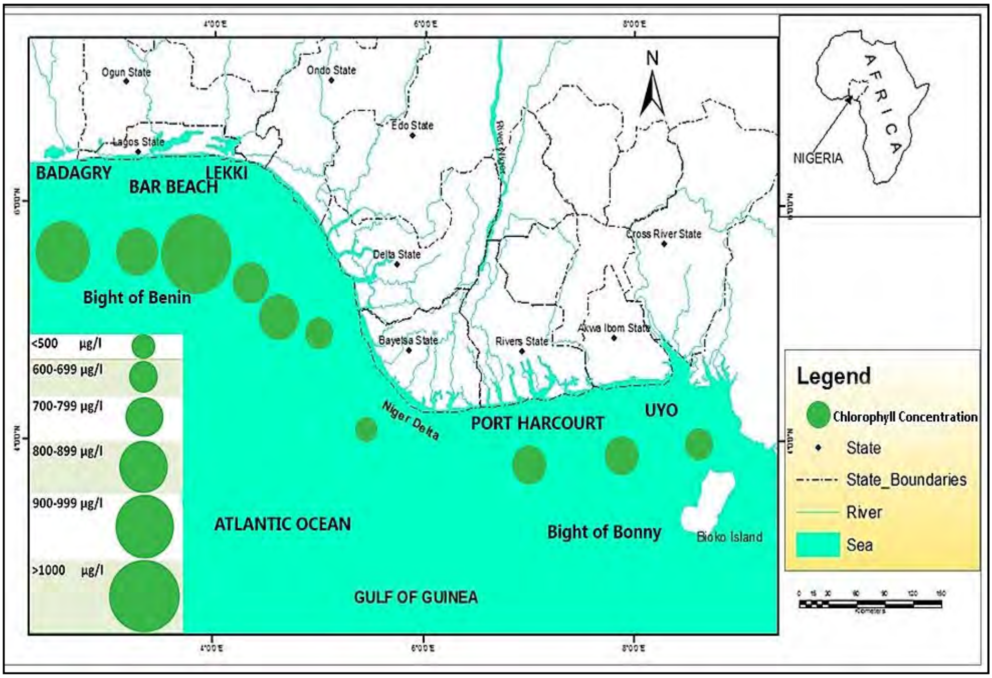

Figure 4. Mean primary production of Atlantic Ocean, Gulf of Guinea, Nigeria

Primary production as a function of mean chlorophyll *a* concentration is depicted in figure 4. There was considerable regional variability in the mean primary production. In this study, mean chlorophyll *a* concentrations varied between 439.85μg L^{-1} and 1051.89μg L^{-1}, and six categories were observed: <500μg L^{-1}; 600-699 μg L^{-1}; 700-799 μg L^{-1}; 800-899 μg L^{-1}; 900-999 μg L^{-1} and > 1000μg L^{-1}.

Spatially, highest values of primary production as particulate chlorophyll *a* were associated with Lekki and Badagry axis of Lagos, falling within the range of 900 μgL^{-1} to >1000 μgL^{-1}, whereas the lowest was recorded in Bayelsa State (<500 μgL^{-1}). In general primary production was higher and more variable in South-West locations (600 μgL^{-1} - >1000 μgL^{-1}) compared to the South-South locations where mean primary production was <500 μgL^{-1} to 799 μgL^{-1}.

Relationship between chlorophyll *a* concentration and environmental variables

Principal Component Analysis was performed using 17 environmental and biological variables. The results as shown in figure 5 illustrate the PCA biplot of some divisions of algae (Bacillariophyta, Dinophyta and Cyanophyta) and environmental variables in the Nigerian coastal waters. The first two components accounted for approximately 40% of the total variance and displayed moderate phytoplankton-environmental variables correlation. Bacillariophyta, Dinophyta,

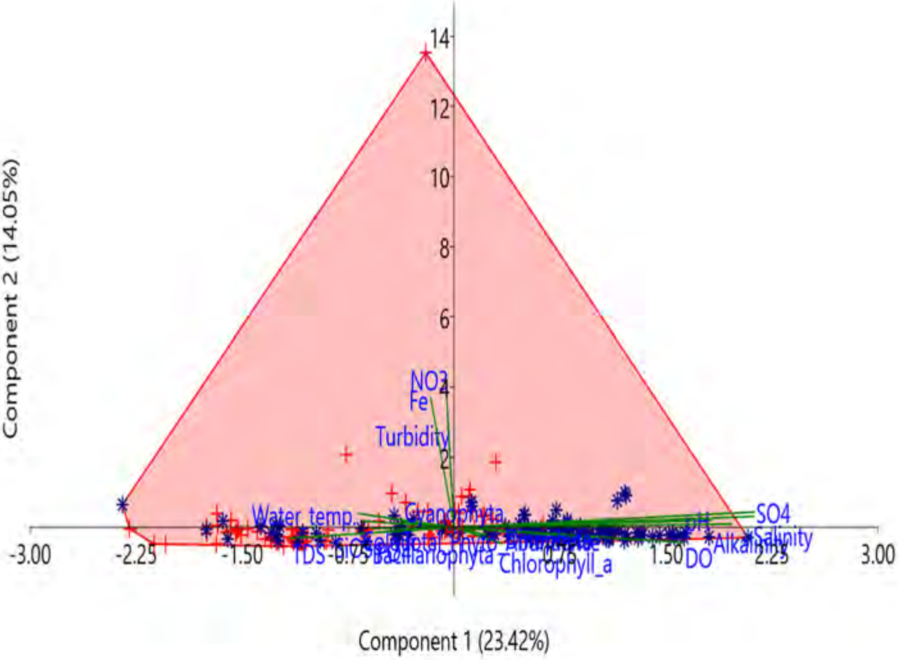

Figure 5. PCA biplot of environmental variables, chlorophyll *a* and phytoplankton divisions in the Nigerian coast.

Cyanophyta, chlorophyll *a*, pH, alkalinity, salinity, dissolved oxygen (DO), and SO_4 correlated and contributed positively to principal component 1 (PC1) while turbidity, water temperature, colour, Fe, NO_3, PO_4, TDS and SiO_3 correlated and contributed negatively to PC1. However, Fe, NO_3 and turbidity contributed and correlated positively to principal component 2 (PC2). Overall, the result revealed that the phytoplankton present in sites with low turbidity in the water column and nutrients had little or no effect in the distribution of phytoplankton communites. Additionally, DO, chlorophyll *a* and the phytoplankton biomass of Cyanophyta, Dinophyta and Bacillariophyta correlated positively. Furthermore, the South-West region of the Nigerian coast had high phytoplankton density when compared to the South-South region.

Chlorophyll concentration is often used as an indicator of algal biomass in aquatic ecosystems. In this study, highest chlorophyll *a* concentrations were recorded in South-West locations where higher phytoplankton biomass of major phytoplankton groups was also recorded. As a measure of trophic state, chlorophyll *a* levels in this study were high based on Horne and Goldman's (1994) trophic categorization of chlorophyll *a* of $<8\ \mu gL^{-1}$ for oligotrophic, 8-25 μgL^{-1} for mesotrophic, 26-75 μgL^{-1} for eutrophic and $>75\ \mu gL^{-1}$ for hypereutrophic waters. Following this classification, all the locations in the Gulf of Guinea, Nigeria studied are considered hypereutrophic. High chlorophyll *a* concentration is considered to signal eutrophication and as an indication of areas affected by eutrophication (HELCOM 2009). Also, employing the criteria of Havens (1994) which stipulates that algal bloom corresponds to 40 $\mu g\ L^{-1}$ of chlorophyll *a*, all the locations studied produced algal bloom. Chlorophyll *a* concentrations can act as an indicator of phytoplankton abundance and biomass in the coastal waters. The results obtained in the Atlantic ocean, Nigeria are quite higher than those reported elsewhere (maximum chlorophyll *a* level of 2 μgL^{-1}) in the Gulf of Guinea by Lefèvre (2009) and Caspian Sea (1.3–2.1 mg m^{-3} by Jamshidi and AbuBakar (2011) and maximal and surface chlorophyll *a* concentrations are 0.15-2.5 and 0.015-2.0 mg m^{-3} respectively, off North West African upwelling areas (Agusti and Duarte, 1999).

Chlorophyll *a*, which is a measure of phytoplankton population density, is influenced by a multitude of intrinsic and extrinsic factors such as internal and external nutrient fluxes, grazing etc. The negative correlation of, water temperature, colour, Fe, NO_3, PO_4, and SiO_3 to chlorophyll *a* observed in this study has been reported by some authors (Gnanamorthy *et al*., 2013; Chen *et al*., 2018). Chlorophyll *a* concentrations in aquatic environments is affected by the seasonal and spatial variations of the sea surface temperature (Jouanno *et al.,* 2011); trace metals like iron (Mills *et al.,* 2004), hydrographic features such as residence time and upwelling (Wieters *et al.,* 2003), nutrients load, bioavailability and form (Heisler *et al.,* 2008), pH (Hinga 2002), grazing rates and selectivity (Irigoien *et al.,* 2005), salinity (Chan and Hamilton, 2001) and light availability (Spilling *et al.*, 2015). The above factors interact in a very complex manner at influencing phytoplankton and chlorophyll *a*. Even when physical and chemical conditions are conducive, intrinsic factors like certain anatomical or metabolic adaptations confer competitive advantage on some phytoplankton species, enabling proliferations and bloom formation. The differential chlorophyll levels in the oceanic waters and adjoining rivers is ascribable to the meso-and macro-tidal systems with strong tidal flushing and micro-tidal coastal systems with limited flushing (Canavate *et al.,* 2015). The observation of the negative correlation between water temperature and chlorophyll *a* in this study negates the findings of Grodsky *et al*. (2008) of the congruence between temperature and chlorophyll *a* trends in the Gulf of Guinea, suggesting that a common process of South Equatorial Current (SEC), and of the Guinea Current takes cool and nutrient-rich waters to the surface (Jouanno *et al.,* 2011).

Conclusions

The study investigated the chlorophyll *a* levels in the Bights of Bonny and Benin in the Gulf of Guinea, Nigeria and some adjoining coastal waterbodies. Results revealed spatio-temporal variation and influence of nutrients. The spatio-temporal distribution of pigment profiles consequent on phytoplankton groups can influence aquatic ecosystem structure and functioning. Chlorophyll *a* is sensitive to ecosystem drivers (stressors, particularly nutrient load) and its measurement is useful for the screening, monitoring, early detection and warning of harmful algal bloom. Routine monitoring of chlorophyll *a* levels can thus provide very useful information about the productivity, water quality, nutrient load and fisheries potential of Nigerian coastal waters. In view of the dynamics of the Gulf of Guinea region, coupled with scarcity of measurements of this nature in this region, the present study has provided a pivot for subsequent future studies in chlorophyll *a,* production and algal blooms in the area.

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REFERENCES

Agusti, S., & Duarte, C. (1999). Phytoplankton chlorophyll *a* distribution and water column stability in the central Atlantic Ocean. *Oceanologia* 22(2)*,* 193–203.

Anderson, D.M., Glibert, P.M., & Burkholder, J.M. (2002). Harmful algal blooms and eutrophication: nutrient sources, composition, and consequences. *Estuaries Coasts* 25 (4), 704–726.

Ayeni, A.O., & Adesalu, T.A. (2018). Validating chlorophyll a concentrations in the Lagos Lagoon using remote sensing extractions and laboratory fluoremetric methods. *MethodsX* **5**, 1204–1212.

Boyer, J.N., Kelble, C.R., Ortner, P.P, & Rudnick, D.T. (2009). Phytoplankton bloom status: chlorophyll *a* biomass as an indicator of water quality condition in the southern estuaries of Florida, USA. *Ecol. Indic.* **9**, 56–67.

Canavate, J., Perez-Gavilan, C., Mazuelos, N., & Machado, M. (2015). Flushing-related changes of phytoplankton seasonal assemblages in marsh ponds of the warm temperate Guadalquivir river estuary (SW Spain). *Hydrobiologia,* 744, 15–33.

Chan, T.U., & Hamilton, D.P. (2001). The effect of freshwater flow on the succession and biomass of phytoplankton in a seasonal estuary. *Mar. Freshwater Res*. 52, 869–884.

Chen, R., Ju, M., Chu, C., Jing, W., & Wang, Y. (2018). Identification and quantification of physicochemical parameters influencing chlorophyll-*a* concentrations through combined principal component analysis and factor analysis: a case study of the Yuqiao Reservoir in China. *Sustainability*,10, 1–15.

Crossland, C., Baird, D., Ducrotoy, J.P., Lineboom, H., Buddemeier, R., & Dennison, W. (2005). The coastal zone - a domain of global interactions. In: *Coastal Fluxes in the Anthropocene,* Crossland, J., Kremer, H., Lineboom, H., Tissier, M.A., (eds). Springer, Berlin, Heidelberg pp. 1–37.

Gnanamorthy, P., Sahu, S.K., & Prabu, A.V. (2013). Multivariate analysis in relation to physico-chemical parameters disparity in Parangipettai waters, southeast coast of India. *Asian J. Biol. Sci.* 6(1), 1 – 20.

Grodsky, S.A., Carton, J.A, & McClain, C.R. (2008). Variability of upwelling and chlorophyll in the equatorial Atlantic. *Geophys. Res. Lett.* 35, 1–6

Haven, K.E. (1994). Relationships of annual chlorophyll *a* means, maxima and algal bloom frequencies in a shallow eutrophic lake (Lake Okeechobee, Florida, USA). *Lake Reserv. Manage*. 10, 133–138.

HELCOM (2009). Eutrophication in the Baltic Sea – an integrated thematic assessment of the effects of nutrient enrichment and eutrophication in the Baltic Sea region. Balt Sea Environ Proc. No. 115 Helsinki Commission. pp. 148.

Heisler, J., Glibert, P.M., Burkholder, J.M., Anderson, D.M., Cochlan, W., Dennison, W.C., Dortch, Q., Gobler, C.J., Heil., C.A., Humphries, E., Lewitus, A., Magnien, R., Marshall, H.G., Sellner, K., Stockwell, DA., Stoecker, D.K., & Suddleson, M. (2008). Eutrophication and harmful algal blooms: a scientific consensus. *Harmful Algae* **8**, 3–13.

Hinga, K.R. (2002). Effects of pH on coastal marine phytoplankton. *Mar. Ecol. Prog. Ser*. 238, 281–300.

Horne, A.J., & Goldman, C.R. (1994). *Limnology* (2nd Edition). McGraw Hill, New York, pp. 464.

Irigoien, X., Flynn, K.J., & Harris, R.P. (2010). Phytoplankton blooms: a 'loophole' in microzooplankton grazing impact? *J. Plankton Res.* 27(4), 313–321.

Jamshidi, S., & AbuBakar, N.B. (2011). A study on distribution of chlorophyll-*a* in the coastal waters of Anzali Port, south Caspian Sea. *Ocean Sci. Discuss.* **8**, 435–451.

Jamshidi, S., Abu Bakar, N.B., & Yousefi, M. (2010). Concentration of chlorophyll-a in the coastal waters of Rudsar, *Res. J. Environ. Sci.* 10**,** 132–138.

Jouanno, J., Marin, F.D.R., Du Penhoat, Y., Molines, J.M., & Sheinbaum, J. (2011). Seasonal modes of surface cooling in the Gulf of Guinea. *J. Phys. Oceanogr.* 41, 1408–1416.

Kadiri, M.O. (1993). Seasonal changes in the phytoplankton biomass of a shallow tropical reservoir. *Niger. J. Bot*. **6**, 167–175.

Kuruk, P. (2004). Customary Water Laws and Practices: Nigeria [Accessed 18 April 2018] http://www.fao.org/ legal/adviserv/FOA?UCNCS. Nigeria.pdf.

Lefèvre, N. (2009). Low CO_2 concentrations in the Gulf of Guinea during the upwelling season in 2006. *Mar. Chem*. 113, 93–101.

Mills, M.M., Ridame, C., Davey, M., La Roche, J., & Geider, R.J. (2004). Iron and phosphorus co-limit nitrogen fixation in the eastern tropical North Atlantic. *Nature* 429, 292–294.

Neumann, B., Vafeidis, A.T., Zimmermann, J., & Nicholls, R.J. (2015). Future coastal population growth and exposure to sea-level rise and coastal flooding – a global assessment. *PLoS ONE* 10(3), e0118571.

Nwadiaro, C.S., & Oji, E.O. (1985). Phytoplankton productivity and chlorophyll *a* concentration of Oguta Lake in Southeastern Nigeria. *Hydrobiol. Bull.* 12(2), 123 – 131.

Nwankwo, D.I., Adesalu, T.A., Amako, C.C., Akagha, S.C., & Keyede, J.D. (2013). Temporal variations in water chemistry and chlorophyll a at the Tomaro creek Lagos, Nigeria. J. Ecol. Nat. Environ. 5(7), 144 - 151.

Ogbeibu, E.A. (2005). Biostatistics: a Practical Approach to Research and Data Handling. Mindex Publishing Ltd., Benin City, Nigeria 264 p.

Onyema, I.C., & Akingbulugbe, G.E. (2017). Water chemistry and chlorophyll-a variations in a perturbed mangrove ecosystem in Lagos. *N. J. Fish. Aqua.* 5(1), 50 – 56.

Patterson, M., & Hardy, D. (2008). Drivers of change and their oceanic-coastal ecological impacts. In: *Ecological economics of the oceans and coasts* Patterson, M., Glavovic, B.C. (eds.). Edward Elgar Publishing, Cheltenham UK. pp. 187-215.

Rakocevic-Nedovic, J., & Hollert, H. (2005). Phytoplankton community and chlorophyll a as trophic state indices of Lake Skadar (Montenegro, Balkan). *Env. Sci. Poll. Res. Int*. 12(3), 146–152.

Spilling, K., Ylöstalo, P., Simis, S., & Seppälä, J. (2015). Interaction effects of light, temperature and nutrient limitations (N, P and Si) on growth, stoichiometry and photosynthetic parameters of the cold-water diatom *Chaetoceros wighamii*. *PLoS ONE* 10(5), e0126308.

Wieters, E.A., Kaplan, D.M., Navarrete, S.A., Sotomayor, A., Largier, J., Nielsen, K.J., & Veliz, F. (2003). Alongshore and temporal variability in chlorophyll a concentration in Chilean nearshore waters. *Mar. Ecol. Prog. Ser*. 249:93–105.