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All authors are responsible for submitting manuscripts in comprehensible US or UK English and ensuring scientific accuracy.

Original picture on front cover: European green lizard *Lacerta viridis*, adult male with nuptial blue throat © Cristian Mihai

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The influence of osmo-priming on germination parameters of *Telfairia occidentalis* Hook f. (fluted pumpkin)

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Abstract. Fluted pumpkin (*Telfairia occidentalis* Hook F.) seed germination proceeds once adequate temperature and moisture content have been reached and dormancy is broken. Seed priming is a technique in which seeds are hydrated (control hydration) and dried to their original moisture content while preventing radicle emergence. The study aims to investigate the consequence of osmo-priming on the germination parameters of fluted pumpkin (*Telfairia occidentalis*). Laboratory studies were carried out using 36 seeds of fluted pumpkin which was osmoprimed with NaCl (0.05, 0.1 and 1 millimolar), $MgCl_2$ (0.05, 0.1 and 1 millimolar) and KCl (0.05, 0.1 and 1 millimolar). Data on germination percentage, growth parameters, and chlorophyll content showed a significant difference in germination percentages between osmoprimed seeds and control seeds. The time of germination in osmoprimed seeds was significantly reduced when compared with control. The germination rate index (64%) was different between controls and osmoprimed seeds with 0.05 millimolar KCl and 0.10 millimolar $MgCl_2$ (). The growth parameters of seedlings 15 days after sowing showed significant increase in the number of leaves, number of root branches and chlorophyll content. Seed osmopriming may be a sustainable method to increase crop production in *T.ccidentalis*.

Keywords: Fluted pumpkin, seed priming, germination, seedling, vegetable.

Introduction

Germination of fluted pumpkin (*Telfairia occidentalis* Hook F.) begins when the temperature and moisture content are appropriate and the dormancy is broken. For embryo growth, seeds must first absorb water to start their metabolism and boost their respiration (Fincer, 1989). Three phases can be differentiated during seed germination/sprouting, all of which are connected to water intake (Bewley *et al*., 2013). Rapid water uptake p(hase I) starts the metabolic process, which includes DNA and mitochondrial repair as well as protein synthesis using existing mRNA (Bewley *et al*., 2013). Further water uptake is limited during phase II because the grain's water potential is nearly equal to that of its surroundings. The activation or lag phase is another name for this stage. Major metabolic changes, such as the manufacture of hydrolytic enzymes (such as-amylase, endoxylanase, and phytase) and other activities required for embryo growth, occur during this phase. A second rapid water uptake occurs in phase III (Bewley *et al*., 2013; Nonogaki *et al*., 2010). The appearance of radicle is known as germination (Nonogaki *et al*., 2010) and is also known as sprouting (Lemmens *et al*., 2019). Plant hormones such as abscisic acid (ABA), gibberellic acid (GA3), ethylene, auxins, cytokinins, and brassinosteroids influence germination and sprouting (Nonogaki *et al*., 2010; Miransari and Smith, 2014). The expression of important genes regulates their synthesis and activity. ABA and GA3 are the most significant plant hormones for seed germination. They are created in the embryo and then transferred to the aleurone (Nonogaki *et al*., 2010; Ma *et al*., 2017). As response to GA3 aleurone cells produce and secrete hydrolytic enzymes in order to mobilize grain reserves and germination, whereas ABA inhibits these processes (Ma *et al*., 2017).

As previously noted, phase II of the germination process is critical due to the significant metabolic changes that occur there (Di and Barbanti, 2012). Seed priming, a pre-sowing therapy, has an impact on this period. To begin metabolic activity, seeds are soaked in an osmotic solution and then dried to their original moisture level before sprouting/sowing (Di and Barbanti, 2012).

Hydro-priming and osmo-priming are two common priming methods (Ventura *et al.*, 2012). Grain imbibitions with water during a limited time period (714 h) is referred to as hydro-priming. It starts the above-mentioned phase II metabolism without putting the seeds under too much stress. The emergence of the radicle (phase III) is prevented during the process by drying the seeds to their original moisture level (Ventura *et al*., 2012). Osmo-priming is a regulated procedure that restores 10 to 20% of complete hydration. Inducing abiotic stress conditions, in phase II physiological and biochemical activities are sustained. The applied negative water potential prevents the development of the radicle

(phase III) during the procedure (Bewley *et al*., 2013; Di and Barbanti, 2012; Rehman *et al*., 2010; Khan *et al*., 2014). Higher water potentials (0.3 to 1.5 MPa) and short priming times (12 h to 2 days) are best for osmo-priming (Rehman *et al*., 2010; Ghiyasi *et al*., 2008; Salehzadeh *et al*., 2009; Yari *et al*., 2010). Oxidative processes occur when using more negative water potentials and/or longer priming times (Basra *et al*., 2005). High molecular weight polyethylene glycol is the most commonly utilized solute for osmo-priming (PEG); it causes high osmotic pressure, which alters the availability of water in the germination medium (Hameed *et al*., 2014). As a result, the amount of cellular damage caused by rapid water entry into a seed is minimized (Ventura *et al*., 2012).

The seeds are soaked in aerated osmotic solutions containing potassium nitrate, potassium phosphate, potassium chloride salts, or polyethylene glycol (PEG) with varying water potentials and time lengths in osmopriming, the most popular priming method. The applied solutes are usually dissolved in water at a concentration that allows the seeds to drink a small amount of water to start the pre-germination metabolism. Before the major root or radicle emerges, the primed seeds are extracted from the osmoticum (Paparella *et al*., 2005).

The use of PEG during priming minimizes toxicity because it is not taken up by seeds due to its high molecular weight (Di and Barbanti 2012). High PEG concentrations, on the other hand, result in high viscosity, which inhibits oxygen transport and requires efficient aeration during priming (Paparella *et al*., 2015). Several factors are thought to play a role in priming beneficial effects on sprouting. First, the increased water content is critical for activating enzymes involved in embryo growth and starchy endosperm mining (Mirza *et al*., 2015). Second, priming activates biochemical cell repair processes, increases RNA content, and improves DNA replication (Di and Barbanti 2012; Khan *et al*., 2014; Salehzadeh *et al*., 2009; Mirza *et al*., 2015). Third, increased activity of antioxidant enzymes such as superoxide dismutase, catalase, and glutathione reductase seems to improve the defense system after priming (Di and Barbanti 2012; Zhang *et al*., 2015). Primed crops absorb water and recover grain metabolism more quickly after sowing than non-primed grains (Hameed *et al*., 2014). As a result, seed priming improves germination rates (Brocklehurst and Dearman, 1983, Hardegree 1994; Toklu *et al*., 2015), emergence uniformity (Brocklehurst and Dearman, 1983; Toklu *et al*., 2015), yield (Toklu *et al*., 2015), and seedling resistance to unfavourable environmental conditions (Hardegree, 1994).

Following water intake (imbibition) in phase I, adequate water is required in metabolic processes to repair cellular components lost during the maturation drying period. Although various trials have been undertaken to explore the effects of pretreatment on seed germination performance of commercially important

crops. The present study was carried out to evaluate the effects of NaCl, $MgCl_2$ and KCl solutions as osmopriming reagents on seed germination and the seed parameters such as number of leaves, length of leaf, number of roots, branches per root, and length of nodes will be determined, Also the chlorophyll content will be determined. This study therefore seeks to address the extent osmopriming enhances germinability and germination parameters of the test plant.

Farmers of fluted pumpkins in tropical West Africa appear to be facing a huge difficulty in overcoming the problem of delayed germination. Osmopriming aids in dormancy breaking, improved germination and vigour, homogeneity of germination, and better development and early flowering (Mirmazloum *et al.,* 2020). It also makes plants more tolerant of abiotic stress like dehydration. To what extent can osmopriming enhance germinability and germination characteristics of the test plant? These are the questions that this research aims to solve.

Materials and methods

Plant materials

Seeds of fluted pumpkin (*Telfairia occidentalis* Hook F.) were obtained from local farmers in Benin City, Edo State, Nigeria.

Seed osmopriming treatment

A total of 36 seeds of fluted pumpkin were treated. Three priming media at varied concentration levels were used as described below: NaCl (0.05, 0.1 and 1 mM, millimolar), $MgCl_2$ (0.05, 0.1 and 1 mM) and KCl (0.05, 0.1 and 1 mM). Osmoproming was done by soaking the seeds inside a plastic bottle containing the three media for 3 hours at room temperature, on the laboratory bench. The unprimed seeds were soaked in tap water for 3 hours (controls). The primed and unprimed seeds were planted right away in plastic bottles with the top half cut off and filled with sandy loamy soil. The plastics were then laid out in block design replicated three times. The plants were watered daily.

Germination analysis

Seed germination analysis was carried out for a period of two weeks. Data was collected on germination from day 1 to day 4 after sowing. A seed is considered germinated when the radicle emerges through the seed coat. Germination percentage was computed using the following formula:

$$\text{Germination percentage} = \frac{\text{Total number of seeds germinated}}{\text{Total number of seeds in all replicates}} \times 100$$

Determination of growth parameters

Analyzed growth parameters were: number of leaves , length of leaf, number of roots, branches per root, and length of nodes. Data was collected for a period of 15 days after sowing. To determine plant moisture content (%), difference between plant fresh and dry weight was expressed as a percentage of the fresh weight. Plants were initially dried in an electric laboratory cabinet drying oven (Model - CAH-550, manufactured by Sepor, Wilmington) at 85^{o}C for 4 hours. Chlorophyll content was also assessed. To do this, three leaves per plant were collected and placed in a chlorophyll meter to ascertain the chlorophyll content of the leaf. Chlorophyll content, measured as Chlorophyll Content Index (CCI) was determined by the hand-held Chlorophyll Concentration Meter (Model MC-100, by Apogee Instruments Inc., USA). Data was collected for a period of 15 days after sowing.

Germination indices

Germination indices calculated were done according the methods of Abdul-Baki and Andersen (1972); AOSA (1983); Scott *et al*. (1984); ISTA (1993); Al-Mudaris (1998); ISTA (1999); Sadeghi *et al*. (2001); Josep and Maria (2002).

First day of germination (or Germinability) (FDG)
It is the time when the first germination was recorded

Last day of germination (LDG)
This is the last day when the seed germination was reported

Final germination percentage (FGP)
This is the germination percentage attained by the plant even beyond the time period

Peak period of germination (PPG) or Modal time of germination (MTG)
It is the time in which highest frequency of germinated seeds are observed and need not be unique.

Median germination time (MeGT), or Days required for 50% germination, T503,
Days required for 50% germination, T50
T50 = Days required for 50% germination of the total number of seeds

Time spread of germination, or Germination distribution (TSG)
TSG = LDG - FDG
This is the time (in days) taken between the first and last germination events.

Mean germination time, MGT

$$T = \frac{\sum_{i=1}^{k} N_i T_i}{\sum_{i=1}^{k} N_i}$$

Where S1, S2, S3, Sn are number of seeds that germinated per lot (or petri dish) at day 1, day 2, day 3 … day n. The lower the MGT, the faster a seed population has germinated. It is also called Length of Germination Time (LGT) or Germination Resistance (GR) or Sprouting Index (SI). It is the average length of time required for maximum germination of a seed lot.

Mean germination rate, MGR

MGR = 1 / MGT

Germination rate index (GRI)

GRI = [GP1/1 + GP2/2 + GP3/3 + … + GPn/n]

Where GP1 is germination percentage at 1st day, GP2 is germ percent at 2 days, GPn is germ percent at n days. GRI reflects the percentage of germination on each day of the germination period.

Speed of accumulated germination, SAG

SAG = [(GP1/1 + (GP1+GP2)/2 + (GP1+GP2+GP3)/3 + … + (GP1+GP2+GP3+…+GPn)/n]

Where GP1 is germination percentage at 1st day, GP2 is germ percent at 2 days, GPn is germ percent at n days. GRI reflects the percentage of germination on each day of the germination period.

Corrected germination rate index (GRI corrected)

$S_{corrected}$ = GRI / FGP

Timson's Index, (TI) or Germination Energy Index

$$\boldsymbol{n} = \sum_{\boldsymbol{i=1}}^{\boldsymbol{t}} \boldsymbol{G_i}$$

GEI = (GP1 + GP2 + GP3 + … + GPn)

Where GP1, GP2, …,GPn are the germination percentages at day 1, 2, … and *n* respectively.

Modified Timson's Index, (TI_{mod})

TI_{mod} = Timson's index (*TI*) divided by the number of intervals (t).

$$T_{mod} = \frac{T}{t}$$

Germination index, GI

GI = 10 x (S1+S2+S3+ … +Sn) / (1*S1 + 2*S2 + 3*S3 + … + n * Sn)

Where S1, S2, S3, Sn are number of seeds that germinated per lot (or petri dish) at day 1, day 2, day 3 … day n

Mean daily germination, MDG

MDG = FGP / d, where d is the number of days it took to first arrive at the FGP

Daily germination speed, DGS

DGS = 1 / MDG. This is the reciprocal of MDG

Germination Value (Czabator)

GV = PV x MDG

Where, PV is the peak value and MDG is the mean daily germination percentage from the onset of germination.

Coefficient of velocity of germination, CVG

CVG = [(G1+G2+G3+ … +Gn) / (1*G1 + 2*G2 + 3*G3 + … + n * Gn)] * 100

Where G1, G2, G3, Gn are germination percent per lot (or petri dish) at day 1, day 2, day 3 ... day n. CVG gives an indication of the rapidity of germination.

Germination capacity, GC

GC = FGP / N

Where N is number of seeds used in the bioassay

Statistical analysis

The data collected on seed germination analysis, number of leaves, length of leaf, number of roots, branches per root length, length of nodes and chlorophyll content were subjected to two-way analysis of Variance (ANOVA).

Results

The effect of osmopriming of *Telfairia occidentalis* seeds on germinability and germinating percentage is shown in Fig. 1. Germination began after four days of sowing when compared to the control, germination increased to 100%.

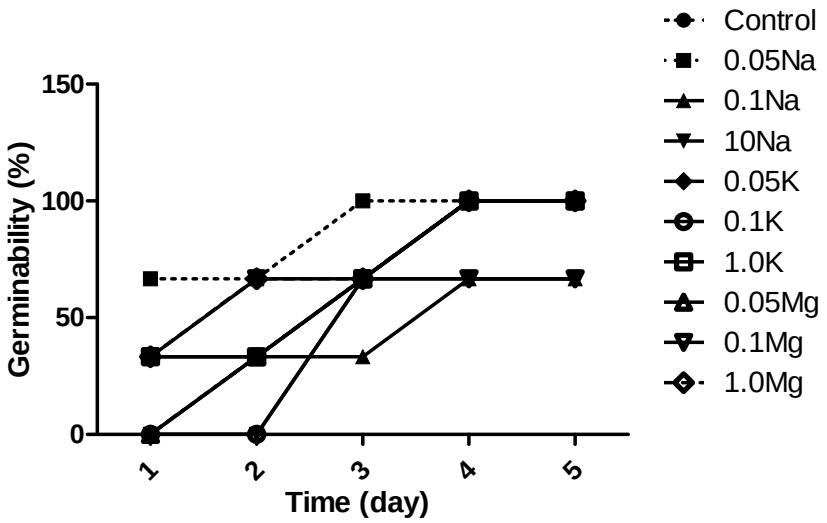


Figure 1. Osmopriming effects on the germinability of *Telfairia occidentalis*

Table 1. ANOVA summary Table to show source of variation due to germination and experimental treatment (osmopriming concentrations)

Source of Variation	Sum-of-squares	Mean square	F	% of total variation	P value
Treatments	7756	861.7	3.277	13.58	***0.0052****
Days	39890	9973	37.93	69.85	***< 0.0001****
Error	9466	262.9			

It was important to show if variations observed in the study were either due to time of germination or to the osmotic treatments applied. Results showed that treatment application accounted for 13.58% of total variations observed in the study (Table 1). Germination time accounted for 69.85% variation.

The effects of the treatments on germination time have been presented in Table 2. In the control group, germination occurred after 47.93 hours. However, when the seeds were osmoprimed with 0.05mM NaCl, the time it took for the seeds to germinate was significantly reduced to 23.96 hours. When

seeds were primed with 0.1 millimolar NaCl, germination time was also 23.96 hours. Seeds primed with 1.00 mM KCl, 0.10 mM $MgCl_2$, and 1.00 mM $MgCl_2$ all took the same amount of time to germinate (23.96 hours). There were no significant differences when seeds were primed with KCl irrespective of the concentration applied. However, with the application of 0.10 millimolar and 1.00 millimolar NaCl as osmoprimer the time taken for the last germination period was significantly reduced to 60.58 hours. The peak period of germination was less (47.93 hours) when seeds were primed with 0.10 millimolar$MgCl_2$ compared to 95.85 hours in the control.

Table 2. Effects of treatments on germination time

Day	Control	0.05 Na	0.1 Na	10 Na	0.05 K	0.1 K	1.0 K	0.05 Mg	0.1 Mg	1.0 Mg	LSD (0.05)	p-value
First Day of Germination	47.93	23.96*	23.96*	71.89*	47.93	71.89*	23.96*	47.93	23.96*	23.96*	19.3	0.006
Last Day of Germination	95.85	71.89*	95.85	71.89*	95.85	95.85	95.85	95.85	47.93*	95.85	22.31	0.031
Final Germination Percent	90.87	90.87	60.58*	60.58*	90.87	90.87	90.87	90.87	60.58*	90.87*	28.62	0.022
Peak period of Germination	95.85	71.89*	95.85	71.89*	95.85	95.85	95.85	95.85	47.93*	95.85	22.31	0.031
Median Germination Time	67.9	19.97*	23.96*	23.96*	61.91	67.9	67.9	64.9	23.96*	39.94*	26.73	0.013
Time Spread of Germination	47.93	47.93	71.89*	46.66	47.93	23.96*	71.89*	47.93	23.96*	71.89*	19.5	<0.001
Mean Germination Time	3.33	0.21	0.28	3	3.33	3.58	3	3.33	1.67	2.87	NA	NA
Mean Germination Rate	0.29	4.75	3.56	0.3	0.29	0.28	0.33	0.29	0.59	0.35	NA	NA

Table 3 shows the impact of seed priming on germination indexes. The germination rate index was minimally differed between the control and those of seeds osmoprimed with 0.05 millimolar KCl and 0.10 millimolar$MgCl_2$ (63.65). Modified Timson's index was the least (22.22) in seeds primed with 1.00 millimolar NaCl and the highest in both the control (77.77) and seeds primed with 0.05 millimolar NaCl. Coefficient of velocity of germination was 29 in the control, 30 in seeds primed with 1.00 millimolar NaCl and 476 when seeds were primed with 0.05 millimolar NaCl.

The growth parameters of the seedlings at 15 days after sowing (Table 4) showed significant increases in the number of leaves for those seeds that were primed with 0.10 millimolar NaCl (16 leaves) and those primed with 1.00

millimolar $MgCl_2$ (9 leaves), compared to the control (4 leaves). Although there were minimal differences in chlorophyll content index in seeds osmoprimed with Na, K and Mg chloride respectively as compared to the control (21.50), were significantly increased in chlorophyll content index in seeds osmoprimed with 0.05 millimolar KCl, 1.00 millimolar KCl and 1.00 millimolar $MgCl_2$ (30.20 – 36.40) ($P < 0.05$). The number of roots branches increased significantly from 31 in seeds primed with 1.00 millimolar NaCl to 4 in seeds primed with 0.05 millimolar NaCl.

Table 3. Impact of seed priming on germination Indices

Day	Control	0.05Na	0.1Na	10Na	0.05K	0.1K	1.0K	0.05Mg	0.1Mg	1.0Mg
Germination Rate Index	63.65	133.33	77.67	22.22	63.67	47	96.98	97.22	66.67	113.66
Speed of accumulated Germination	99.96	211.12	141.65	22.22	83.3	64.1	169.36	100	83.33	160.5
Corrected Germination Rate Index	0.64	1.33	1.16	0.33	0.64	0.47	0.97	0.97	1	1.13
Timson's Index	233.3	233.34	166.66	66.67	200	166.67	233.33	200	100	266.67
Modified Timson's Index	77.77	77.78	41.65	22.22	50	41.67	58.33	50	50	66.67
Germination Index	2.99	46.67	35.7	3.33	2.99	2.78	3.3	2.99	5.99	3.48
Mean Daily Germination	25	33.33	16.67	22.22	25	25	25	25	33.33	25
Daily Germination Speed	0.04	0.03	0.06	0.05	0.04	0.04	0..04	0.04	0.03	0.04
Germination Value	2500	3333	1111	1481	2500	2500	2500	2500	2222	2500
Coefficient of velocity of germination	29	476	357	30	29	28	33	29	59	35
Germination capacity	33.33	33.33	22.22	22.22	33.33	33.33	33.33	33.33	22.22	33.33

Figure 2 shows the treated and control seedlings with roots exposed at 13 days after sowing (Figure 2a), while the treated and controlled seedlings without root exposure have been presented in Figure 2b. By the 16 days, results showed improved growth capacity for the osmoprimed seedlings at 16 days after planting (Figure 2c).

Table 5 shows the two-way analysis of variance set out to determine the sources of variation in number of leaves, chlorophyll content, number of root branches, moisture content as well as dry weight measurement. For each of

these parameters, the primary treatments were compared against the measured parameters and results as presented in Table 5 show a mean square of 1.31 for number of leaves, 0.08 for chlorophyll content and 1.74 for dry weight measurement.

Table 4. Growth parameters of seedling 15 days after sowing

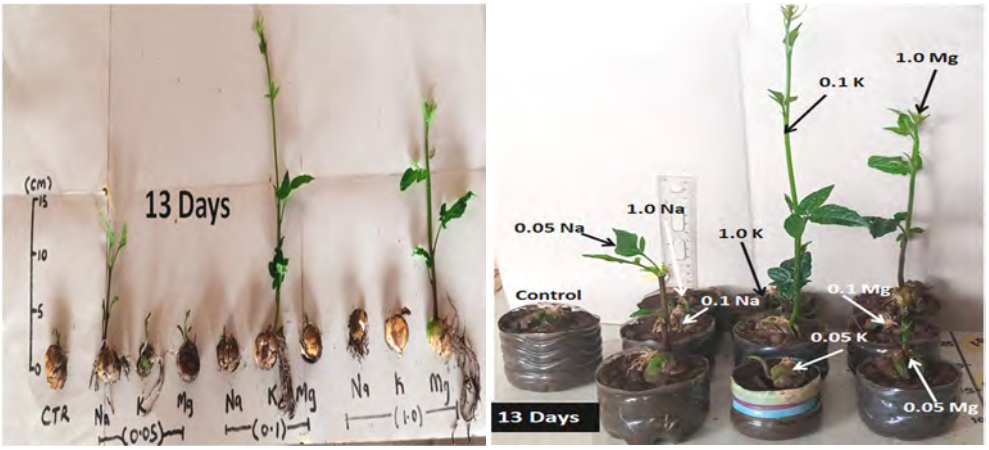
Groups	#No of leaves	Chlorophyll content (CCI)	Internode (cm)	#No primary root branches	#Av. Number of secondary root branches	Plant moisture content (%)	Plant dry wt. (g)
Control	4	21.5	0.4	5	10	2.8	7
0.05Na	7	29.3	1.1	4	9	18.9*	2.4*
0.1Na	3	27.2	0.3	5	14*	9.7	6.5
10Na	1	21	0.5	31*	3*	15.6	2.7*
0.05K	5	30.2*	2.3	5	15*	18.2*	4.5
0.1K	16*	27.1	1.1	18*	7	27.5*	4
1.0K	2	35.3*	2.8*	2	9	15.4	5.5
0.05Mg	8	23.1	1.2	6	11	2.3	8.5
0.1Mg	3	29.3	0.5	15*	8	14.0	4.3
1.0Mg	9*	36.4*	1.1	28*	5*	17.1*	2.9*
Mean	6	28.1	1.1	11.9	9.1	14.1	4.7
Variance	19	27.4	0.6	112	14	56.3	4.1
LSD(0.05)	5	8.7	1.6	8	4	13.2	3.7
p-value	0.043	0.007	0.293	0.001	0.062	<0.001	0.173

#Means are presented to the nearest integer
* Means differ significantly from the control ($p<0.05$)

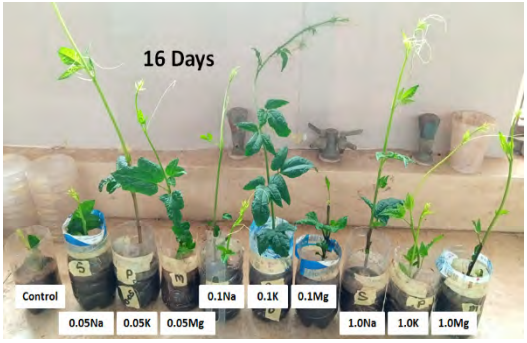
Discussion

The study on the effects of osmo-priming on the germination parameters of fluted pumpkin (*Telfairia occidentalis*) was carried out. This study revealed that osmopriming of seed of *Telfairia occidentalis* caused significant increases in the germination percentage compared to the control. The significant increase in germination percentage on osmoprimed seeds could be related to the activation of physiological germination processes in primed seeds, resulting in better germination through seed coat activation and softening. This finding was similar to that of Gurusinghe *et al*. (1999), who found that during priming, radical tip cells of some tomato seed lots proceeded through the cell cycle.

Several osmotica have been proven to have beneficial effects on germination (Lemrasky and Husseini, 2012). The effect of osmopriming on enhanced germination percentage could be explained by an increase in the activity of essential enzymes such amylase and proteases (Dell-Aquila and Tritto, 1990), which play a critical part in the seed embryo's growth and development. On the appropriate application of osmopriming treatment, Dell-Aquila *et al*. (1984) found a link between the pattern of water absorption, the reactivation of mitotic activity, and the initiation and synchronization of germination.



(a) (b)



(c)

Figure 2. Treated and control plant seedlings at different times after exposure to osmopriming regimes**:** (a) Treated and control seedlings (roots exposed) 13 days after sowing**;** (b) Treated and control seedlings 13 days after sowing; (c) Treated and control seedlings 16 days after sowing.

Seed priming approaches improved germination percentage, emergence, and seedling stand, according to Basra *et al*. (2003). In fact, priming causes a variety of biochemical changes in the seed that are essential to start the germination process, such as dormancy breaking, inhibitor hydrolysis or metabolism, imbibitions, and enzyme activation (Ajouri *et al*., 2004). Some earlier research has suggested that priming triggers some or all of the processes that occur prior to germination and that these processes continue after the seed is re-desiccated (Asgedom and Becker, 2001). As a result, primed seed can quickly ingest and revive the seed metabolism after sowing, resulting in a higher germination percentage and less physiological variability in germination (Rowse, 1995).

Table 5. Two way ANOVA to determine sources of variation

Source of Variation	% of total variation	Sum-of-squares	Mean square	F	P value
Number of leaves	0.52	2.621	1.31	17.05	< 0.0001
Priming treatments	99.2	495.5	55.06	716.2	< 0.0001
Residual		1.384	0.07687		
Chlorophyll content	61.25	284.5	8.09	< 0.0001	30.63
Priming treatments	694.1	716.2	91.66	< 0.0001	77.12
Residual	1.938				0.1077
No primary root branches	11.03	12.63	0.39	0.0004	5.516
Priming treatments	2815	716.2	99.33	< 0.0001	312.8
Residual	7.86				0.4367
Plant moisture	15.46	35.44	1.08	< 0.0001	7.731
Priming treatments	1411	718.7	98.64	< 0.0001	156.8
Residual	3.927				0.2182
Dry weight measurement	1.817	57.27	1.74	< 0.0001	0.9087
Priming treatments	102.3	716.2	97.99	< 0.0001	11.36
Residual	0.2856				0.01587

In the control group, the first day of germination took 47.93 hours. When the seed was osmoprimed with NaCl at 0.05 miligrams per kilogram, the time it took for the seed to germinate on the first day was dramatically reduced to 23.96 hours. Primed seeds with 0.1 millimolar NaCl, first germination took 23.96 hours. Seeds primed with 1.00 millimolar KCl, 0.10 millimolar $MgCl_2$, and 1.00 millimolar $MgCl_2$ all took the same amount of time to germinate on the first day (23.96 hours). When seeds were primed with KCl, regardless of the concentration

used, there was no significant difference. The time taken for the last germination period was significantly reduced to 60.58 hours when 0.10 millimolar and 1.00 millimolar NaCl were used as osmoprimers. Seeds primed with 0.10 millimolar $MgCl_2$ had a shorter peak period of germination (47.93 hours) compared to 95.85 hours in the control. Seed priming resulted in an increase in anti-oxidants such as glutathione and ascorbate in seed, according to Huns and Sung (1997). By reducing lipid proxidation activity, these enzymes increase germination speed. Priming has been shown to increase the speed of germination in sorghum, sunflower, and melon (Foti *et al*., 2002; Sivritepe *et al*., 2003; Demir Kaya *et al*., 2006).

When comparing the growth parameters of the seedlings 15 days after sowing, those primed with 0.10 millimolar NaCl (16 leaves) and those primed with 1.00 millimolar $MgCl_2$ (9 leaves) showed significant increases in the number of leaves compared to the control (4 leaves). Although there were minimal differences in chlorophyll content in seeds osmoprimed with Na, K, and Mg chloride as compared to the control (21.50), chlorophyll content index in seeds osmoprimed with 0.05 millimolar KCl, 1.00 millimolar KCl, and 1.00 millimolar $MgCl_2$ (30.20 – 36.40) ($P<0.05$) was significantly increased. The number of root branches increased significantly from 31 with 1.00 millimolar NaCl-primed seeds to 4 in 0.05 millimolar NaCl-primed seeds. The primed seed's early emergence could be attributable to the completion of pregermination metabolic activities, preparing the seed for radicle protrusion, and the primed seed germinated quickly after planting when compared to untreated dry seed (Arif, 2005). Chemically primed seeds have been shown to have a better germination pattern and vigor level than nonprimed seeds (Ruan *et al*., 2002). Nascimento and West (1998) found that seed coat adhesion was reduced during the emerging of muskmelon seeds. The improvement in germination and vigor of normal/low-vigor seed could be related to food reserve mobilization, activation and re-synthesis of certain enzymes, and the commencement of DNA and RNA synthesis during osmotic priming (Basra *et al*., 2003). When the barrier to germination was eliminated, the embryos grew quickly (Basra *et al*., 2003).

Priming can help to improve seed quality (Arif *et al*., 2008). Sung *et al*. (1993) found that priming reduced seed secretion and, as a result, decreased EC, which was consistent with Xiang *et al*. (1995) findings. Priming has been proved to be an effective approach in numerous experiments, particularly for seeds with low vigor (Varier *et al*. 2010, Flors *et al*. 2007, Soeda *et al*. 2005). The effects of priming applied to seeds of many domesticated crop species have helped agriculture. Good examples include barley (Ajouri et al., 2004), soybean (Arif, 2005), canola (Basra *et al*., 2003), carrot (Brocklehurst and Dearman, 1983), and sorghum (Foti *et al.,* 2002).

Conclusions

The results of this study revealed that germination in osmoprimed seedlings increased to 100%. The number of leaves, root branches, and chlorophyll content were all higher than in control seeds. This knowledge can be applied to fluted pumpkin farming on a larger scale. Osmopriming causes the vegetative growth of the plant to increase; this thus improves food security by increasing production.

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Occurrence of community-acquired Panton-Valentine leukocidin-producing and enterotoxin-producing methicillin-resistant staphylococci in companion dogs

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Abstract. In Nigeria, available data on drug-resistant bacterial infections that are caused by companion dogs are scarce. Hence the present study evaluated the occurrence of some community-acquired toxigenic methicillin-resistant staphylococci (MRS) on companion dogs harboured in Nigerian homes, as a pointer to the extent of exposure of humans to these pathogens. Samples were collected from 70 healthy companion dogs during dry and rainy season periods by swabbing a 125 cm^2 fur area on the lumbar and thoracic sites. Phenotypic tests, Kirby Bauer disc diffusion test and 16S rRNA gene analysis were used to identify presumptive colonies of staphylococci and MRS. Molecular methods were employed to detect Panton-Valentine leukocidin (PVL) and prototypic enterotoxin B in MRS isolates. The counts of staphylococci on fur of companion dogs during the rainy season exceeded usual limits of bacteria (≤ 2.54 log_{10} CFU cm^{-2}) on a healthy dog, thus, suggesting that companion dogs harboured in homes situated in Nigeria may be reservoirs of bacteria, especially during rainy season. The mean counts of staphylococci during the rainy season were estimated at 3.09 ± 2.78 log_{10} CFU cm^{-2} and 2.77 ± 2.43 log_{10} CFU cm^{-2} in Edo and Delta States, respectively. The main *Staphylococcus* species that were

carried on fur of companion dogs included *S. pseudintermedius*, *S. aureus*, *S. epidermidis*, *S. simulans* and *S. saprophyticus*. Amongst the staphylococci, expression of methicillin and multidrug resistance was mainly exhibited by *S. pseudintermedius* and *S. aureus*, while enterotoxigenicity was mainly expressed by methicillin-resistant *S. aureus*. Enterotoxigenic *S. aureus* was carried on the fur of companion dogs during the rainy season at estimated prevalence of 8.57% in both Edo and Delta States, respectively; while PVL-producing *S. aureus* was estimated at 5.71% and 2.86%, with PVL-producing *S. pseudintermedius* estimated at 25.71% and 34.29%, respectively. The high prevalence of toxigenic-producing isolates seen on the fur of companion dogs, especially during rainy season, could pose a risk for humans, particularly those that harbour pet dogs at their homes.

Keywords: Panton-Valentine toxin, Enterotoxin B, Methicillin resistance, *Staphylococcus aureus*, *Staphylococcus pseudintermedius.*

Introduction

There is a surge in companion animals harboured within households. In European countries, it is estimated that the population of dogs and cats within households exceeds 127 million (FEDIAF, 2012). Household companion animals refer to animals that are harboured within homes by people for company, psychological support or enjoyment (Damborg *et al.*, 2016). The skin of companion animals, such as dogs and cats, is covered by dense hair referred to as fur. The fur performs vital roles in dogs, some of which include protecting the skin of dogs against chemical, microbial and physical damage, as well as insulating the skin (Miller *et al*., 2013; Cuscó*et al*., 2017). The fur on the skin of these companion animals is often colonized or infected with myriads of pathogenic bacteria. Thus, the close contact between companion animals and humans may cause zoonotic transmission by direct human contact or indirectly by cross-contamination of food. Though, bacterial zoonoses directly associated with companion animals are relatively negligible when compared to foodborne zoonoses (Damborg *et al.*, 2016). Various studies (Chah *et al*., 2014; Rodrigues-Hoffmann *et al*., 2014; Bradley *et al*., 2016; Chermprapai *et al*., 2019; Suepaul *et al*., 2021) have reported that *Staphylococcus, Pseudomonas*, *Corynebacterium* and *Microbacterium* were the dominant bacterial genera that often colonize healthy companion dogs. Staphylococci, the dominant bacteria that colonize the fur and skin of dogs, have been categorized into two main groups, namely, coagulase-positive staphylococci and coagulase-negative staphylococci. Some

common coagulase-negative staphylococci that colonize the skin of dogs include *S. schleiferi*, *S. epidermidis*, *S. simulans*, *S. haemolyticus* and *S. sciuri* (Kloos and Bannerman, 1994; Chah *et al*., 2014), while the most dominant coagulase-positive staphylococci colonizer and infectious agent in dogs is *S. pseudintermedius* (Lynch and Helbig, 2021; Suepaul *et al*., 2021). Coagulase-positive *S. aureus*, the most clinically important coagulase-positive species in humans, is often found colonizing the skin of companion dogs (Wang *et al*., 2019; Suepaul *et al*., 2021). The coagulase-positive staphylococci are often associated with virulence because of their ability to express coagulase-mediated clotting of blood, thereby, evading the host's immune responses (Lamers *et al*., 2012). Coagulase-negative staphylococci have been widely associated with negligible virulence and are often regarded as contaminants (Kloos and Bannerman, 1994; Chah *et al*., 2014). Various studies (Lloyd, 2007; Findik *et al*., 2018; Li *et al*., 2021) have reported that staphylococci, particularly the coagulase-positive staphylococci, can develop resistance to antibiotics, such as the methicillin, in companion dogs due to their close contact with humans and the extensive use of broad-spectrum antibiotics to treat companion dogs. Methicillin-resistant *S. aureus* and methicillin-resistant *S. pseudintermedius* have been found to colonize both humans and dogs, though methicillin-resistant *S. aureus* may only be temporary colonizers of dogs (Gortel *et al.*, 1999; Gronthal *et al*., 2014; Ventrella *et al*., 2017). The carriage of these methicillin-resistant strains on the skin of companion dogs plays a fundamental role in the pathogenesis and epidemiology of community-associated infections. The infections caused by methicillin-resistant *S. aureus* and *S. pseudintermedius* could progress to become severe due to necrotizing processes mediated by the production of Panton-Valentine leukocidin (PVL) by these strains (Weese *et al*., 2009; Reyes-Robles *et al*., 2013; Maali *et al*., 2018). PVL-producing *S. aureus* and *S. pseudintermedius* are dominant causative agents of skin infections, such as furnicles, in canine and human subjects (Prevost *et al*., 1995; Weese *et al*., 2010). *S. aureus* carried by companion dogs has also been reported to produce enterotoxins that are the causative agents of staphylococcal food poisoning outbreaks in humans (Abdel-Moein and Samir, 2011). From both human and veterinary perspectives, understanding the prevalence/occurrence of toxigenic-producing drug-resistant staphylococci among companion dogs is imperative. However, available data on drug-resistant bacterial infections that are caused by companion dogs are scarce, particularly because cases of these pet-related infections are not often recorded and monitored. Hence the present study evaluated the occurrence of community-acquired Panton-Valentine leukocidin-producing and enterotoxin-producing methicillin-resistant staphylococci in companion dogs harboured in Nigerian homes, as a pointer to the extent of exposure of humans, particularly dog owners, to these pathogens.

Materials and methods

Recruitment of companion dogs

Healthy companion dogs with no clinical symptoms were enrolled for this study, while ill dogs with vivid infections were excluded. The breeds of companion dogs that were recruited include Caucasian Shepherd, German Shepherd, Doberman Pinscher, American Eskimo, Lhasa Apso and Alsatian. The registers in 12 veterinary clinics within the study localities were used to locate the homes of companion dog owners. Informed consent was obtained from owners of companion dogs before the participation of their dogs in the study. As an incentive, dog owners were promised that the results of the investigation on their dogs will be transmitted to their veterinarian who will implement any probable treatment regime.

Sample collection

The sample collection was performed in homes of dog owners and experimental analysis of the samples were done in the facility of Igbinedion University, Okada from January 2020 to October 2020 to cover dry and rainy seasons. A total of 35 companion dogs were recruited from 27 homes situated in Edo State (latitude: 6.5438^{o}N, 5.8987^{o}E). Other 35 companion dogs were recruited from 23 homes situated in Delta State (latitude: 5.7040^{o}N, 5.9339^{o}E). Overall, a total of 70 companion dogs were recruited from 50 homes situated in Edo (latitude: 6.5438^{o}N, 5.8987^{o}E) and Delta (latitude: 5.7040^{o}N, 5.9339^{o}E) states of Nigeria. Samples were initially collected from each of the companion dogs during the dry season period (January – March 2020) and then repeated during the rainy season period (July – October 2020). Sampling was done according to the previously prescribed techniques (Cuscó *et al.,* 2017). A 125 cm^2 fur area on the lumbar and thoracic sites of each dog was swabbed with 10 sterile swab sticks moistened with sterile phosphate-buffered saline. After swabbing each dog, the swab sticks were put into a sterile bottle containing 30 ml of sterile phosphate-buffered saline and it was stored on ice while being transported to the laboratory. The bacterial analysis took place in the laboratory within six hours of sample collection.

Isolation and enumeration of staphylococci and methicillin-resistant Staphylococci (MRS)

Presumptive isolation of staphylococci and MRS was performed with the spread plate method (Public Health England, 2014). Each sterile bottle containing swab sticks was thoroughly agitated to disperse the contents in the

fur into the saline diluents and serial dilutions ranging from 10^{-1} to 10^{-4} were made. Sterile mannitol salt agar plates, containing 75 g sodium chloride per litre of agar medium, were prepared and used for isolation of *Staphylococci*; while sterile mannitol salt agar plates containing 4 µg oxacillin per millilitre of the agar medium were used to isolate MRS. Twenty-five millilitre portion of the fur contents in each bottle was mixed with 225 ml of sterile phosphate-buffered saline to obtain the 10^{-1} dilution. Serial dilutions up to 10^{-4} were subsequently prepared from the fur contents of the 10^{-1} dilution. One hundred microlitres (100 µl) aliquots of each of the serial dilutions and the undiluted sample were spread on the duplicate agar medium (HiMedia Laboratories, Mumbai, India) and the inoculated Petri dishes were incubated at 35°C for 48 hours. After incubation, colonies on the Petri plates were presumptively identified as *Staphylococci* and were counted. The colony counts on the Petri plates were subsequently used to deduce the count of presumptive staphylococci and MRS on the fur of dog samples with the following equation and expressed as colony-forming units per square centimeter (CFU/cm^2).

$$CPS \text{ or } CPMS\ (CFU\ cm^{-2}) = \frac{CFU \times Df \times V}{v \times A} \quad (1)$$

CPS: count of presumptive staphylococci on the fur of dog; *CPMS*: count of presumptive MRS on the fur of dog; *CFU*: bacterial colony-forming units on the Petri plates; *Df*: reciprocal of the sample dilution selected for counting; *V*: total volume of diluents (30 ml); v: unit volume of diluents inoculated on each of the plates (0.1 ml); A: surface area of the dog's fur sampled (125 cm^2).

Genus-level identification of Staphylococci and MRS

Genus-level identification of presumptive staphylococci and MRS colonies were performed with standard methods (Krieg and Holt, 1984). The phenotypic tests included the bacterial colony examination, Gram-stain, tube coagulase, catalase, oxidase, haemolysis and mannitol fermentation, including maltose fermentation that was used to presumptively distinguish between *S. aureus* and *S. pseudintermedius*. Cefoxitin antibiotic susceptibility test was further performed for confirmation of MRS colonies using the cefoxitin disc (30 µg) diffusion method (CLSI, 2014). Colonies with zones of inhibition greater than or equal to 22 mm (≥ 22 mm) were suspected to be methicillin-sensitive or susceptible *S. aureus* while those that had zones of inhibition less than or equal to 21 mm (≤ 21 mm) were suspected to be methicillin-resistant *S. aureus*. However, for suspected *S. pseudintermedius* colonies, 1 µg oxacillin disc was

specifically used to carry out the disc diffusion test, and colonies with zones of inhibition greater than or equal to 20 mm (≥ 20 mm) were suspected to be methicillin-susceptible *S. pseudintermedius* while those that had zones of inhibition less than 20 mm (< 20 mm) were suspected to be methicillin-resistant *S.pseudintermedius*.

Species-level identification of staphylococci and MRS

The presence of methicillin resistance phenotype in the suspected MRS was confirmed by using PCR to detect the *MecA* gene in their DNA templates. The PCR was performed according to previously described methods (Adhikari *et al.*, 2017). *MecA* gene amplification was done with the specific primers:*mecA*F (5'-AAA ATC GAT GGT AAA GGT TGG C -3') and *mecA*R(5'-AGT TCT GGA GTA CCG GAT TTG C -3'), with amplicon size of 533 base pairs. The PCR protocol was performed with a 24 µL reaction mixture containing 1 µL of DNA template, 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM of each of the deoxynucleoside triphosphates (dNTPs) (Fermentas Inc., Burlington, Canada), 0.1% Triton X-100, 2 U of GoTaq Hot Start DNA Polymerase (Promega Corporation, Madison, WI) and 20 µM of each primer. PCR was run on a GeneAmp PCR system 9700 (Applied Biosystems, Waltham, MA). The amplification program involved 30 cycles, with each cycle consisting of denaturation at 95°C for 1 minute, followed by annealing at 55°C for 1 minute and extension at 72°C for 1 minute. The PCR products were analyzed by gel electrophoresis on a 1.5% agarose gel (Sigma-Aldrich, Taufkirchen, Germany). DNA bands in the gel were then visualized and documented on the gel documentation system (Applied Biosystems).

Partial 16S rRNA gene analysis confirmed the presence of staphylococci and its methicillin-resistant strains on the fur of companion dogs. The gene analysis was done by polymerase chain reaction (PCR) and sequencing methods (Lane, 1991; Schuurman *et al.*, 2004). Zymo-spin column (Zymo Research Corporation, Irvine, CA) was used to extract ultra-pure DNA templates from suspected staphylococci and MRS colonies at the Microbiology Laboratory of Igbinedion University according to prescriptions of the manufacturer. The DNA templates were subsequently used to perform PCR and sequencing of PCR amplicons. Universal 16S rRNA primers (27F [forward primer]: AGA GTT TGA TCM TGG CTC AG; 1492R [reverse primer]: GGT TAC CTT GTT ACG ACT T) were used for the taxonomic classification. PCR protocol was performed with a 50-µl reaction mixture containing 10mmol/l Tris–HCl (pH 8.3), 2mmol/l $MgCl_2$, 2µl of template DNA, 200mmol/l of each of the deoxynucleoside triphosphates (dNTPs) (Fermentas Inc., Burlington, Canada), 50mmol/l KCl,

2 U of GoTaq Hot Start Polymerase (Promega Corporation, Madison, WI) and 0.5 µmol/l of each primer. Amplification was done on a GeneAmp PCR system 9700 (Applied Biosystems, Waltham, MA) with the following cycling conditions: initial denaturation at 95°C for 2 minutes, followed by 40 cycles, with each cycle consisting of denaturation at 94°C for 45 seconds; annealing at 55°C for 1 minute; extension at 72°C for 2 minutes; and a final extension at 72°C for 5 minutes. DNA sequencing of PCR amplicons was carried out with the dideoxy chain termination method (Sanger *et al*., 1977). Query nucleotide sequence comparison with a database of reference nucleotide sequence to confirm the identity of suspected *Staphylococci* was done with the BLASTN 2.8.0+ program (National Center for Biotechnology Information [NCBI]).

Phylogenetic analysis

16S rRNA gene sequences of some staphylococci strains that colonized companion dogs harboured in Nigerian homes were compared with reference strains from other environmental sources to infer their ancestral lineages. Phylogenetic analysis was constructed with the neighbour-joining method using MEGA software, version 6 (Tamura *et al*., 2013), and the robustness of the groupings in the tree was assessed with 1000 bootstrap iterations.

Molecular detection of Panton-Valentine leukocidin (PVL) toxin in MRS isolates

PCR was used to detect the presence of PVL genes in the genomic DNA templates of MRS isolates according to previously described methods (Lina *et al*., 1999). Specific primers employed for amplification of the PVL genes were *luk-PV-1* (5'- ATCATTAGGTAAAATGTCTGGACATGATCCA-3') and *luk-PV-2* (5′-GCATCAASTGTATTGGATAGCAAAAGC-3′). The amplification program involved 30 cycles, consisting of denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds and extension at 72°C for 1 minute. The PCR products were analyzed by gel electrophoresis on a 1.5% agarose gel (Sigma-Aldrich, Taufkirchen, Germany) and the bands were visualized as previously described. DNA sequencing of the PCR products was carried out with the dideoxy chain termination method (Sanger *et al*., 1977). Comparison of query nucleotide sequences against sequence database (non-redundant protein sequences) was done with BLASTX 2.8.0+ program (NCBI) to confirm the presence of PVL in the MRS isolates. All the databases of non-redundant GenBank CDS translations + PDB + SwissProt + PIR + PRF excluding environmental samples from WGS projects were searched for reference sequences that were homologous to the query translated nucleotide sequences of the MRS isolates.

Molecular detection of enterotoxin B in MRS isolates

The PCR was done with specific primers that targeted the prototypic enterotoxin B gene (Machado *et al*., 2020). Specific primers used for amplifying enterotoxin B gene were *SEB*-F (5'-ACATGTAATTTTGATATTCGCACTG-3') and *SEB*-R (5′-TGCAGGCATCATGTCATACCA-3′), with amplicon size of 667 base pairs. The DNA templates were amplified with the following cyclic conditions: initial denaturation for 5 minutes at 94°C followed by 30 cycles of denaturation, with each cycle consisting of denaturation at 94°C for 2 minutes; annealing at 50°C for 1 minute; extension at 72°C for 1 minute; and a final extension step at 72°Cfor 5 minutes. The amplified products were subsequently run on a 2% agarose gel and the amplicons were sequenced and compared using BLASTX 2.8.0+ program as previously described.

Antibiotic susceptibility testing of MRS isolates

Susceptibility of confirmed MRS isolates to other classes of antibiotics was performed with the Kirby Bauer disc diffusion test as earlier described (CLSI, 2014). The bacterial colony suspension adjusted to 0.5 McFarland turbidity standard was inoculated on a Petri dish containing sterile Mueller-Hinton agar medium (HiMedia Laboratories, Mumbai, India) and antibiotic discs attached to the agar surface. The Petri dish was incubated at 35°C for 16 – 18 hours. Inhibitory zone diameter was interpreted as sensitive (susceptible), intermediate or resistant based on zone diameter interpretive standards set by CLSI. *S. aureus* ATCC 25923 and *Escherichia coli* ATCC 25922 were used as control. Ciprofloxacin (5 µg), pefloxacin (5 µg); cotrimoxazole (30 µg), erythromycin (15 µg) and gentamycin (10 µg) were the antibiotic discs that were used.

Calculation of the confirmed (actual) count of staphylococci, MRS, PVL-producing and enterotoxin-producing MRS

Each presumptive staphylococci or MRS colony that was subjected to phenotypic and molecular tests was confirmed to be staphylococci if it was shown to be Gram-positive coccus, coagulase-positive, catalase-positive, oxidase negative, beta-haemolytic and if its 16S rRNA gene sequence (query sequence) significantly matched with a reference *Staphylococci* 16S rRNA gene sequence in the NCBI GenBank database. Presumptive MRS colonies that were first confirmed as staphylococci and also produced cefoxitin zone diameter of less than or equal to 21 mm (≤ 21 mm) and whose DNA templates also contained the *MecA* gene were ultimately confirmed as MRS. The relative occurrence (*F*) of staphylococci and MRS colonies in each of the dog samples was deduced as follows:

$$F = \frac{\text{Number of bacterial colonies that were confirmed to be staphylococci or MRS}}{\text{Total number of presumptive staphylococci or MRS colonies examined}} \quad (2)$$

The counts of confirmed staphylococci (*CS*) or confirmed MRS (*CMS*) in each of the dog samples was deduced with equation 3.

$$CS/CMS = H \times CPS/CPMS \quad (3)$$

H is the relative occurrence of staphylococci/MRS in each of the dog samples. *CPS* is the count of presumptive staphylococci while *CPMS* is the count of presumptive MRS in each of the dog samples.

The count of PVL-producing MRS (*CLS*) in each of the dog samples was deduced as follows:

$$CLS = P \times CMS \quad (4)$$

CMS is the count of confirmed MRS. *P* is the ratio of PVL-producing MRS colonies to the total methicillin-resistant bacterial colonies examined.

The count of enterotoxigenic-producing MRS (*CES*) in each of the dog samples was deduced as follows:

$$CES = O \times CMS \quad (5)$$

CMS is the count of confirmed MRS. *O* is the ratio of enterotoxigenic-producing MRS colonies to the total methicillin-resistant bacterial colonies examined.

Probability of exposure

The probability of exposure of humans, especially dog owners, to staphylococci, MRS, PVL-producing MRS and enterotoxigenic-producing MRS isolated from the companion dogs was mathematically deduced from the prevalence of companion dogs that are colonized by these microbes (FDA/CFSAN/JIFSAN/RSI 2021).

Statistical analysis

Descriptive statistics of staphylococci counts and relative occurrence datasets was done with NCSS ver. 12 data analysis software. Levene test of homogeneity, Shapiro–Wilk test, Kruskal–Wallis nonparametric one-way ANOVA test, Fisher (F) one-way ANOVA test for normally distributed datasets with equal variances, Welch's one-way ANOVA test for normally distributed datasets with unequal variances and one-tailed Student's t-test were also performed with NCSS ver. 12 data analysis software. The test of the hypothesis was considered statistically significant if the achieved level of significance (p) was less than 0.05. The accuracy of the phylogenetic tree implemented with MEGA software was evaluated by Monte Carlo simulation of the original tree-building dataset using the bootstrap sampling technique.

Results

Identification of bacterial colonies on MSA plates

Tab. 1 and 2 present the phenotypic and molecular characterization of bacterial colonies on MSA Petri plates with oxacillin (4 μg ml^{-1}) and without oxacillin. Results of phenotypic and molecular analysis performed on the bacterial colonies in MSA Petri plates without oxacillin (Tab. 1) indicated that staphylococci and non-staphylococci were isolated from companion dogs harboured in homes situated in Edo State, Nigeria during the dry season. The isolated staphylococci were classified as *S. pseudintermedius*, *S. aureus*, *S. epidermidis*, *S. simulans* and *S. saprophyticus*; while the non-staphylococci were found to be mainly *Micrococcus* spp. and *Bacillus* spp. Representative *S. pseudintermedius* strain ADEOLAAKINNIBOSUN 200, *S. epidermidis* strain ADEOLAAKINNIBOSUN 202, *S. simulans* strain ADEOLAAKINNIBOSUN 203, *S. pseudintermedius* strain ADEOLAAKINNIBOSUN 201, *S. aureus* strain ADEOLAAKINNIBOSUN 204, *S. pseudintermedius* strain ADEOLAAKINNIBOSUN 215, *S. aureus* strain ADEOLAAKINNIBOSUN 231, *S. aureus* strain ADEOLAAKINNIBOSUN 232, *S. pseudintermedius* strain ADEOLAAKINNIBOSUN 230, *S. simulans* strain ADEOLAAKINNIBOSUN 240 and *S. saprophyticus* strain ADEOLAAKINNIBOSUN 219 isolated from the fur of companion dogs harboured in homes situated in Edo and Delta States, Nigeria were deposited in the GenBank (NCBI) database under accession numbers MW965474, MW965579, MW965587, MW965574, MW965613, MZ008355, MZ461457, MZ461460, MZ461942, MZ488580, and MZ021337.

All staphylococcal colonies on MSA plates without oxacillin that were isolated from companion dogs during the dry season were susceptible to cefoxitin, with *MecA* prevalence estimates of 0.00%. However, during the rainy season, some staphylococci isolates were resistant to cefoxitin. Cefoxitin/oxacillin resistance was observed only in staphylococci isolates that were identified as *S. pseudintermedius* and *S. aureus*, with *MecA* prevalence of 53.06% and 34.52% for *S. aureus* and *S. pseudintermedius* isolates obtained from dog samples situated in Edo State, Nigeria.

No bacterial growth was seen in all the dry season samples from Edo State that were cultured on MSA plates with oxacillin (Tab. 2), but bacterial growth was seen in the rainy season samples. The bacteria that were identified on MSA plates with oxacillin were similar to the bacteria that were found on MSA plates without oxacillin. Unlike the case with MSA plates without oxacillin, the staphylococci on MSA plates with oxacillin were all resistant to cefoxitin/oxacillin, with *MecA* prevalence estimates of 100%, thus, were all termed as methicillin-resistant staphylococci.

The bacterial species that were isolated from companion dogs in homes situated in Delta State, Nigeria during dry and rainy season samplings significantly conformed to those isolated from companion dogs harboured in homes situated in Edo State, Nigeria.

Table 1. Phenotypic and molecular characterization of bacterial colonies on MSA Petri plates without oxacillin

Sampling locations	Period of sampling	Representative bacterial colonies	Colonial and morphological characteristics		Biochemical characteristics of bacterial colonies										Molecular analysis				Identified bacteria
					CO	CA	OX	MA	HT	MR	VP	FOX			16S homology	16S identity	*Mec4* prevalence		
			Growth on MSA Petri plates	Gram staining								Z (mm)	I				(F)	(%)	
Edo State, Nigeria	January – March 2020 (Dry season)	1	Yellow colonies	Positive cocci	-	+	+	-	v	+	v	23 - 33	S		NP	NP	NP		*Micrococcus* spp.
		2	Dry/mucoid colonies	Positive bacilli	NP	+	v	-	v	-	+	23 - 36	S		NP	NP	NP		*Bacillus* spp.
		3	Mucoid colony	Positive cocci	-	-	-	-	-	-	+	28 - 35	S		98 - 100%	95 - 99%	0/5	0.00	*Staphylococcus simulans*
		4	White colonies	Positive cocci	+	+	-	+	+	-	+	25 - 33	S		99 - 100%	95 - 100%	0/23	0.00	*Staphylococcus pseudintermedius*
		5	Yellow colonies	Positive cocci	+	+	-	-	+	-	+	23 - 26	S		94 - 99%	92 - 99%	0/3	0.00	*Staphylococcus aureus*
		6	Mucoid colonies	Positive cocci	-	+	-	-	-	-	+	24 - 33	S		96 - 100%	96 - 100%	0/20	0.00	*Staphylococcus epidermidis*
	July – September 2020 (Rainy season)	1	Yellow colonies	Positive cocci	-	+	+	-	v	+	v	17 - 28	S/R		NP	NP	NP		*Micrococcus* spp.
		2	Dry/mucoid colonies	Positive bacilli	NP	+	v	-	v	-	+	23 - 33	S		NP	NP	NP		*Bacillus* spp.
		3	Yellow colonies	Positive cocci	+	+	-	-	+	-	+	12 - 33	S/R		93 - 100%	92 - 100%	26/49	53.06	*Staphylococcus aureus*
		4	Mucoid colonies	Positive cocci	-	+	-	-	-	-	+	23 - 29	S		97 - 100%	99 - 100%	0/7	0.00	*Staphylococcus epidermidis*
		5	Mucoid colonies	Positive cocci	-	+	-	-	-	-	+	30	S		99%	100%	0/1	0.00	*Staphylococcus saprophyticus*
		6	White colonies	Positive cocci	+	+	-	+	+	-	+	23 - 33	S/R		98 - 100%	97 - 99%	29/84	34.52	*Staphylococcus pseudintermedius*
		7	Mucoid colonies	Positive cocci	-	-	-	-	-	-	+	23 - 27	S		99 - 100%	100%	0/3	0.00	*Staphylococcus simulans*
Delta State, Nigeria	January – March 2020 (Dry season)	1	Yellow colonies	Positive cocci	-	+	+	-	v	+	v	22 - 35	S		NP	NP	NP		*Micrococcus* spp.
		2	Dry/mucoid colonies	Positive bacilli	NP	+	v	-	v	-	+	22 - 36	S		NP	NP	NP		*Bacillus* spp.
		3	White colonies	Positive cocci	+	+	-	+	+	-	+	24 - 30	S		95 - 100%	95 - 99%	0/22	0.00	*Staphylococcus pseudintermedius*
		4	Mucoid colony	Positive cocci	-	-	-	-	-	-	+	29 - 30	S		96 - 100%	98 - 99%	0/6	0.00	*Staphylococcus simulans*
		5	Mucoid colonies	Positive cocci	-	+	-	-	-	-	+	25 - 33	S		99 - 100%	100%	0/17	0.00	*Staphylococcus epidermidis*
		6	Mucoid colonies	Positive cocci	-	+	-	-	-	-	+	28 - 29	S		99 - 100%	99 - 100%	0/3	0.00	*Staphylococcus saprophyticus*
	July – October 2020 (Rainy season)	1	Yellow colonies	Positive cocci	-	+	+	-	v	+	v	12 - 28	S/R		NP	NP	NP		*Micrococcus* spp.
		2	Dry/mucoid colonies	Positive bacilli	NP	+	v	-	v	-	+	23 - 25	S		NP	NP	NP		*Bacillus* spp.
		3	Mucoid colonies	Positive cocci	-	+	-	-	-	-	+	23 - 25	S		97 - 100%	97 - 100%	0/11	0.00	*Staphylococcus epidermidis*
		4	White colonies	Positive cocci	+	+	-	+	+	-	+	12 - 25	S/R		99 - 100%	97 - 100%	36/72	50.00	*Staphylococcus pseudintermedius*
		5	Mucoid colonies	Positive cocci	-	-	-	-	-	-	+	23 - 27	S		95 - 99%	97 - 99%	0/4	0.00	*Staphylococcus simulans*
		6	Yellow colonies	Positive cocci	+	+	-	-	+	-	+	17 - 26	S/R		94 - 99%	92 - 99%	27/43	62.79	*Staphylococcus aureus*

Voges-Proskauer test. FOX: cefoxitin/oxacillin antibiotic test; Z: zone inhibition diameter; I: interpretive criteria; S: sensitive, R: resistant; F: fractional prevalence of *MecA* gene; NP: not performed; +: positive result; -: negative result; v: variable result.

Table 2. Phenotypic and molecular characterization of bacterial colonies on MSA Petri plates containing 4 µg oxacillin per millilitre

Sampling locations	Period of sampling	Representative bacterial colonies	Colonial and morphological characteristics		Biochemical characteristics of bacterial colonies										Molecular analysis				Identified bacteria
					CO	CA	OX	MA	HT	MR	VP	FOX			16S	16S	*Mec A*		
			Growth on the MSA Petri plates	Gram staining								Z (mm)		I	homology	identity	prevalence (F)	(%)	
Edo State, Nigeria	January – March 2020 (Dry season)		No bacterial growth in all MSA plates examined																
	July – September 2020 (Rainy season)	1	Yellow colonies	Positive cocci	-	+	+	-	v	+	v	8 - 13	R		NP	NP	NP		*Micrococcus* spp.
		2	Dry/mucoid colonies	Positive bacilli	NP	+	v	-	v	-	+	12	R		NP	NP	NP		*Bacillus* spp.
		3	Yellow colonies	Positive cocci	+	+	-	-	+	-	+	0 - 8	R		98 - 100%	99 - 100%	29/29	100.00	*Staphylococcus aureus*
		4	White colonies	Positive cocci	+	+	-	+	+	-	+	0 - 10	R		94 - 99%	95 - 99%	34/34	100.00	*Staphylococcus pseudintermedius*
		5	Mucoid colonies	Positive cocci	-	-	-	-	-	-	+	13	R		98%	100%	1/1	100.00	*Staphylococcus similans*
		6	Mucoid colonies	Positive cocci	-	+	-	-	-	-	+	12	R		100%	99%	1/1	100.00	*Staphylococcus epidermidis*
Delta State, Nigeria	January – March 2020 (Dry season)		No bacterial growth in all MSA plates examined																
	July – October 2020 (Rainy season)	1	Yellow colonies	Positive cocci	-	+	+	-	v	+	v	0 - 13	R		NP	NP	NP		*Micrococcus* spp.
		2	Dry/mucoid colonies	Positive bacilli	NP	+	v	-	v	-	+	12	R		NP	NP	NP		*Bacillus* spp.
		3	Mucoid colonies	Positive cocci	-	+	-	-	-	-	+	12 - 13	R		100%	100%	3/3	100.00	*Staphylococcus epidermidis*
		4	White colonies	Positive cocci	+	+	-	+	+	-	+	0 - 10	R		96 - 100%	97 - 99%	38/38	100.00	*Staphylococcus pseudintermedius*
		5	Mucoid colonies	Positive cocci	-	-	-	-	-	-	+	12 - 13	R		99 - 100%	99%	5/5	100.00	*Staphylococcus similans*
		6	Yellow colonies	Positive cocci	+	+	-	-	+	-	+	0 - 8	R		97 - 100%	99 - 100%	30/30	100.00	*Staphylococcus aureus*

Staphylococcal phylogeny

Phylogenetic tree highlighting the evolutionary relatedness of some staphylococci strains that colonized companion dogs harboured in Nigerian homes and reference strains that were isolated from other environmental sources in the world is shown in Fig. 1. *S. epidermidis* strain ADEOLAAKINNIBOSUN 223 (MZ021414), *S. epidermidis* strain ADEOLAAKINNIBOSUN 229 (MZ021508), *S. epidermidis* strain ADEOLAAKINNIBOSUN 208 (MZ971554) and *S. epidermidis* strain ADEOLAAKINNIBOSUN 207 (MW971519) isolated from the fur of the companion dogs in Nigeria were found to have shared a common ancestry with *S. epidermidis* strain 100911 (NR 113957) collected from Japan, with a 97% likelihood. There was 88% likelihood that *S. aureus* strain ADEOLAAKINNIBOSUN 209 (MW971572), *S. aureus* strain ADEOLAAKINNIBOSUN 210 (MW978696), *S. aureus* NBRC 100910 (NR 113956) and *S. aureus* strain S33 R (NR 037007) evolved from a common ancestor. A common ancestor was also inferred to be the origin from where *S. pseudintermedius* strain ADEOLAAKINNIBOSUN 221 (MZ021339), *S. pseudintermedius* strain ADEOLAAKINNIBOSUN 205 (MW970345), *S. pseudintermedius* strain ADEOLAAKINNIBOSUN 206 (MW971449), *S. pseudintermedius* strain ADEOLAAKINNIBOSUN 215 (MZ008355) and *S. intermedius* strain H11/68 (NR 036829) evolved, with a 99% likelihood.

Occurrence and counts of staphylococci and non-staphylococci on the companion dogs

The occurrence and counts of bacteria seen on MSA plates without oxacillin are presented in Tab. 3. *Bacillus* spp., a non-staphylococci, was found to be the most abundant bacteria that were seen on the companion dogs harboured in homes situated in Edo State, Nigeria during the dry season because they had a relative occurrence estimated at 65.96% and a mean count estimated at 0.79 ± 0.15 $\log_{10}$ CFU cm^{-2}.

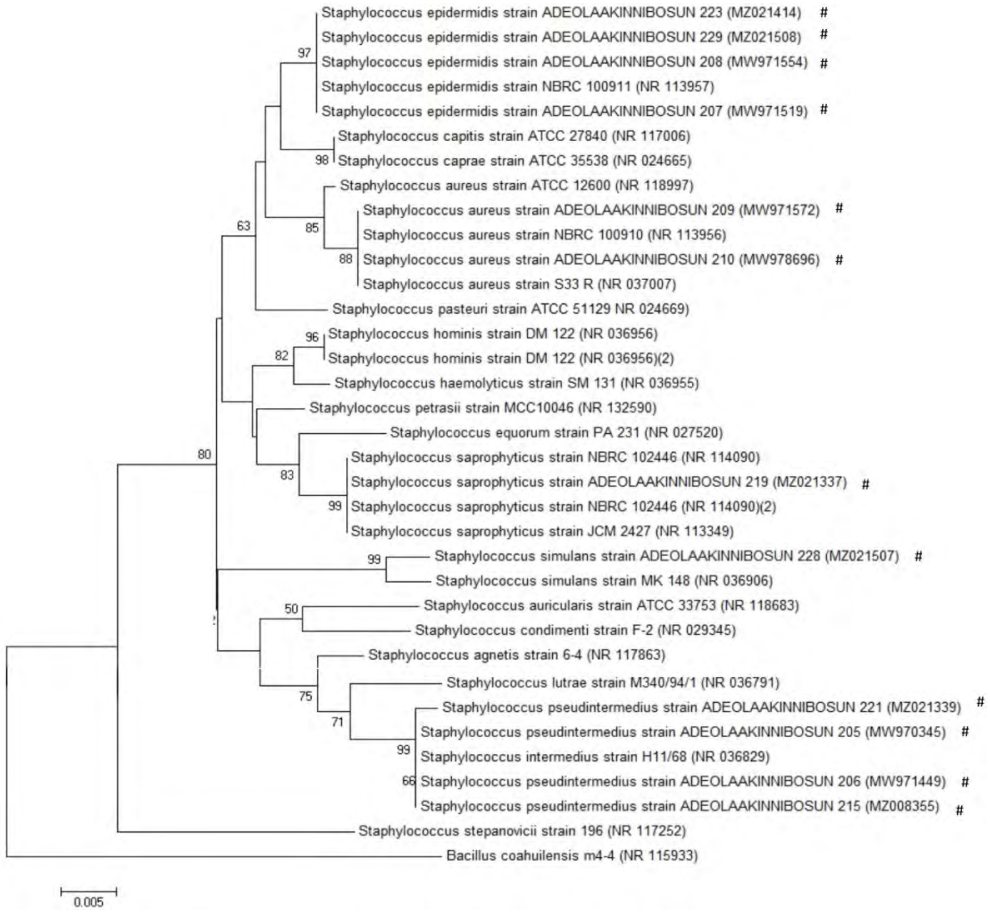

Figure 1. Phylogenetic tree constructed with the neighbour-joining method (# is used to indicate some novel *Staphylococcus* strains on the fur of the companion dogs examined in this study. GenBank accession numbers of all the strains used to implement the phylogenetic tree are indicated in parenthesis. The tree was rooted on midpoint and only bootstrap values that were above 50 % are displayed on branches).

Table 3. Occurrence and counts of bacteria seen on MSA plates without oxacillin

Sampling locations	Period of sampling	Counts of presumptive staphylococci (*CPS*)		Counts of identified bacteria					Counts of confirmed staphylococci (*CS*)	
		Mean ± SE (Log_{10} CFU cm^{-2}) (N = 35)	95% CI (Log_{10} CFU cm^{-2}) (N = 35)	Identified bacterial colonies	Relative occurrence of bacterial colonies		Relative counts		Mean ± SE (Log_{10} CFU cm^{-2}) (N = 35)	95% CI (Log_{10} CFU cm^{-2}) (N = 35)
					(*H*)	(%)	Mean ± SE (Log_{10} CFU cm^{-2}) (N = 35)	95% CI (Log_{10} CFU cm^{-2}) (N = 35)		
Edo State, Nigeria	January – March 2020 (Dry season)	1.20 ± 0.23	0.87 – 1.53						0.29 ± 0.06	0.21 – 0.37
				Micrococcus spp.	18/188	9.57	0.12 ± 0.02	0.09 – 0.15		
				Bacillus spp.	124/188	65.96	0.79 ± 0.15	0.57 – 1.01		
				Staphylococcus simulans	4/188	2.13	0.03 ± 0.01	0.02 – 0.04		
				Staphylococcus pseudintermedius	21/188	11.17	0.12 ± 0.03	0.09 – 0.17		
				Staphylococcus aureus	3/188	1.60	0.02 ± 0.00	0.01 – 0.03		
				Staphylococcus epidermidis	18/188	9.57	0.12 ± 0.02	0.09 – 0.15		
	July – September 2020 (Rainy season)	4.00 ± 3.59	2.56 – 5.44						3.09 ± 2.78	1.97 – 4.21
				Micrococcus spp.	38/181	20.99	0.84 ± 0.75	0.54 – 1.15		
				Bacillus spp.	3/181	1.66	0.07 ± 0.06	0.05 – 0.09		
				Staphylococcus aureus	48/181	26.52	1.06 ± 0.95	0.68 – 1.44		
				Staphylococcus epidermidis	7/181	3.87	0.16 ± 0.14	0.10 – 0.22		
				Staphylococcus saprophyticus	1/181	0.55	0.02 ± 0.02	0.01 – 0.03		
				Staphylococcus pseudintermedius	82/181	45.30	1.81 ± 1.63	1.15 – 2.47		
				Staphylococcus simulans	2/181	1.11	0.04 ± 0.04	0.02 – 0.06		
Delta State, Nigeria	January – March 2020 (Dry season)	1.17 ± 0.10	0.88 – 1.46						0.27 ± 0.02	0.22 – 0.34
				Micrococcus spp.	15/190	7.90	0.09 ± 0.01	0.07 – 0.11		
				Bacillus spp.	131/190	68.95	0.81 ± 0.07	0.61 – 1.01		
				Staphylococcus pseudintermedius	20/190	10.53	0.12 ± 0.01	0.09 – 0.15		
				Staphylococcus simulans	5/190	2.63	0.03 ± 0.00	0.02 – 0.04		
				Staphylococcus epidermidis	15/190	7.90	0.09 ± 0.01	0.07 – 0.11		
				Staphylococcus saprophyticus	4/190	2.11	0.03 ± 0.00	0.02 – 0.04		
	July – October 2020 (Rainy season)	4.21 ± 3.68	2.74 – 5.68						2.77 ± 2.43	1.30 – 3.24
				Micrococcus spp.	44/182	24.18	1.02 ± 0.89	0.66 – 1.38		
				Bacillus spp.	18/182	9.89	0.42 ± 0.36	0.27 – 0.57		
				Staphylococcus epidermidis	11/182	6.04	0.25 ± 0.22	0.16 – 0.34		
				Staphylococcus pseudintermedius	72/182	39.56	1.67 ± 1.46	1.09 – 2.25		
				Staphylococcus simulans	4/182	2.20	0.09 ± 0.08	0.06 – 0.12		
				Staphylococcus aureus	33/182	18.13	0.76 ± 0.67	0.49 – 1.03		

Amongst the staphylococci that were found to colonize the companion dogs during the dry season, *S. pseudintermedius* was the most abundant, with a relative occurrence of 11.17% and a mean count of 0.12 ± 0.03 log_{10} CFU cm^{-2} while *S. aureus* was the least abundant, with a relative occurrence of 1.60% and a mean count estimated at 0.02 ± 0.00 log_{10} CFU cm^{-2}. The mean count of presumptive staphylococci and confirmed staphylococci were respectively estimated at 1.20 ± 0.23 log_{10} CFU cm^{-2} and 0.29 log_{10} CFU cm^{-2} during the dry season. The count datasets of presumptive and confirmed staphylococci were non-normally distributed ($p = 0.01$ for both presumptive and confirmed staphylococci) with unequal variances ($p = 0.04$ for presumptive staphylococci and $p = 0.03$ for confirmed staphylococci). Kruskal-Wallis ANOVA tests indicated no significant difference in the median counts of presumptive and confirmed staphylococci ($p = 0.49$ for presumptive staphylococci and $p = 0.42$ for confirmed staphylococci).

Unlike the findings reported on companion dogs that were harboured in homes situated in Edo State, Nigeria during the dry season, the staphylococci were the most abundant bacteria on the companion dogs during the rainy season, with *S. pseudintermedius* accounting for 45.30% of the total viable bacteria on the fur of companion dogs and *S. aureus* accounting for 26.52%. Mean counts of presumptive staphylococci and confirmed staphylococci during the rainy season were estimated at 4.00 ± 3.59 $\log_{10}$ CFU cm^{-2} and 3.09 ± 2.78 $\log_{10}$ CFU cm^{-2}, respectively. The count datasets of presumptive and confirmed staphylococci were non-normally distributed ($p = 0.00$ for both presumptive and confirmed staphylococci) with equal variances ($p = 0.70$ for presumptive staphylococci and $p = 0.43$ for confirmed staphylococci). Kruskal-Wallis ANOVA tests showed that there was no significant difference in the median counts of presumptive and confirmed staphylococci ($p = 0.41$ for presumptive staphylococci and $p = 0.34$ for confirmed staphylococci). The mean count of presumptive staphylococci on companion dogs harboured in homes situated in Delta State, Nigeria during the dry season was estimated at 1.17 ± 0.10 $\log_{10}$ CFU cm^{-2} while the mean count of confirmed staphylococci was estimated at 0.27 ± 0.02 $\log_{10}$ CFU cm^{-2}. The presumptive and confirmed staphylococci count datasets were normally distributed ($p = 0.06$ for presumptive staphylococci and $p = 0.08$ for confirmed staphylococci) with equal variances ($p = 0.52$ for presumptive staphylococci and $p = 0.43$ for confirmed staphylococci). Fisher one-way ANOVA tests indicated that there was no significant difference in the mean counts of presumptive and confirmed staphylococci ($p = 0.85$ for presumptive staphylococci and $p = 0.65$ for confirmed staphylococci). *Bacillus* spp. was also the most abundant bacterium on companion dogs harboured in homes situated in Delta State, Nigeria during the dry season. *S. pseudintermedius*, with a relative occurrence of 10.53% and a mean count of 0.12 ± 0.01 $\log_{10}$ CFU cm^{-2}, was also found to be the most abundant staphylococcum on companion dogs harboured in homes situated in Delta State, Nigeria during the dry season. Unlike companion dogs harboured in homes situated in Edo State, Nigeria, no *S. aureus* was seen in samples obtained from companion dogs harboured in homes situated in Delta State, Nigeria during the dry season.

In Delta State, the staphylococci were also the most abundant bacteria seen on the fur of the companion dogs during the rainy season, with *S. pseudintermedius* accounting for 39.56% of the total viable bacteria on the fur of companion dogs. Mean counts of presumptive and confirmed staphylococci during the rainy season were estimated at 4.21 ± 3.68 $\log_{10}$ CFU cm^{-2} and 2.77 ± 2.43 $\log_{10}$ CFU cm^{-2} respectively. The count datasets of presumptive and confirmed staphylococci were non-normally distributed ($p = 0.00$ for both

presumptive and confirmed staphylococci) with equal variances ($p = 0.30$ for presumptive staphylococci and $p = 0.21$ for confirmed staphylococci). Kruskal-Wallis ANOVA tests showed that there was no significant difference in the median counts of presumptive and confirmed staphylococci ($p = 0.94$ for presumptive staphylococci and $p = 0.71$ for confirmed staphylococci). Student's t-test showed that the count datasets of presumptive and confirmed staphylococci on companion dogs in Edo State during the dry season did not significantly differ ($p = 0.49$ for presumptive staphylococci and $p = 0.63$ for confirmed staphylococci) from those that were obtained from companion dogs in Delta State, Nigeria. No significant difference ($p = 0.16$ for presumptive staphylococci and $p = 0.24$ for confirmed staphylococci) was also observed during the rainy season samplings. However, there was a significant difference in the counts of presumptive and confirmed staphylococci when the count datasets obtained during the dry and rainy seasons were compared ($p = 0.01$ and $p = 0.03$ for counts of presumptive and confirmed staphylococci in Edo State; $p = 0.01$ and $p = 0.00$ for counts of presumptive and confirmed staphylococci in Delta State).

Occurrence and counts of methicillin-resistant staphylococci and their virulent strains in companion dogs

The occurrence and counts of bacteria seen on MSA plates with oxacillin are presented in Tab. 4. Due to the absence of bacterial growth on the Petri plates, no counts of presumptive methicillin-resistant staphylococci were reported on the fur of the companion dogs harboured in homes situated in Edo State, Nigeria during the dry season. However, during the rainy season in which bacteria was seen on the Petri plates, methicillin-resistant *S. pseudintermedius* was the most abundant methicillin-resistant bacterial species, with a relative occurrence of 43.59% and a relative mean count of 1.38 ± 1.31 $\log_{10}$ CFU cm^{-2}. The least frequently occurred methicillin-resistant species was reported as *S. simulans* and *S. epidermidis*, with relative occurrences of 1.28% and relative mean counts of 0.04 ± 0.04 $\log_{10}$ CFU cm^{-2}. Mean counts of presumptive and confirmed methicillin-resistant staphylococci were respectively estimated at 3.16 ± 3.00 $\log_{10}$ CFU cm^{-2} and 2.63 ± 2.50 $\log_{10}$ CFU cm^{-2} during the rainy season. The count datasets of presumptive and confirmed staphylococci were non-normally distributed ($p = 0.00$ for both presumptive and confirmed staphylococci) with unequal variances ($p = 0.00$ for both presumptive and confirmed staphylococci). Kruskal-Wallis ANOVA tests showed that there was no significant difference in the median counts of presumptive and confirmed staphylococci ($p = 0.72$ for presumptive staphylococci and $p = 0.64$ for confirmed staphylococci).

No counts of presumptive methicillin-resistant staphylococci were reported on companion dogs that were sampled in Delta State, Nigeria during the dry season. Methicillin-resistant *S. pseudintermedius* were also the most abundant methicillin-resistant bacterial species seen on the fur of the companion dogs during the rainy season. Mean counts of presumptive methicillin-resistant staphylococci during rainy season was estimated at 3.17 ± 3.01 $\log_{10}$ CFU cm^{-2} while the mean count of confirmed methicillin-resistant staphylococci was estimated at 2.48 ± 2.37 $\log_{10}$ CFU cm^{-2}. The count datasets of presumptive and confirmed staphylococci were non-normally distributed (p = 0.00 for both presumptive and confirmed staphylococci) with equal variances (p = 0.33 for presumptive staphylococci and p = 0.51 for confirmed staphylococci). Kruskal-Wallis ANOVA tests showed that there was no significant difference in the median counts of presumptive staphylococci (p = 0.69) and median counts of confirmed staphylococci (p = 0.48). Student's t-test indicated that the count datasets of presumptive and confirmed staphylococci obtained during rainy season sampling of companion dogs in Edo State did not significantly differ (p = 0.49 for presumptive staphylococci and p = 0.61 for confirmed staphylococci) from those obtained from companion dogs in Delta State.

Table 4. Occurrence and counts of methicillin-resistant staphylococci (MRS) and its virulent strains on MSA plates containing 4 µg per millilitre

Sampling locations	Period of sampling	Counts of presumptive MRS (*CPMS*)		Counts of identified methicillin-resistant bacteria					Counts of confirmed MRS (*CMS*)		PVL-producing MRS (*P*)			Enterotoxigenic-producing MRS (*O*)		
		Mean ± SE (Log_{10} CFU cm^{-2}) (N = 35)	95% CI (Log_{10} CFU cm^{-2}) (N = 35)	Identified bacterial colonies	Relative occurrence of bacterial colonies (*H*)	(%)	Relative counts Mean ± SE (Log_{10} CFU cm^{-2}) (N = 35)	95% CI (Log_{10} CFU cm^{-2}) (N = 35)	Mean ± SE (Log_{10} CFU cm^{-2}) (N = 35)	95% CI (Log_{10} CFU cm^{-2}) (N = 35)	Relative counts	Mean ± SE (Log_{10} CFU cm^{-2}) (N = 35)	95% CI (Log_{10} CFU cm^{-2}) (N = 35)	Relative counts (*CES*)	Mean ± SE (Log_{10} CFU cm^{-2}) (N = 35)	95% CI (Log_{10} CFU cm^{-2}) (N = 35)
Edo State, Nigeria	January – March 2020 (Dry season)			None												
	July – September 2020 (Rainy season)	3.16 ± 3.00	1.91 – 4.41						2.63 ± 2.50	1.59 – 3.67						
				Micrococcus spp.	11/78	14.10	0.45 ± 0.42	0.27 – 0.63			NP			NP		
				Bacillus spp.	2/78	2.56	0.08 ± 0.08	0.05 – 0.11			NP			NP		
				Staphylococcus aureus	29/78	37.18	1.17 ± 1.11	0.71 – 1.63			2/78	0.07 ± 0.06	0.04 – 0.10	11/78	0.37 ± 0.35	0.19 – 0.55
				Staphylococcus pseudintermedius	34/78	43.59	1.38 ± 1.31	0.84 – 1.92			29/78	0.98 ± 0.93	0.52 – 1.44	0/78	0.00 ± 0.00	0.00 – 0.00
				Staphylococcus simulans	1/78	1.28	0.04 ± 0.04	0.02 – 0.06			0/78	0.00 ± 0.00	0.00 – 0.00	0/78	0.00 ± 0.00	0.00 – 0.00
				Staphylococcus epidermidis	1/78	1.28	0.04 ± 0.04	0.02 – 0.06			0/78	0.00 ± 0.00	0.00 – 0.00	0/78	0.00 ± 0.00	0.00 – 0.00
Delta State, Nigeria	January – March 2020 (Dry season)			None												
	July – October 2020 (Rainy season)	3.17 ± 3.01	1.92 – 4.42						2.48 ± 2.37	1.50 – 3.46						
				Micrococcus spp.	20/102	19.61	0.62 ± 0.59	0.38 – 0.87			NP			NP		
				Bacillus spp.	2/102	1.96	0.06 ± 0.06	0.04 – 0.08			NP			NP		
				Staphylococcus epidermidis	3/102	2.94	0.09 ± 0.09	0.05 – 0.13			0/102	0.00 ± 0.00	0.00 – 0.00	0/102	0.00 ± 0.00	0.00 – 0.00
				Staphylococcus pseudintermedius	40/102	39.22	1.24 ± 1.18	0.75 – 1.73			37/102	0.90 ± 0.86	0.45 – 1.35	0/102	0.00 ± 0.00	0.00 – 0.00
				Staphylococcus simulans	5/102	4.90	0.16 ± 0.15	0.10 – 0.22			0/102	0.00 ± 0.00	0.00 – 0.00	0/102	0.00 ± 0.00	0.00 – 0.00
				Staphylococcus aureus	32/102	31.37	1.00 ± 0.94	0.61 – 1.38			1/102	0.02 ± 0.02	0.01 – 0.03	9/102	0.22 ± 0.21	0.11 – 0.33

N: total number of dogs examined during the dry or rainy season; MRS: methicillin-resistant staphylococci; SE: standard error of the mean; CI: confidence interval of the mean; NP: PVL or enterotoxin B detection not performed on bacterial isolates.

Amongst the isolated methicillin-resistant staphylococci, methicillin-resistant *S. pseudintermedius* was the most prevalent PVL-producing methicillin-resistant staphylococci. Of the 34 isolated methicillin-resistant *S. pseudintermedius* in Edo State, 29 methicillin-resistant *S. pseudintermedius* was found to produce PVL toxin, thus, constituting 85.29% prevalence. In Delta State, 37 methicillin-resistant *S. pseudintermedius* was found to produce PVL toxin out of the 40 methicillin-resistant *S. pseudintermedius* isolated from the companion dogs, with an estimated prevalence of 92.50%. Only a few methicillin-resistant *S. aureus* were found to produce PVL toxin in both Edo and Delta States samples.

Twenty-nine methicillin-resistant *S. aureus* were reported in companion dogs from Edo State, with only 2 of these isolates producing PVL; corresponding to a 6.90% prevalence. Of the 32 isolated methicillin-resistant *S. aureus* reported in Delta State samples, only 1 methicillin-resistant *S. aureus* produced PVL, with an estimated prevalence of 3.13%. The mean count of PVL-producing methicillin-resistant staphylococci on the fur of the companion dogs harboured in homes situated in Edo State, Nigeria was estimated at 1.05 ± 0.99 $\log_{10}$ CFU cm^{-2}, and in Delta State, an estimate of 0.92 ± 0.88 $\log_{10}$ CFU cm^{-2} was reported. Representative PVL toxins produced by some of these strains and their respective translated gene were deposited in the GenBank under accession numbers QWX21626, QWX21629, QWX21628, QZW25256 for the PVL toxins, and MZ230623, MZ230626, MZ230625, MZ682632 for the translated gene.

Methicillin-resistant *S. aureus* accounted for all of the enterotoxigenic-producing staphylococci on the fur of companion dogs selected from both Edo and Delta States. In Edo State, 11 methicillin-resistant *S. aureus* produced enterotoxin B, out of the 29 methicillin-resistant *S. aureus* isolated from the companion dogs, with an estimated prevalence of 37.93%. In Delta State, 9 methicillin-resistant *S. aureus* produced enterotoxin B, out of the 32 methicillin-resistant *S. aureus* that was isolated from the companion dogs, thus, given prevalence estimates of 28.13%. The mean count of enterotoxin-producing methicillin-resistant staphylococci on the fur of the companion dogs harboured in homes situated in Edo State, Nigeria was estimated at 0.40 ± 0.38 $\log_{10}$ CFU cm^{-2} and in Delta State, it was estimated at 0.22 ± 0.21 $\log_{10}$ CFU cm^{-2}. Representative enterotoxin B produced by some of these strains were also deposited in the GenBank under accession numbers QZW25259, QZW25258 and their respective translated gene deposited under accession numbers MZ682635, MZ682634.

Exposure estimates

The prevalence of companion dogs colonized by *Staphylococcus* species is presented in Tab. 5. During the dry season, humans, especially dog owners, were mostly exposed to *S. pseudintermedius* carried by companion dogs harboured in homes situated in Edo State (17.14%) and Delta State (20.00%), Nigeria.

During the dry season in Edo State, the likelihood of exposure of humans to coagulase-positive *S. aureus* was estimated at 5.71%. In the rainy season, exposure to *S. pseudintermedius* was estimated at 80.00% in Edo State and 71.43% in Delta State; while the likelihood of exposure to *S. aureus* was estimated at 51.43% in Edo State and 34.29% in Delta State.

Unlike the dry season, it was found that humans were likely exposed to methicillin-resistant staphylococci, as well as PVL- and enterotoxigenic-producing staphylococci, carried on companion dogs during the rainy season. In Edo State, exposure to methicillin-resistant *S. pseudintermedius* and methicillin-resistant *S. aureus* was 31.43% and 25.71%, respectively, as well as 37.14 ± 8.29% and 28.57 ± 7.75% in Delta State. Only *S. aureus* was found to produce enterotoxins, and the likelihood of exposure of humans to enterotoxigenic-producing methicillin-resistant *S. aureus* was estimated at 8.57 ± 4.80% in Edo and Delta States, respectively. Exposure to PVL-producing methicillin-resistant *S. aureus* and PVL-producing methicillin-resistant *S. pseudintermedius* was respectively estimated at 5.71% and 25.71% in Edo State, as well as 2.86% and 34.29% in Delta State.

Table 5. Prevalence of companion dogs colonized by *Staphylococcus* species

Period of sampling	Identified *Staphylococcus* species	Companion dogs			
		Prevalence of colonized companion dogs from Edo State N = 35		Prevalence of colonized companion dogs from Delta State N = 35	
		Mean ± SE (%)	95% CI (%)	Mean ± SE (%)	95% CI (%)
January – March 2020 (Dry season)	*Staphylococcus simulans*	5.71 ± 3.98	0.00 – 13.51	5.71 ± 3.98	0.00 – 13.51
	Staphylococcus pseudintermedius	17.14 ± 6.46	4.47 – 29.81	20.00 ± 6.86	6.56 – 33.44
	Staphylococcus aureus	5.71 ± 3.98	0.00 – 13.51		
	Staphylococcus epidermidis	14.29 ± 6.00	2.53 – 26.05	14.29 ± 6.00	2.51 – 26.07
	Staphylococcus saprophyticus			5.71 ± 3.98	0.00 – 13.51
July – October 2020 (Rainy season)	*Staphylococcus aureus*	51.43 ± 8.57	34.63 – 68.23	34.29 ± 8.14	18.34 – 50.25
	Staphylococcus epidermidis	8.57 ± 4.80	0.00 – 17.98	11.43 ± 5.46	0.74 – 22.12
	Staphylococcus saprophyticus	2.86 ± 2.86	0.00 – 8.46		
	Staphylococcus pseudintermedius	80.00 ± 6.86	66.56 – 93.44	71.43 ± 7.75	56.24 – 86.62
	Staphylococcus simulans	2.86 ± 2.86	0.00 – 8.46	5.71 ± 3.98	0.00 – 13.51
	Methicillin-resistant *Staphylococcus aureus*	25.71 ± 7.50	11.02 – 40.40	28.57 ± 7.75	13.38 – 43.76
	Methicillin-resistant *Staphylococcus pseudintermedius*	31.43 ± 7.96	15.83 – 47.03	37.14 ± 8.29	20.90 – 53.38
	Methicillin-resistant *Staphylococcus simulans*	2.86 ± 2.86	0.00 – 8.46	5.71 ± 3.98	0.00 – 13.51
	Methicillin-resistant *Staphylococcus epidermidis*	2.86 ± 2.86	0.00 – 8.46	2.86 ± 2.86	0.00 – 8.46
	PVL-producing methicillin-resistant *Staphylococcus aureus*	5.71 ± 3.98	0.00 – 13.51	2.86 ± 2.86	0.00 – 8.46
	PVL-producing methicillin-resistant *Staphylococcus pseudintermedius*	25.71 ± 7.50	11.02 – 40.40	34.29 ± 8.14	18.34 – 50.25
	Enterotoxigenic-producing methicillin-resistant *Staphylococcus aureus*	8.57 ± 4.80	0.00 – 17.98	8.57 ± 4.80	0.00 – 17.98

N: total number of dogs examined during the dry or rainy season;
SE: standard error of the mean; CI: confidence interval of the mean

Antibiotic resistance profile

Tab. 6 shows the antibiotic resistance profile of methicillin-resistant staphylococcal colonies obtained from the companion dogs. Methicillin-resistant staphylococci that exhibited resistance to at least two other antibiotics from the different antibiotic classes examined were termed multidrug-resistant. Thirty-eight methicillin-resistant staphylococci were found to be multidrug-resistant out of the 141 methicillin-resistant staphylococci that were isolated from the companion dogs selected from Edo and Delta States. Coagulase-positive methicillin-resistant *S. aureus* and *S. pseudintermedius* accounted for all the multidrug-resistant staphylococci. Methicillin-resistant *S. aureus* was mostly resistant to ciprofloxacin, as indicated by percentage resistance of 13.79% and 13.33%, in isolates obtained from Edo and Delta States, respectively. Methicillin-resistant *S. aureus* also had the highest prevalence of resistance to all antibiotics tested, except for erythromycin. The coagulase-negative methicillin-resistant staphylococci were mostly susceptible to all the classes of antibiotics tested.

Table 6. Antibiotic resistance profile of methicillin-resistant staphylococcal colonies obtained from the companion dogs

Sampling locations	Period of sampling	Identified methicillin-resistant staphylococci on the MRSA plates	Relative count of methicillin-resistant staphylococci	Prevalence of antibiotic resistance										Relative count of multidrug-resistant staphylococci
				CIP 5 µg		PF 5 µg		C 30 µg		E 15 µg		GM 10 µg		
				Q	T (%)	Q	T (%)	Q	T (%)	Q	T (%)	Q	T (%)	
Edo State, Nigeria	January – March 2020 (Dry season)	No identified methicillin-resistant staphylococci												
	July – September 2020 (Rainy season)	Coagulase-positive *Staphylococcus aureus*	29	4/29	13.79	4/29	13.79	11/29	37.93	7/29	24.14	13/29	44.83	13
		Coagulase-positive *Staphylococcus pseudintermedius*	34	2/34	5.88	3/34	8.82	11/34	32.35	1/34	2.94	11/34	32.35	6
		Coagulase-negative *Staphylococcus simulans*	1	0/1	0.00	0/1	0.00	0/1	0.00	1/1	100.00	0/1	0.00	0
		Coagulase-negative *Staphylococcus epidermidis*	1	0/1	0.00	0/1	0.00	0/1	0.00	0/1	0.00	0/1	0.00	0
Delta State, Nigeria	January – March 2020 (Dry season)	No identified methicillin-resistant staphylococci												
	July – October 2020 (Rainy season)	Coagulase-positive *Staphylococcus aureus*	30	4/30	13.33	7/30	23.33	14/30	4.67	6/30	20.00	12/30	40.00	10
		Coagulase-positive *Staphylococcus pseudintermedius*	38	1/38	2.63	3/38	7.90	16/38	53.33	5/38	13.16	13/38	34.21	9
		Coagulase-negative *Staphylococcus epidermidis*	3	0/3	0.00	0/3	0.00	0/3	0.00	0/3	0.00	0/3	0.00	0
		Coagulase-negative *Staphylococcus simulans*	5	0/5	0.00	0/5	0.00	0/5	0.00	0/5	0.00	0/5	0.00	0

CIP: Ciprofloxacin; PF: Pefloxacin; C: Cotrimoxazole; E: Erythromycin; GM: Gentamycin; Z: fractional prevalence of resistance; I: percentage prevalence of resistance

Discussion

Bacterial transmissions associated with companion dogs are largely dependent on the cleanliness of the shelter/homes where these pet animals are kept (Song *et al.*, 2013). Upon examination of swabbed fur samples that were inoculated on MSA Petri plates with and without oxacillin, both *Staphylococcus*

and non-*Staphylococcus* species were found to colonize the fur of companion dogs that were harboured in Nigerian homes (Tables 1 and 2). *Bacillus* and *Micrococcus* species were the dominant non-*Staphylococcus* species, while the main *Staphylococcus* species that were carried on the fur of companion dogs included *S. pseudintermedius*, *S. aureus*, *S. epidermidis*, *S. simulans* and *S. saprophyticus*. Amongst the *Staphylococcus* species, *S. pseudintermedius* was the most frequently detected (up to 39.56% in Delta State and 45.30% in Edo State) followed by *S. aureus* (up to 18.13% in Delta State and 26.52% in Edo State). Suepaul *et al*. (2021) also reported that *S. pseudintermedius* was the most frequently detected species on companion dogs, accounting for 87.40% of the isolates that they examined. The findings of the present study were also consistent with the work of Janos *et al.* (2021) that detected *S. pseudintermedius* and *S. intermedius* in 48.83% and 27.90% of all canine isolates, followed by *S. aureus* in 11.62% of all isolates carried by the skin of healthy companion dogs.

Some *Staphylococcus* species that colonized the fur of the healthy companion dogs selected for this study were found to express resistance phenotype to methicillin, as confirmed by *MecA* PCR (Tab. 1 and 2), and were then regarded as methicillin-resistant *Staphylococcus* species. The effects of seasonal variations may have also resulted in the absence of methicillin-resistant staphylococci on the fur of the companion dogs during the dry season (Tab. 4). Amongst the *Staphylococci*, the expression of methicillin resistance was mostly exhibited by *S. pseudintermedius* and *S. aureus* during the rainy season. Interestingly, one of the earliest reports on methicillin resistance in companion dogs was recorded in Nigeria in 1972 (Ojo, 1972). The relative occurrence of methicillin-resistant *S. pseudintermedius* carried by the companion dogs harboured in Nigerian homes (39.22% in Delta State and 43.59% in Edo State) was higher than those reported in Australia [11.80%] (Saputra *et al.*, 2017) and even significantly higher when compared to those reported in Sweden, estimated at 0.4% (SWEDRES/ SVARM, 2015), and Norway, estimated at 0.5% (Simonsen and Urdahl, 2017).

26.95% of all the coagulase-positive methicillin-resistant *S. aureus* and *S*. *pseudintermedius* were found to account for all the multidrug-resistant *Staphylococcus* species carried by the fur of the companion dogs examined in the present study (Tab. 6). Coagulase-positive *Staphylococcus* species accounted for most of the multidrug-resistant staphylococci (25.40%) in canine samples examined by Suepaul *et al*. (2021), thus agreeing with the values reported in the present study. Unlike the study of Chah *et al*. (2014) that found a high rate of multidrug resistance (81.3%) amongst the coagulase-negative *Staphylococci* carried by clinically healthy dogs in Enugu State, Nigeria, no coagulase-negative *Staphylococci* obtained from the present study were regarded as multidrug-resistant.

In the present study, only the methicillin-resistant *S. aureus* was found to produce enterotoxin (Tab. 4), with an estimated prevalence of 37.93% and 28.13% in Edo and Delta States, respectively. In a study carried out by Abdel-Moein and Samir (2011), enterotoxigenic *S. aureus* was also detected at an estimated prevalence of 10.00% in pet dog samples collected from Egypt. The high prevalence of enterotoxigenic *S. aureus* on the fur of companion dogs in the present study is a pointer to a probable zoonotic transmission to human contacts.

PVL-producing methicillin-resistant *S. aureus* and *S. pseudintermedius* were also carried on the fur of companion dogs sampled in the present study (Tab. 4). This was consistent with the study of Findik *et al*. (2018) that reported the carriage of PVL-producing methicillin-resistant *S. aureus* and *S. pseudintermedius* on healthy dogs in Turkey. Futagawa-Saito *et al*. (2004) also reported the colonization of pet dogs harboured in Japanese homes with PVL-producing methicillin-resistant *S. intermedius*. These PVL-producing strains could pose a probable risk of transmission between humans and dogs that share the same household. The PVL toxins produced by these strains are pore-forming toxins that are capable of necrotizing plasma membranes, thus, resulting in cell lysis (Prévost *et al*., 2001; Reyes-Robles *et al*., 2013; Spaan *et al*., 2013; Maali *et al*., 2018).

The present study revealed that amongst staphylococci species, humans were mostly exposed to *S. pseudintermedius* during dry [17.14%] and rainy [80.00%] seasons (Tab. 5). Exposure of humans to methicillin-resistant *Staphylococci*, as well as PVL- and enterotoxigenic-producing *Staphylococci*, was reported only during the rainy season (Tab. 5). The companion dogs carried methicillin-resistant *S. pseudintermedius* at the rates of 31.43% in Edo State and 37.14% in Delta State, Nigeria. Rates ranging from 1.5% to 2.1%, as it relates to the prevalence of healthy dogs colonized by methicillin-resistant *S. pseudintermedius* and methicillin-resistant *S.aureus*, have been reported by several researchers (Griffeth *et al*., 2008; Hanselman *et al*., 2008; Loeffler *et al*., 2011; Chah *et al*., 2014; Suepaul *et al*., 2021); thus, revealing a significant difference from the rates reported during the rainy season in the present study. In the present study, the colonization of companion dogs by methicillin-resistant staphylococci during the rainy season is worrisome. This is because these staphylococcal strains could potentially transfer the resistance genes between dogs and humans.

The prevalence of companion dogs (8.57%) that carried enterotoxigenic *S. aureus* in the present study was similar to that reported by Abdel-Moein and Samir (2011). In the present study, 25.71% and 34.29% of companion dogs from Edo and Delta States, Nigeria were respectively colonized by PVL-producing

methicillin-resistant *S. pseudintermedius* (Tab. 5). These colonization rates reported in the present study appeared to be lower than that reported by Futagawa-Saito *et al*. (2004) where all 8 healthy dogs (100%) examined were colonized by PVL-producing *S. intermedius*.

Conclusions

This study revealed the high exposure of humans to PVL toxins and enterotoxins on the fur of companion dogs, mainly during the rainy season. The high prevalence of toxigenic-producing isolates seen on the fur of companion dogs, especially during the rainy season, could pose a risk for humans, particularly those that harbour pet dogs at their homes.

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Cuticle structure of Carpathian endemic species: *Trachelipus trilobatus* (Crustacea, Isopoda, Oniscidea) described with the scanning electron microscope

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Abstract. The cuticle is the interface between an animal and its environment; thus, it has a special importance. In Arthropods, the cuticle is not uniform, having numerous formations, which is also the case of epigeic terrestrial isopods. Our study presents data on cuticle surface morphology, obtained with a scanning electron microscope, of an endemic terrestrial isopod species, *Trachelipus trilobatus*. Here we present SEM images of some external morphological features of this species, which were previously described only at the light microscope. Although *T*. *trilobatus* was frequently encountered in caves, the aspect of its cuticle is characteristic for an epigeic isopod, presenting numerous micro-scales, spines, and tricorn sensilla, which are considered hygroreceptors. This fact proves that originally *T*. *trilobatus* is an epigeic species, which secondarily adapted to karst areas with caves. Nevertheless, it regularly leaves the caves and the limestone cracks and emerges on the soil surface. Therefore, *T*. *trilobatus* is able to receive information about environmental humidity, especially on the soil surface, which, when unfavorable, causes the species retreat into caves or cracks. Climatic fluctuations from the glacial periods could direct *T*. *trilobatus* to this environment and way of life, modifying its morphology, but not its cuticle.

Keywords: SEM, cuticle surface, endemic species, ecology, sensory structures.

Introduction

Many terrestrial isopod species in Romania are endemic; their percentage is much higher than in other regions in Europe, except for the Mediterranean area (Sfenthourakis and Hornung, 2018). Thus, according to a recent review, in the country, 96 terrestrial isopod species are present, and 42 of them are endemic (Giurginca, 2022). In the case of terrestrial isopods in Romania, the genus *Trachelipus* contains numerous endemic species, some even endemic to the country (Tomescu *et al*., 2015). Among them, large sized species *T. trilobatus*, *T. ater,* and *T. vareae* have limited distribution to only some regions in the Carpathians (Tomescu *et al*., 2015), and they are considered to have a Mediterranean aspect (Radu, 1958). The larger and the most outstanding is *T. trilobatus*, the species with the smallest distribution range, as it is present only in the region of the Herculane Spa, in south-western Romania (Tomescu *et al*., 2015). Even in the last years, *T. trilobatus* has been mentioned in the Herculane Spa area (Pop *et al*., 2019), although upstream from its previous records (Ferenţi *et al*., 2020). Its small distribution range should facilitate the knowledge of the species, but this is not a fact, as *T. trilobatus* is an elusive species, related to restrictive habitats, either gorges and steep limestone walls from forested areas, or caves (e.g., Tabacaru and Giurginca, 2013; Tomescu *et al*., 2015; Pop *et al*., 2019; Ferenţi *et al*., 2020). Thus, neither its' distribution nor its ecology, is fully understood. Recent morphological data are also scarce and presented with classic light microscopy methods (Tomescu *et al*., 2015), although scanning electron microscope is considered an appropriate method to investigate the cuticle surface of terrestrial isopods (Hornung, 2011). Considering that SEM was often used to describe the cuticle of terrestrial isopods (Holdich and Lincoln, 1974; Schmalfuss, 1978, 2011; Ziegler and Altner, 1995; Giurginca *et al*., 2016; Vittori and Gantar, 2020), and due to the zoogeographic and conservation importance of *T. trilobatus* (Tomescu *et al.,* 2015; Ferenţi *et al.,* 2020), we proposed to study the cuticle morphology of this species with SEM. We hypothesized that SEM would offer useful information about *T. trilobatus* morphology, which could help in understanding its ecology, and its relationship with the epigeic or cavernicolous environment. Our objective was to describe in detail, for the first time, the morphological characters, and the cuticle of this species with SEM.

Materials and methods

The study with SEM was performed in the summer of the year 2021. We analyzed two *T. trilobatus* individuals from Herculane Spa, which were utilized in the previous study (Tomescu *et al*., 2015). The studied individuals were conserved in test tubes with ethylic alcohol. The methods used in the SEM study

were the same used for terrestrial isopods by other authors (Holdich and Lincoln, 1974; Schmalfuss, 1978, 1998; Meyer-Rochow, 1980; Ziegler and Altner, 1995; Csonka *et al*., 2018). Thus, the isopods were dehydrated, fixed, and then covered with a 2 nm layer of gold using the Quorum Q T150 ES Magnetron Sputtering. The Electron Microscope used is a Leo 438 VP SEM, with an acceleration voltage of 20 kV at various magnifications according to the size of the structure studied. We investigated both the cuticle external morphology, but also anatomic elements with taxonomic characters, which were previously used for determining the species (Tomescu *et al*., 2015). Thus, we tried to obtain clear images of the appendages, head, eyes, etc. Also, we investigated the structure of the cuticle and observed its structures, compared them with the data from the literature (Holdich and Lincoln, 1974; Schmalfuss, 1978; Ziegler and Altner, 1995; Giurginca *et al*., 2016; Csonka *et al*., 2018). Totally, we analyzed 234 SEM images of different body segments and cuticles of the two studied *T. trilobatus* individuals.

Results

The head, like the entire dorsal part of the isopod, is covered with numerous micro-scales and tricorn sensilla (Fig. 1). The eyes consist of 24 ommatidia, between which tricorn sensilla can be present, especially at the marginal ones (Fig. 1). The cephalic lobes are long. The length of the lateral lobes is almost half of the total distance between the lobes' tip and the posterior edge of the head. The frontal lobes' length exceeds half of the lateral lobes' length. The frontal lobe ends with a short rostrum. Even with these long cephalic lobes, the head width is larger than its length, and clearly exceeded anteriorly by the epimers of the first thoracic segment (Fig. 1).

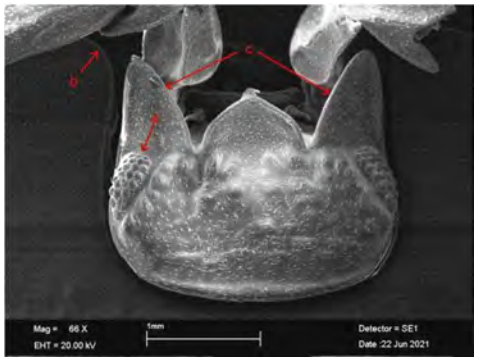

Figure 1. *T. trilobatus* head a. eyes, b. anterior margin of the first epimer, c. lateral lobes

The first antennae are situated in the space between the lateral lobes and the frontal lobe, formed by three segments, of which the last one has numerous sensilla (Fig. 2 a). The second antennae segments are covered with small micro-scales and tricorn sensilla, and the antennae flagellum presents spines (Fig. 2 b). The flagellum is shorter than the segment before and continues with the terminal organ, which has the aspect of a short extension, with slightly widened tip. The terminal organ has a smooth tip (it is the only component of the second antennae with smooth cuticle) (Fig. 2 c, d).

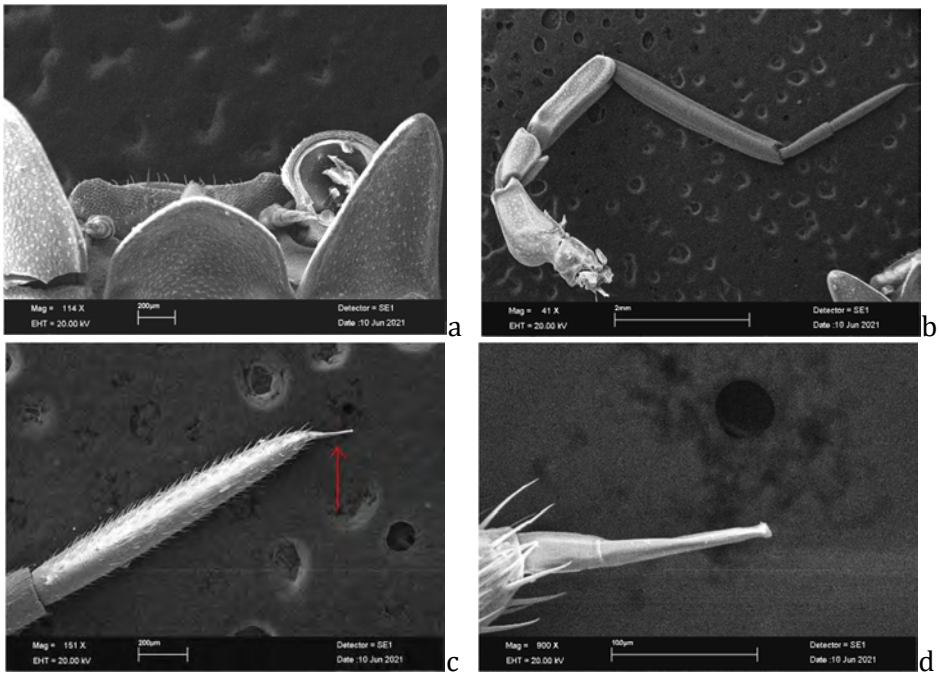

Figure 2. *T. trilobatus* a. first antennae, b. second antennae,
c. second antennae tip with the terminal organ (shown by the arrow),
d – terminal organ of the second antenna

On the dorsal side of the pereon *T. trilobatus* presents numerous micro-scales, which form a continuous layer (Fig. 3 a). Beside scales, numerous uniformly distributed tricorn sensilla are present on the dorsal surface of the animal (Fig. 3 a). The pleon is covered with tricorn sensilla, just as the rest of the dorsal side of the animal. The tip of some pleonal epimers can present relatively large-sized terminal setae (Fig. 3 b).

On the anterior part of the thoracic epimers, there are glandular pore fields with a regular disposition present at the edges of the epimers (Fig. 3 c).

The glandular pore fields are surrounded by micro-scales and tricorn sensilla. In many cases, the product of the glandular pore fields is also visible. Fields with numerous, supposedly glandular openings are also present on the tergal face of the pleon (Fig. 3 d).

Also on the thoracic segments the noduli laterals are present in their posterior and external part. They have the aspect of long, filiform extensions, connected with the deeper layers of the cuticle (Fig. 3 e). The noduli laterals are present on the thoracic segments 2-7 and are oriented in the caudal direction.

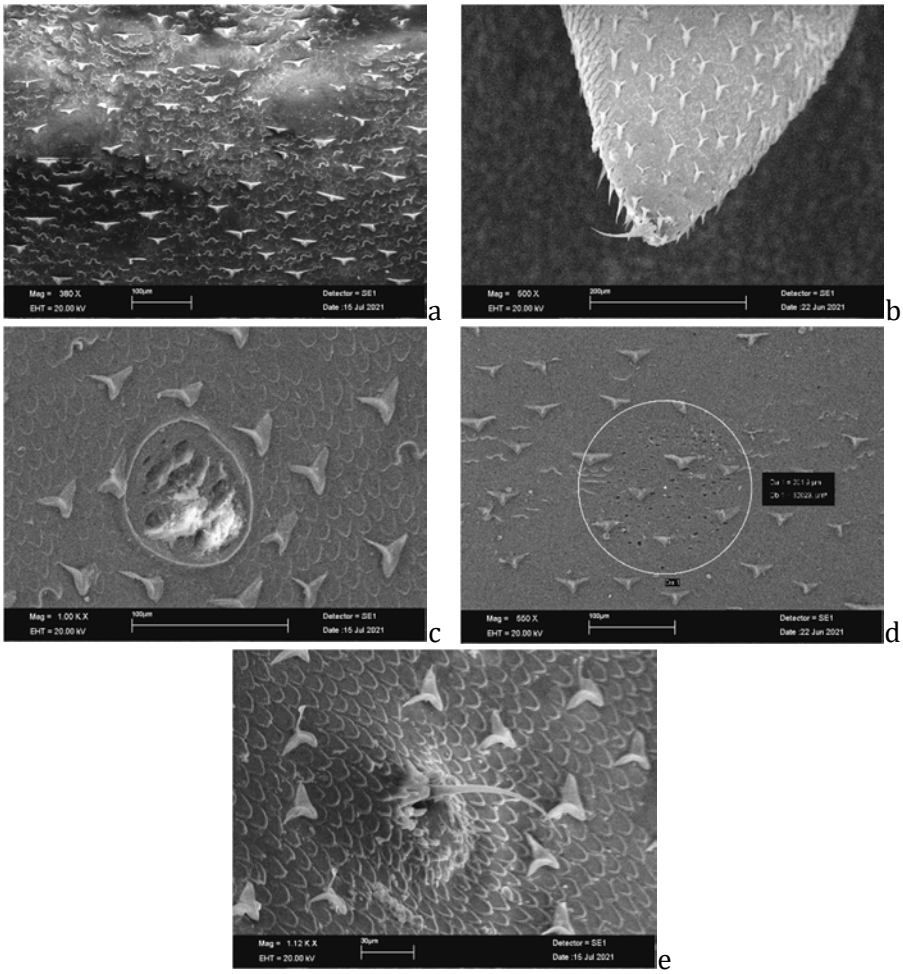

Figure 3. *T. trilobatus* a. the surface of the pereonal segments, b. the edge of a pleonal segment with terminal setae, c. glandular field on the pereonal epimers, d. surface with pores on the mediodorsal part of the pleonal segments, e. nodulus lateralis

Pereopods. In their turn, the legs are covered with micro-scales. The first legs present numerous spines on their distal segments, but also numerous micro-scales which are very evident and compact (Fig. 5 a). The surface of the legs is covered with tricorn sensilla. Unlike the first leg, the seventh leg pair does not present so many spikes on their distal segments, but the micro-scales are equally numerous (Fig. 5 b). The basis does not present numerous cuticular structures, micro-scales are present on the dorsal part, where they form a continuous layer, partially covering one another, and a few tricorn sensilla and thin setae on the rest of the segment. The ischium presents on its ventral part a field with numerous triangular scales, and four very well-developed setae (Fig. 5 c). On the merus, carpus, and propodus the setae are mostly situated dorsally, while the tricorn sensilla are more evenly distributed (Fig. 5 d, e, f, g). The dactyl has some simple elongated scales on its external part, and a few well-developed setae (Fig. 5 h).

Cuticular structures are also present on the pleopods, mostly on the margin as simple setae, the first endopod has mostly a smooth surface, and the first male exopodite presents some tricorn sensilla (Fig. 6 a, b, c).

On the telson the distribution of tricorn sensilla is similar to the one observed on the dorsal part of the body, uropods end with a filiform structure (Fig. 6 d).

Discussion

Differences in the cuticular structures could be explained by the differences in ecology and behavior of different terrestrial isopod species (Schmalfuss, 1978). Thus, description of cuticle structures could help in understanding the ecology of *T. trilobatus*, an endemic species with a very small distribution range (e.g., Tomescu *et al*., 2015; Ferenţi *et al*., 2020). The structures present on the surface of the antenna of *T. trilobatus* are similar to the ones that are present on the antenna of other terrestrial isopod species (e.g., Khisametdinova and Schmalfuss, 2012). This resemblance is even higher in comparison with other species of *Trachelipus* genus, including the terminal organ at the tip of the second antenna (Schmalfuss and Khisametdinova, 2015). In the case of terrestrial isopods, the second antenna is considered the most important sensory organ (Schmalfuss, 1998), thus it is expectable to have a similar structure within the same genus.

Micro-scales are common elements in the cuticle of terrestrial isopods (Holdich and Lincoln, 1974; Schmalfuss, 1978; Price and Holdich, 1980; Wood *et al*., 2017; Štrus *et al*., 2019), thus their abundance in this species is not surprising. Tricorn sensilla are numerous and uniformly distributed on the dorsal

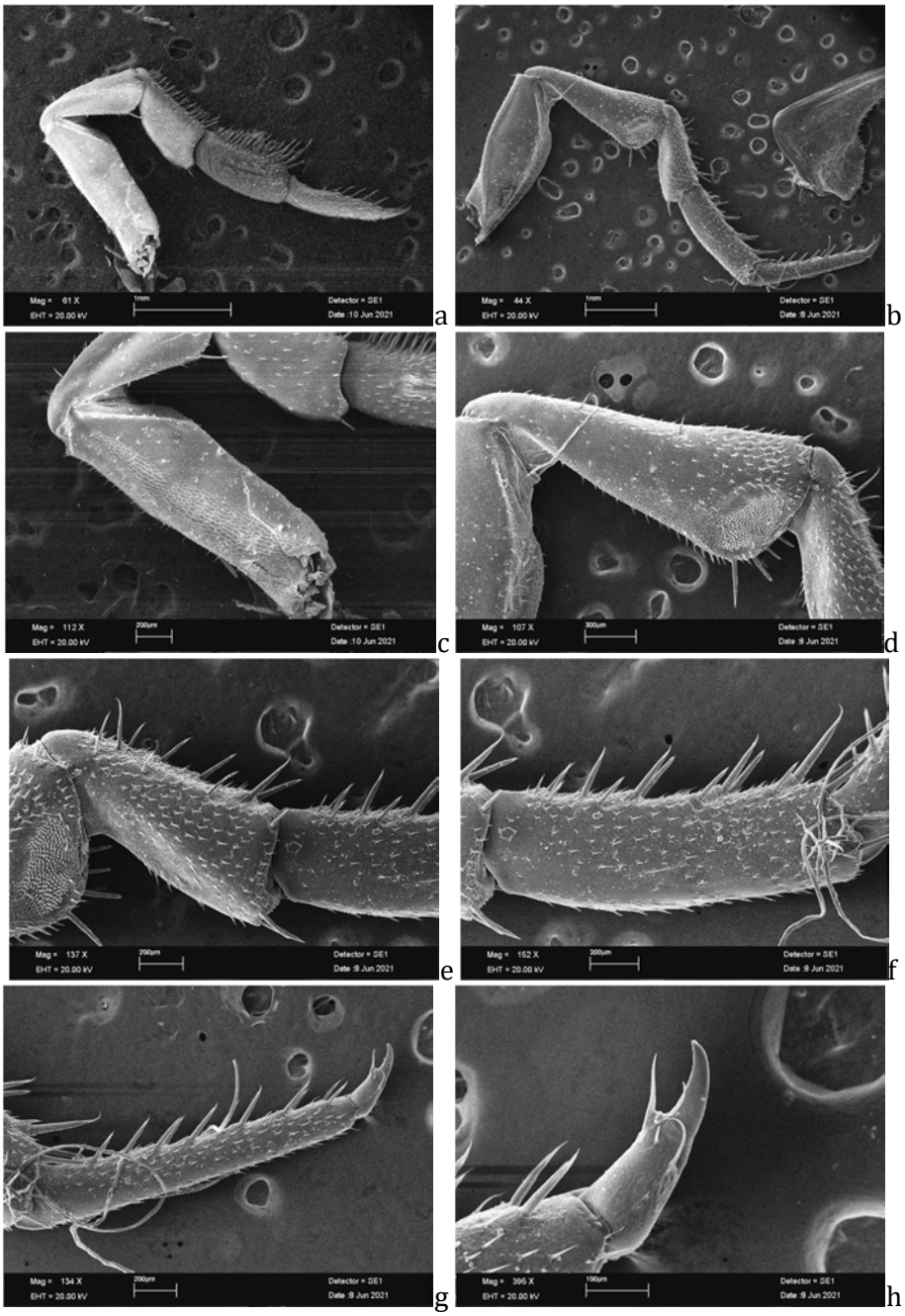

Figure 5. *T. trilobatus* a. first pereiopod, b. 7th pereiopod, and its segments: c. basis, d. ischium, e. merus, f. carpus, g. propodus, h. dactyl

side of *T. trilobatus*. These structures are present in terrestrial isopods (e.g., Holdich and Lincoln, 1974; Price and Holdich, 1980; Hatanaka, 1989; Csonka *et al*., 2018; Štrus *et al*., 2019; Seidl *et al*., 2021), but they lack in aquatic isopods (e.g., Schmalfuss, 1978; Powell and Halcrow, 1982). They were assigned the role of hygroreceptors (Price and Holdich, 1980), although there are uncertainties about their exact function (Hatanaka, 1989; Ziegler and Altner, 1995). The high number of these cuticular formations indicated that *T. trilobatus*, at least in one phase during its past, evolved in environments in which the level of humidity changed rapidly.

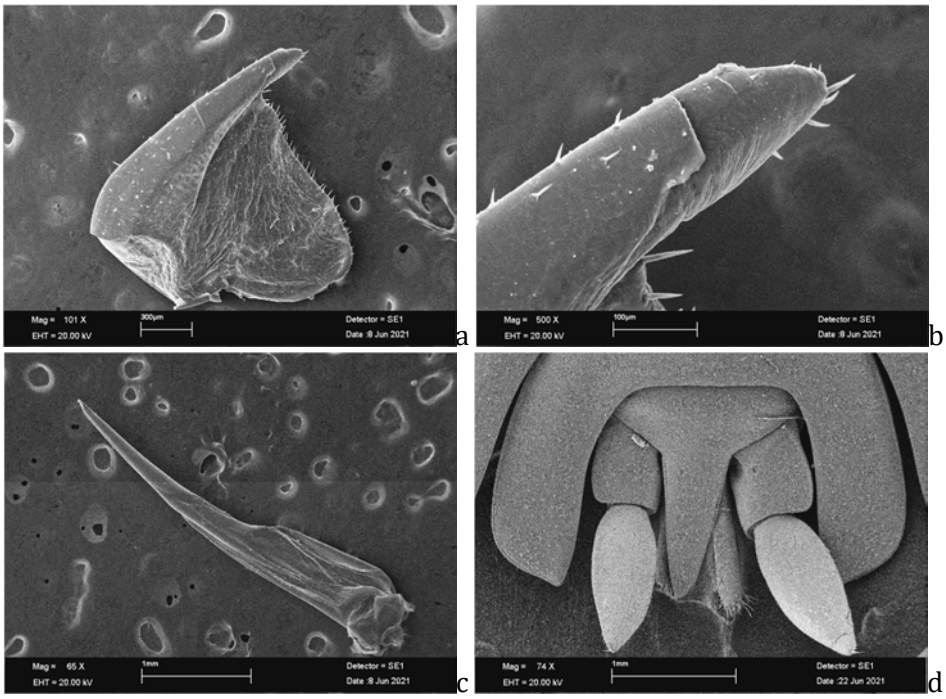

Figure 6. *T. trilobatus* a. first male exopod and b. the exopod's tip, c. first male endopod, d. telson and uropods

Thus, for the species, it was very important to react quickly to those changes in order to avoid dehydration. It seems that in the case of isopods the water loss occurs passively, and the larger size reduces this loss (Dias *et al*., 2012), and *T. trilobatus* is a larger species (Radu, 1985; Tomescu *et al*., 2015). This fact probably indicates that initially, *T. trilobatus* was an epigeic animal, because in the case of epigeic terrestrial isopods this type of sensilla are numerous (Holdich and Lincoln, 1974; Price and Holdich, 1980; Csonka *et al*.,

2018; Štrus *et al*., 2019; Seidl *et al*., 2021). Thus, although *T. trilobatus* was frequently identified in caves (Tabacaru and Giurginca, 2013; Tomescu *et al*., 2015), and was even considered by some authors as a troglobitic species (Boitan and Negrea, 2001), it was probably initially epigeic.

Probably even nowadays *T. trilobatus* moves between the numerous limestone cracks and caves from Herculane Spa and the soil surface, where it can be detected only sometimes and in certain habitat types (Tabacaru and Giurginca, 2013; Tomescu *et al*., 2015; Pop *et al*., 2019; Ferenţi *et al*., 2020). It is known that many terrestrial isopod species migrate due to fluctuations in environmental factors (Warburg *et al*., 1984). Therefore, this species' annual cycle should be studied in its surface habitats. Also, from the biogeographic point of view, remains to be seen what determined the species to retreat in the limestone areas in which it has contact with the underground environment. Probably in the glacial periods, once the weather cooled, *T. trilobatus* was "lucky enough" and found the possibility to avoid the effects of climate cooling. Probably, *T. trilobatus* exits and enters the caves and cracks depending on the surface hydric regime. Thus, in the dry and cold periods, the species descends in caves or cracks because it has numerous tricorn sensilla which detect the decrease of humidity. This assumption is sustained by the complete absence of tricorn sensilla in the case of *Mesoniscus graniger* (Giurginca *et al*., 2016), which is a species usually present in caves and rarely endogeic (e.g., Tabacaru and Giurginca, 2013; Giurginca, 2000-2001, 2009; Ferenţi and Covaciu-Marcov, 2018; Pop *et al*., 2021). Thus, in those extremely and constantly humid conditions, it does not need tricorn sensilla (Giurginca e*t al*., 2016). The presence of numerous tricorns in *T. trilobatus* indicates that this is an epigeic species. The climate conditions had a great impact on the species. This impact was particularized by the peculiarities of the Herculane Spa region, which because of the karst (e.g., Povară and Conovici, 2013; Ponta *et al*., 2013; Povară *et al*., 2015) offered access and shelter in caves and cracks to this species.

At the same time, it seems that in the case of the genus *Trachelipus*, there is a high cuticle uniformity, as in the congeneric species *T. rathkii*, the cuticle morphology is very similar, with numerous micro-scales and tricorn sensilla (Csonka *et al*., 2018). The uniformity of the cuticle in the case of the genus *Trachelipus* indicates a high morphological conservatism at this level, despite the obvious morphological differences between the two species (Tomescu *et al*., 2015). At the same time, the two species are present in very different habitats, as *T. rathkii* is considered a eurytopic species (Tomescu *et al*., 2015). However, also in this case the humidity is the connecting factor, hence the function of tricorn sensilla as hygroreceptors (Price and Holdich, 1980). Thus, in different environments, with different humidity, the existence of a high number of

hygroreceptors is very important, and *T. rathkii* was identified starting in the vicinity of thermal waters (Ferenți *et al*., 2013), going in urban areas (e.g., Vilisics and Hornung, 2009; Giurginca *et al*., 2017) and reaching even to Siberia (Khisametdinova *et al*., 2016) and Finland (Vilisics and Terhivuo, 2009). Unlike this, *T. trilobatus* is related to restrictive habitats (Tomescu *et al*., 2015; Pop *et al*., 2019; Ferenți *et al*., 2020) but nevertheless, it has equally numerous tricorn sensilla.

The surface structures and the cuticle formations of terrestrial isopods are considered to have an anti-adhesive function (e.g., Schmalfuss, 1977, 1978; Powell and Halcrow, 1982; Wood *et al*., 2017). This function is maintained also in the case of a cave species, like *Mesoniscus graniger* (Giurginca *et al*., 2016). Probably those structures have the same function also in the case of *T. trilobatus*, moreover because this species shelters under stones in wet areas, where soil particles could easily adhere to the animals' surface. The large surface and the wide aspect of this isopod (Radu, 1985; Tomescu *et al*., 2015) offer a larger contact surface for different soil particles, thus *T. trilobatus* needs numerous anti-adhesive structures.

Noduli laterals, considered sensilla (Schmidt, 2002, 2008) are situated in the lateral edge of the thoracic segments 2-7, and probably had a tactile function. In the case of other species, such sensilla were considered to be gauge sensors (Jans and Ross, 1963; Holdich and Lincoln, 1974). Thus, they can be extremely useful for a species that shelters in limestone cracks, caves, or under stones. At most, the location of this sensilla is curious, as they are situated on the posterior part of the segments, and lack from the first thoracic segment. It seems that *T. trilobatus* is an exception in its genus, having the noduli laterals situated posterior to the glandular fields, a fact considered related to the life in caves and very wet habitats (Radu and Tomescu, 1970). Probably in that position, they are more useful, and the pressure exercised upon them informs the animal to stop advancing in limestone cracks, if they are too tight. At the same time, in the cephalic region, there are receptors at the level of the terminal organ of the antenna. This is probably an adaptation to a way of life in which the animal shelters in very narrow spaces.

The SEM analysis revealed morphological aspects which now are much clearer than in the previous studies realized with classic light microscopy (Tomescu *et al*., 2015). It is the case of the genital parts, which now could be observed distinctly, or the case of the appendages, in which the dactylus is extremely similar to the one recently described in another terrestrial isopod species (Vittori, 2021). This highlights the utility of SEM in helping to understand the adaptations and the way of life of *T. trilobatus*, an endemic species with a very small distribution range (Tomescu *et al*., 2015; Ferenți *et al*., 2020). Thus,

studies on species from different environments are necessary, and also studies on other endemic species, as most previous studies aimed a common species such as *Porcellio scaber* (Holdich and Lincoln, 1974; Ziegler and Altner, 1995; Csonka *et al*., 2018; Vittori, 2021).

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Global warming and avifauna from the Argeş River dam basins (Southern Romania) – long term study case

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Abstract. An attempt to find a link between the global warming, manifested on local scale, and the dynamics of the winter avifauna recorded on the Vâlcele, Budeasa, Bascov, Piteşti and Goleşti Dam Basins from ROSPA0062 Lacurile de acumulare de pe Argeş was achieved in the paper. Based on the data collected between 1999 and 2020 during the MidWinter (the Winter Census of the Wetland Birds), some major conclusions were drawn: the climate change resulted from the analyse of the air temperature registered in the area and it was noticeable in some measure on the phenology of the birds; it influenced the dynamics of the avifauna, as total number of species and individuals, as well as the strength of every species; also, other local and extern elements, like the process of silting of the dam basins, the direct anthropogenic pressure, were involved here.

Keywords: global warming, dynamics of birds, human pressure.

Introduction

The global warming is a delicate topical issue, because not the whole scientific world agrees that it is caused by the human activities (Niederer, 2013). It menaces both the human health and the wildlife. Many species will have to adapt but other won't be able to do this and some predictions show that an increase in temperature of ca. 2°C would induce an extinction of 20-30% of species (Tekalign and Balakrishinan, 2016). Regarding the birds, the climatic

change is causing advanced spring migration, changes of habitat, higher possibility of disease transmission, earlier egg-laying time, less food availability and a decline in the population (Li *et al.*, 2022). Also, the warming influences the time of the autumn migration that are starting earlier (Cotton, 2003) and the choice of the wintering sites (Li *et al.*, 2022). These places can be used as an indicator, since the bird wintering centre of abundance continuously are moving to the North (https://www.epa.gov/). Particularly, the waterbirds show a high degree of adaptability to these challenges, because they establish new quarters of wintering (Fox *et al.*, 2019). Also, the displacement of the breeding range for some wetland species was documented as a consequence of the changes of the latitudinal temperature and of the corresponding changed pattern of precipitations (Soultan *et al.*, 2022), though the wintering bird communities are tracking the climate change faster than the breeding communities (Lehikoinen *et al.*, 2021). A recent study on the European birds revealed that the rising temperatures are affecting even the morphology of the birds, while some species reduced their body size and other increased it (McLean *et al.*, 2022). The changes of the body length affected mainly the migratory birds and the changes in body mass affected mainly the non-migratory birds (Dubiner and Meiri, 2022).

The study of the avifauna of the artificial reservoirs begun from the premise that it helps to see how the human pressure exercises on the natural environment, because it is known that the construction of the dam basins destroys the old habitats and creates new ones. They can become good places to live for the birds, mainly outside of the breeding season, the more so as the adequate management plans are implemented (Munteanu and Mătieş, 1983).

In this context, the ornithofauna of the Argeş River dam basins was the subject of many papers still 1960s-1970s, when the reservoirs started to be built. Mătieş (1969) published the first paper on the theme, in which he talked about the birds from the Vidraru dam basin. Only a few works have longer issued until 1990 (Munteanu and Mătieş, 1983, Munteanu *et al.*, 1989), though the avifauna of the dam basins was in the core of the researches of Mircea Mătieş until 1982, when he prematurely died. Activated by the founding of the local branch of the Romanian Ornithological Society in 1990, the researches have focused on the dam basins between Vâlcele and Goleşti and a series of papers, mainly about the winter avifauna, appeared (Gava, 1997, Mestecăneanu *et al.*, 2003, Gava *et al.*, 2004a, 2004b, Gava *et al.*, 2007, Conete, 2011, Conete *et al.*, 2012, Mestecăneanu *et al.*, 2013, Mestecăneanu & Gava, 2015, 2016a, 2018, 2019a, etc.). Meanwhile, the ornithofauna of the other dam basins from the Argeş River was occasionally approached (Petrescu, 2005, Mestecăneanu and Mestecăneanu, 2018-2019, Mestecăneanu, 2019).

Our goal is to find in what extent the phenomenon of the global warming, reflected on local scale, affected the birds from the dam basins of the Argeş River. Specifically, we aimed to answer following questions: did the global warming manifest in the researched area during the period of study? did the global warming reflect in the birds' phenology? was there a trend over years of the number of species and of the number of individuals? was there a good correlation between the variation of the average temperature of the air and the dynamics of the avifauna?

Materials and methods

1. The natural setting of the study area

The dam basins where the research-study was performed belong to ROSPA0062 Lacurile de acumulare de pe Argeş (in English, The dam basins from the Argeş River). They are characterized by a large variation in size, from 122 ha (Piteşti) to 649 ha (Goleşti), while Vâlcele has 408 ha, Budeasa, 412 ha, and Bascov, 162 ha. The Argeş River springs from the Făgăraş Mountains and flows to Danube, gathering the waters from the Transilvanian Alps, Sub-Carpathians, Gethic Piedmont, and the Romanian Plain. The uppstream reservoirs (Vâlcele, Budeasa and Bascov) are situated between the Cotmeana Piedmont, Argeş Hills and the Cândeşti Piedmont, while the downstream ones (Piteşti and Goleşti) are located in the Piteşti High Plain, a subunit of the Romanian Plain (Fig. 1).

The climate is temperate continental with hilly features in the North and plain traits in the South. As a result, the yearly average temperature is 9 ^{0}C, at Piteşti (Barco and Nedelcu, 1974).

The vegetation of the area depends on the relief. The broadleaf forests with *Fagus sylvatica*, in the colder valleys, and with *Quercus* sp., in the warmer plateaux, predominate and interfere with meadows, orchards and settlements. Some buildings are situated next to the water bodies, mostly the case of Piteşti Town, Budeasa and Vâlcele Villages. Mainly toward the ends of the basins and on the islands formed by the silting process, the vegetation gets aspect of wetlands, with coppices of *Alnus glutinosa*, *Salix* sp., *Populus* sp. and reed beds of *Typha* sp. and *Phragmites australis*.

Regarding the human pressure, the settlements are interconnected through a dense web of roads. A highway passes by the Bascov, Piteşti and Goleşti Dam Basins and belt roads border the reservoirs. Some supermarkets were also built in the vicinity of Piteşti and Goleşti. The fishing, poaching, clearing of trees, and the nautical sports, particularly on Bascov, more rarely on Piteşti, are practiced in the area. While the basins are provided with concrete slopes on large areas, some birds use them for resting, like some herons, ducks and cormorants, or for feeding, like some Passeriformes or waders (Mestecăneanu and Gava, 2018).

2. The data collection on birds

Systematically observations performed during 1999-2020 lay to the basis of this study. Generally, they were gathered in the second weekend of January, during the Winter Census of the Wetland Birds, known better as the MidWinter. This is coordinated in Romania by the Romanian Ornithological Society and by the Milvus Group and, on the European level, by Wetlands International. It is a synchronous event whose main purpose is to estimate the birds' populations of the continent to take adequate measures to protect them (https://milvus.ro/). The dam basins were visited once every month, during a day, between 9:00 and 16:00. The dependent of wetlands birds were counted and, additionally, the other individuals from the basins' perimeter. The itinerary method and the method of observations from the fixed points, where the first was unapplied, were used and, as tools, binoculars, a spotting scope and a photo device. The data were worked in Excel to compute some ecological indicators (the constancy, the dominancy, the Dzuba index of ecological significance), respectively correlations and regressions, by the conventional methods (Gomoiu and Skolka, 2001, Zamfirescu and Zamfirescu, 2008). Species denominations and their main phenologic belonging correspond to the Hamlin Guide (Bruun *et al.*, 1999).

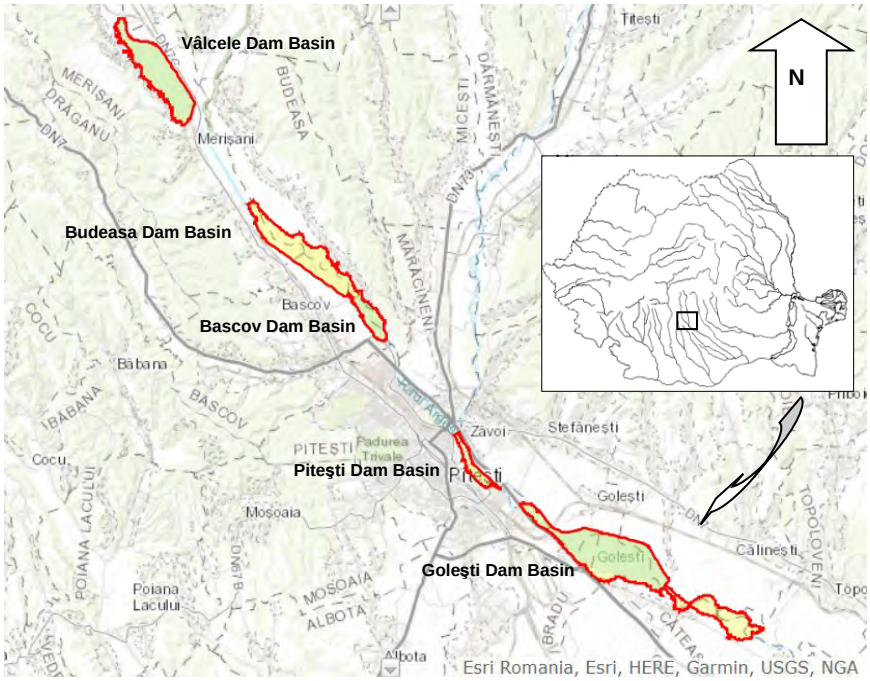

Figure 1. The map of the researched area
(by https://natura2000.eea.europa.eu/, modified).

3. The data collection on clime

The temperature of the air was measured at the Piteşti Weather Station. The values were collected by standard method generally utilised in meteorology. To respond to the questions that underlie this approach, the average temperature of the day was used to calculate the average temperature of January, December, December-January interval, respectively diverse periods of time before the day of observation (3 days, 7 days, 10 days, 14 days, 20 days, 30 days, 45 days, the first 7 days from January, the last 15 days from December, the first 16 days from December), starting from the idea that the temperature of the air is a key factor that influences the dynamics of birds. The trend and the correlation were used to verify this assumption. The data were worked in Excel, too.

Results and discussions

Before discussing the issue of the global warming influence on the dam basins avifauna, we have to establish the context. Thus, during the census, between 1999 and 2020, 88 species of birds were observed in the area and 275,530 individuals. Among them, 38 species (43.8%) represented by 267,759 individuals (97.17%) were dependent on wetlands (Tab. 1). 8 species belong to the Annex I of the Birds Directive 2009/147/CE: most of them (7 species, *Gavia arctica*, *Pelecanus crispus*, *Phalacrocorax pygmeus*, *Cygnus cygnus*, *Egretta alba*, *Haliaeetus albicilla* and *Alcedo atthis*) are dependent on wetlands and 1 species (*Circus cyaneus*) is a terrestrial species.

Table 1. The species of birds observed in the area, the constancy, dominance, Dzuba index of ecological significance, the main phenology in Romania and the percentage increase of strengths from 1999-2009 to 2010-2020.

No.	Species	Constancy	Category of constancy	Dominance	Category of dominance	Dzuba index of ecological significance	Category of Dzuba index of ecological significance	Main phenology in Romania	Percentage increase (1999-2009 – 2010-2020)
1	*Gavia arctica**	9.09	C1	0.00	D1	0.00	W1	W	0
2	*Podiceps cristatus**	72.73	C3	0.10	D1	0.07	W1	S	73.20
3	*Podiceps grisegena**	4.55	C1	0.00	D1	0.00	W1	S	-100
4	*Podiceps nigricollis**	22.73	C1	0.01	D1	0.00	W1	PM	325.00
5	*Tachybaptus ruficollis**	100	C4	0.52	D1	0.52	W2	S	-30.33
6	*Pelecanus crispus**	4.55	C1	0.00	D1	0.00	W1	S	x

7	*Phalacrocorax carbo**	77.27	C4	0.84	D1	0.65	W2	S	523.44
8	*Phalacrocorax pygmeus**	86.36	C4	0.28	D1	0.24	W2	S	73.59
9	*Egretta alba**	77.27	C4	0.08	D1	0.06	W1	S	76.62
10	*Ardea cinerea**	90.91	C4	0.05	D1	0.04	W1	S	-17.65
11	*Cygnus olor**	100	C4	2.18	D3	2.18	W3	PM	-5.91
12	*Cygnus cygnus**	63.64	C3	0.14	D1	0.09	W1	W	498.21
13	*Anser albifrons**	40.91	C2	0.73	D1	0.30	W2	W	151.39
14	*Anas platyrhynchos**	100	C4	41.89	D5	41.89	W5	PM	10.60
15	*Anas strepera**	13.64	C1	0.00	D1	0.00	W1	S	x
16	*Anas acuta**	18.18	C1	0.00	D1	0.00	W1	P	-25.00
17	*Anas penelope**	77.27	C4	0.39	D1	0.30	W2	P	851.46
18	*Anas crecca**	100	C4	5.46	D4	5.46	W4	P	107.13
19	*Anas clypeata**	13.64	C1	0.01	D1	0.00	W1	P	x
20	*Tadorna tadorna**	54.55	C3	0.05	D1	0.02	W1	S	6,050
21	*Netta rufina**	4.55	C1	0.01	D1	0.00	W1	S	x
22	*Aythya marila**	4.55	C1	0.00	D1	0.00	W1	W	-100
23	*Aythya fuligula**	90.91	C4	2.37	D3	2.15	W3	W	110.71
24	*Aythya ferina**	86.36	C4	9.36	D4	8.08	W4	PM	280.72
25	*Aythya nyroca*	27.27	C2	0.00	D1	0.00	W1	S	75.00
26	*Bucephala clangula**	86.36	C4	0.64	D1	0.55	W2	W	572.49
27	*Mergus albellus**	72.73	C3	0.05	D1	0.04	W1	W	-25.93
28	*Haliaeetus albicilla**	4.55	C1	0.00	D1	0.00	W1	PM	-100
29	*Buteo lagopus*	4.55	C1	0.00	D1	0.00	W1	W	-100
30	*Buteo buteo*	90.91	C4	0.03	D1	0.03	W1	PM	50
31	*Accipiter gentilis*	9.09	C1	0.00	D1	0.00	W1	R	-100
32	*Accipiter nisus*	31.82	C2	0.00	D1	0.00	W1	R	-42.86
33	*Circus cyaneus*	27.27	C2	0.00	D1	0.00	W1	W	-25.00
34	*Falco tinnunculus*	59.09	C3	0.01	D1	0.00	W1	PM	33.33
35	*Perdix perdix*	22.73	C1	0.02	D1	0.00	W1	R	-86.96
36	*Phasianus colchicus*	18.18	C1	0.00	D1	0.00	W1	R	700
37	*Gallinula chloropus**	54.55	C3	0.02	D1	0.01	W1	S	-14.71
38	*Fulica atra**	100	C4	10.31	D5	10.31	W5	PM	-1.13
39	*Gallinago gallinago**	18.18	C1	0.00	D1	0.00	W1	P	-83.33
40	*Tringa ochropus**	45.45	C2	0.01	D1	0.00	W1	P	-57.14
41	*Larus argentatus**	100	C4	4.35	D3	4.35	W3	R	110.31
42	*Larus canus**	86.36	C4	5.18	D4	4.48	W3	W	521.84
43	*Larus ridibundus**	100	C4	12.13	D5	12.13	W5	PM	150.32
44	*Columba palumbus*	9.09	C1	0.10	D1	0.01	W1	S	x
45	*Streptopelia decaocto*	63.64	C3	0.02	D1	0.01	W1	R	557.14
46	*Alcedo atthis**	18.18	C1	0.00	D1	0.00	W1	PM	300
47	*Picus viridis*	18.18	C1	0.00	D1	0.00	W1	R	x
48	*Picus canus*	9.09	C1	0.00	D1	0.00	W1	R	0
49	*Dedrocopos major*	27.27	C2	0.00	D1	0.00	W1	R	500
50	*Dendrocopos syriacus*	4.55	C1	0.00	D1	0.00	W1	R	x
51	*Galerida cristata*	31.82	C2	0.01	D1	0.00	W1	R	-81.25
52	*Anthus spinoletta*	68.18	C3	0.05	D1	0.03	W1	S	-65.96
53	*Motacilla cinerea**	9.09	C1	0.00	D1	0.00	W1	S	x
54	*Motacilla alba*	9.09	C1	0.00	D1	0.00	W1	S	-75.00
55	*Lanius excubitor*	36.36	C2	0.00	D1	0.00	W1	PM	-33.33

56	*Sturnus vulgaris*	9.09	C1	0.00	D1	0.00	W1	PM	-100
57	*Garrulus glandarius*	22.73	C1	0.00	D1	0.00	W1	R	600
58	*Pica pica*	95.45	C4	0.27	D1	0.26	W2	R	-13.75
59	*Corvus monedula*	72.73	C3	0.40	D1	0.29	W2	R	12.65
60	*Corvus frugilegus*	90.91	C4	0.66	D1	0.60	W2	R	-24.07
61	*Corvus corone cornix*	90.91	C4	0.06	D1	0.06	W1	R	-38.89
62	*Corvus corax*	81.82	C4	0.07	D1	0.06	W1	R	-40.50
63	*Troglodytes troglodytes*	22.73	C1	0.00	D1	0.00	W1	S	300
64	*Prunella modularis*	9.09	C1	0.00	D1	0.00	W1	S	-100
65	*Regulus regulus*	13.64	C1	0.00	D1	0.00	W1	PM	x
66	*Phoenicurus ochruros*	9.09	C1	0.00	D1	0.00	W1	S	x
67	*Turdus merula*	45.45	C2	0.01	D1	0.00	W1	PM	42.86
68	*Turdus viscivorus*	4.55	C1	0.00	D1	0.00	W1	PM	-100
69	*Turdus pilaris*	31.82	C2	0.07	D1	0.02	W1	PM	92.42
70	*Parus palustris*	4.55	C1	0.00	D1	0.00	W1	R	x
71	*Parus caeruleus*	72.73	C3	0.04	D1	0.03	W1	R	102.78
72	*Parus major*	81.82	C4	0.04	D1	0.03	W1	R	230.43
73	*Aegithalos caudatus*	9.09	C1	0.00	D1	0.00	W1	R	x
74	*Sitta europaea*	9.09	C1	0.00	D1	0.00	W1	R	x
75	*Passer domesticus*	59.09	C3	0.07	D1	0.04	W1	R	548.00
76	*Passer montanus*	68.18	C3	0.12	D1	0.08	W1	R	42.42
77	*Fringilla coelebs*	68.18	C3	0.13	D1	0.09	W1	PM	48.99
78	*Fringilla montifringilla*	9.09	C1	0.01	D1	0.00	W1	W	100
79	*Pyrrhula pyrrhula*	9.09	C1	0.00	D1	0.00	W1	R	x
80	*Coccothraustes coccothraustes*	9.09	C1	0.00	D1	0.00	W1	R	-100
81	*Carduelis chloris*	50.00	C2	0.02	D1	0.01	W1	R	310
82	*Carduelis spinus*	22.73	C1	0.03	D1	0.01	W1	PM	1,660
83	*Carduelis carduelis*	81.82	C4	0.38	D1	0.31	W2	R	-52.99
84	*Carduelis cannabina*	27.27	C2	0.04	D1	0.01	W1	PM	736.36
85	*Emberiza schoeniclus**	59.09	C3	0.02	D1	0.01	W1	PM	-33.33
86	*Emberiza citrinella*	77.27	C4	0.14	D1	0.11	W2	R	383.33
87	*Plectrophenax nivalis*	4.55	C1	0.00	D1	0.00	W1	W	x
88	*Miliaria calandra*	4.55	C1	0.00	D1	0.00	W1	PM	x

Legend: * – species dependent on wetlands; C1 – occasional species, C2 – accessory species, C3 – constant species, C4 – euconstant species; D1, W1 – subrecedent species, D2, W2 – recedent species, D3, W3 – subdominant species, D4, W4 – dominant species, D5, W5 – eudominant species; PM – partial migratory species, W – winter visitor, S – summer visitor, R – resident species; P – species of passage; x – no meaning operation.

The species and the individuals were not equally distributed on the dam basins and the situation do not differ much if we consider the species dependent on wetlands (Tab. 2). In terms of percentual weight, that expresses the percent of species, respectively individuals registered on each dam basin of all species, respectively individuals registered on whole studied area, it is obvious that

the trend of the total number of individuals was strongly increasing from upstream to downstream. As a result, the size of the dam basins and the type of the habitats had a secondary role in the birds' distribution. Instead, the influence of the nautical base from the Bascov Dam Basin was clear and it becomes more obvious if we compare Bascov to Piteşti, the later with even a lower area than the first. While the two are the smallest of all dam basins and have relatively similar habitats, Piteşti is the second as number of individuals, after Goleşti, the largest one, while Bascov is the latest. The position on the river course is less important regarding the number of species, which remained stable around value of 60 and that, however, tended to increase from upstream to downstream in the case of the species dependent on wetlands. The smallest reservoirs remarked through the maximum (the Piteşti Dam Basin), respectively the minimum (the Bascov Dam Basin) number of species, which highlights, again, the importance of the anthropological pressure for the avifauna (Fig. 2). It is worth to mention, too, that Piteşti Dam Basin follows by Bascov Dam Basin on the river course.

Table 2. The weight of species and individuals (%) on each dam basin of all registered species, respectively individuals.

Dam basin	Goleşti	Piteşti	Bascov	Budeasa	Vâlcele
Species weight	69.32	77.27	54.55	64.77	76.14
Individuals weight	54.09	20.62	2.48	14.96	7.86
Species weight*	81.58	81.58	57.89	73.68	78.95
Individuals weight*	54.80	20.12	2.38	15.07	7.63

Legend: * - for the species dependent on wetlands.

The occasional species and the subrecedent species prevailed, both by the dominance index and by the Dzuba index of ecological significance (Tab. 1, 3). It is noticeable that no species was recedent by the dominance index, and all subdominant, dominant and eudominant species, the two considered indices, are also euconstant species. The eudominant species totalised 64.32% of all strengths and the dominant species, 19.99%.

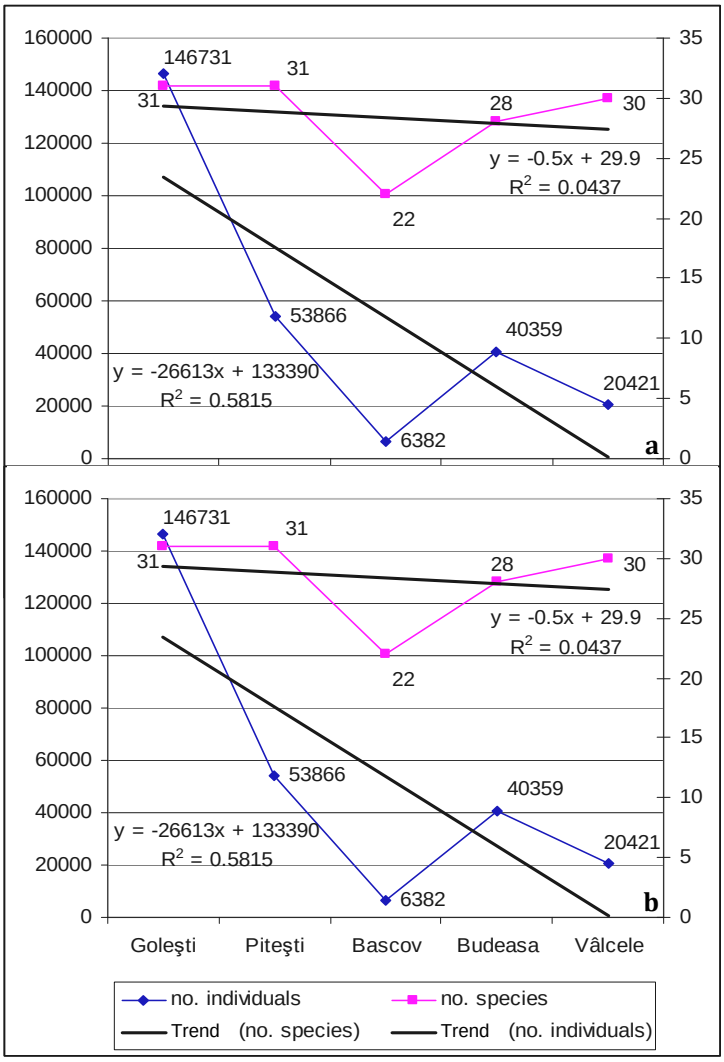

Figure 2. The distribution of species and individuals (a), respectively of species and individuals dependent on wetlands (b) on each dam basin.

Table 3. The distribution of species and their percent of all, by the categories of constancy, dominance and Dzuba index of ecological significance.

Class		1	2	3	4	5
C	**No. species**	38	12	14	24	-
	Weight (%)	43.18	13.64	15.91	27.27	-
D	**No. species**	79	0	3	3	3
	Weight (%)	89.77	0.00	3.41	3.41	3.41
W	**No. species**	68	11	4	2	3
	Weight (%)	77.27	12.50	4.55	2.27	3.41

Legend: constancy (C), 1 – occasional species, 2 – accessory species, 3 – constant species, 4 – euconstant species; dominance (D) and Dzuba index of ecological significance (W), 1 – subrecedent species, 2 – recedent species, 3 – subdominant species, 4 – dominant species, 5 – eudominant species.

Related on the subject of the influence of the global warming on the avifauna of the dam basins, there are some questions we have to respond, as follows.

1. Did the global warming manifest in the researched area?

A strong trend of increase of the average air temperature there was both in January and December, and well as in the period December-January when the observations were performed, but the spreading of the points on the graph around the regression lines is very height, which means a low correlation between the variables. The average temperatures varied very much, even between consecutive years, e.g., the minimum of January (-3.72°C) was recorded in 2006, while the maximum (4.84°C) was recorded in 2007 (Fig. 3). We considered that, for an increase/decrease of more 5% a period, there was a strong increase/decrease, for an increase/decrease of less 5% a period, there was a moderate increase/decrease, and for an increase/decrease of less 1% a period, there was a stable situation.

However, if we compare the period 1998-2008 to the period 2009-2020 (both of 11 years), we see that the average air temperature for December, preceding the time of observations, increased with 1.21°C, from 0,49°C to 1.70°C. For January, when the observations were performed, it decreased with 0.17°C, from -0.20°C to -0.37°C, and for December-January, it increased with 0.52°C, from 0.14°C to 0.66°C. So, the winters seem to arrive later and to become warmer, though January seems to get a little colder, in spite of the increasing trend for the whole interval 1999-2020.

Considering the whole period, the average for December-January was 0.40°C, while for December it was 1.10°C and for January, -0.29°C. By comparison with the average of the air temperature recorded during 1900-1970 (Gava, 2009), the changing is more obvious, the temperature increasing with 0.90°C in December and with 2.30°C in January. Although the Pitesti Weather Station, where the

measurements were made, is located on the outskirts of the city, the increase in average air temperature is probably correlated to some extent with the urban development, too.

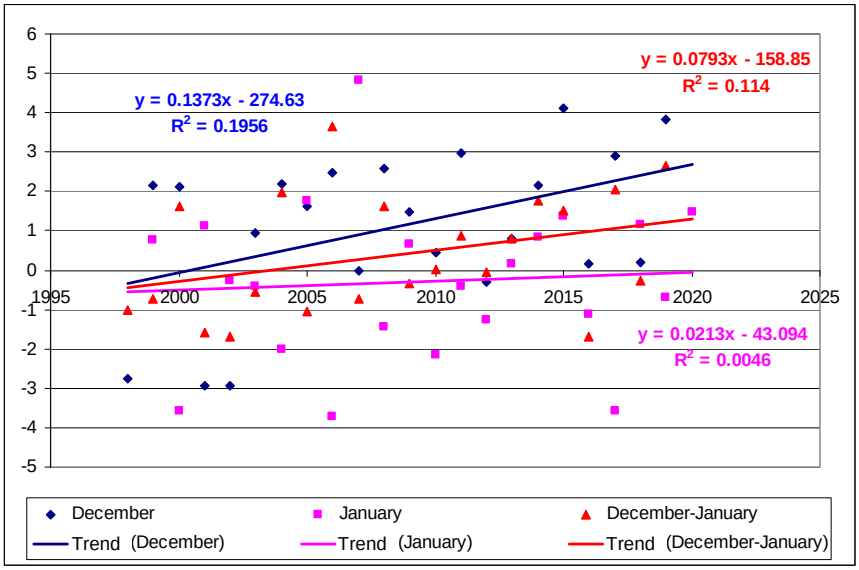

Figure 3. The distribution of the average values of temperature in December, January and in December- January for 1999-2020, at Piteşti.

2. Did the global warming reflect in the birds' phenology?

The weight of the partial migratory species and the weight of the winter visitors decreased from 1999-2009 to 2010-2020, and, instead, the weights of the summer visitors, resident species and species of passage increased. Regarding the species dependent on water, the situation is relatively similar, except for the resident species, for which the weight decreased and not increased. Additionally, we see that the later species had a much smaller importance than in the case of all species and that the resident species, followed by the partial migratory species, had the highest weights among all species, while the summer visitors had the highest weight among the species dependent on wetlands.

By Bruun *et al.* (2009), all eudominant species (*Anas platyrhynchos*, *Fulica atra* and *Larus ridibundus*) are considered migratory species, while, among the dominant species, *Anas crecca* is considered species of passage, *Aythya ferina*, migratory species and *Larus canus*, winter species.

As number of individuals, the partial migratory species decreased as weight, from 1999-2009 to 2010-2020, while the other phenological categories considered to the national level increased, and, here, the winter visitors catch

the eye, because their share of the total increased from 4.74 to 11.91 (Tab. 4). Practically, they increased with 299.52% from 1999-2009 to 2010-2020, while the partial migratory species increased with only 40.24%, the summer visitors increased with 114.57%, the passage species increased with 122.02% and the resident species increased with 58.15%, where the total number of individuals increased with 58.86%. The increase of the winter visitors is mainly owned to *Larus canus*, dominant species, because its strength increased with 521.84%, while *Aythya fuligula*, subdominant species, increased with 110.71%. The other winter visitors are subrecedent species (their importance in the ecosystem is low as number of individuals), and, here, it is worth to mention the species dependent on wetlands: *Cygnus cygnus*, *Anser albifrons*, *Bucephala clangula*, whose strengths increased with 498.21%, 151.39%, respectively 572.49%, and *Aythya marila* and *Mergus albellus*, whose strengths decreased with 100%, respectively 25.93%. The two species of gulls, with relatively similar predilection of habitats, also showed increasing strengths with 110.31%, for *Larus argentatus* (resident species, today divided into several taxa), and with 150.32%, for *Larus ridibundus* (partial migratory species). Huge increasing strengths had *Tadorna tadorna* (6,050%), summer visitor, *Carduelis spinus* (1,660%), partial migratory species, *Anas penelope* (851.46%), passage species, *Carduelis cannabina* (736.36%), partial migratory species, *Phasianus colchicus* (700.00%), resident species, *Garrulus glandarius* (600.00%), resident species etc. (Tab. 1). As a result, it means that the phenology of the species was influenced not only by the global warming, but also by other factors such as the development of the habitats (the apparition of new bare shores, shallow waters, coppices, reed beds etc.).

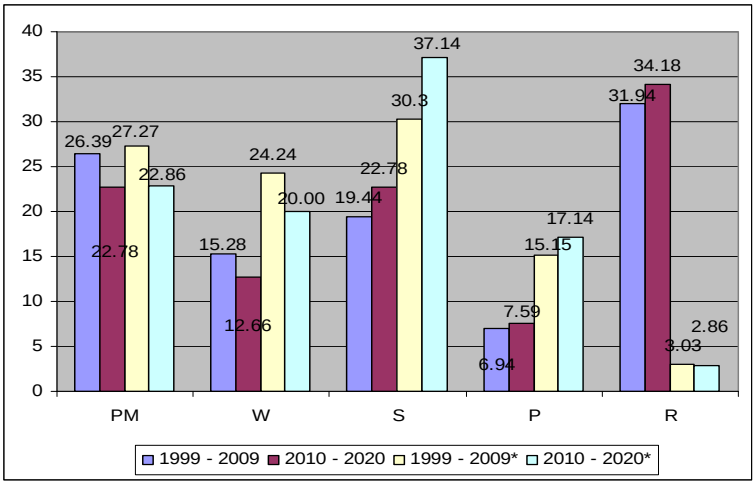

Figure 4. The percent distribution of species and of species dependent on water (*) by the main phenologic categories they belong (PM – partial migratory species, W – winter visitors, S – summer visitors, P – species of passage, R – resident species).

Table 4. The percentage distribution as number of individuals by the main phenological categories.

Main phenology	PM	W	S	P	R
1999-2009	82.13	4.74	1.72	4.72	6.69
2010-2020	72.51	11.91	2.33	6.60	6.67

Legend: PM – partial migratory species, W – winter visitors, S – summer visitors, P – species of passage, R – resident species.

3. Was there a trend over years of the number of species and of the number of individuals?

Not all species from a certain area have the same ecological demands, and while the global warming up to a particular limit is useful for some, it is unfavourable for others and has less relevance for the rest. As a result, the strength of any species will increase for a well delimited interval of temperature, will remain relatively stable for other interval of temperature, and will decrease for another interval of temperature, by a distribution of Gauss type. As a result, the variation of the avicoenose strength depends on variation of the strengths of all component species, which, over time, can have different importance in the ecosystem. So, general trends caused by the temperature change can be distinguishable, although they can have ups and downs.

In our case, a strong increasing trend was noticeable for the number of species, 56.73% of the variables being really correlated, and also for the number of individuals, where only 18.40% of the variables were really correlated (Figs. 4, 5). For the species dependent on wetlands, the graphs are quite similar (Figs. 6, 7). The coefficient of determination, $R^2 = 0.6421$, respectively $R^2 = 0.1804$, shows an increase with ca. 8% of the real correlation between the variable, in the case of the number of species, and, practically, no change, in the case of the number of individuals, because it was only slightly influenced by the contribution of the species that not depend on the wetlands.

It is interesting that, if we split the period 1999-2020 in two intervals, we find out that the trend of the individuals of species dependent on wetlands strongly increasing between 1999 and 2012, 74.72% of the variables being really correlated, although an obvious decrease was noticed between 2006 and 2008 (Fig. 8). Eliminating the values from these years, R^2 gets 0.86 and, consequently, the decline can be linked both to the construction of the road of belt of Piteşti town, that extended the highway Bucureşti-Piteşti and that passes in the proximity of the Goleşti, Piteşti and Bascov dam basins, inaugurated in November 2007 and to the building of two commercial sites, placed, effectively, on the banks of the Piteşti, respectively Goleşti dam basins, finalised in May 2007 and June 2008. For 2012-2020, the trend strongly decreased, although

only 26.78% of the variables were really correlated (Fig. 9). It was caused by the decline of the majority of the eudominant and dominant species, as we will see below.

In Europe, the trend of the common species decreased, mainly in the case of the species from the farmlands, although it increasing after 2007 in the case of forest species (https://www.eea.europa.eu/...chart_1). Most of the species dependent on wetlands that winter in Europe had a favourable trend, too (https://www.eea.europa.eu/...dashboard-01) and, here, the species from the Appendix 1 of the Birds Directive manifested a strong increase, except *Mergus albellus* and *Cygnus columbianus*. The species from the Appendix 2 had different trends, for some, like *Anas strepera*, they increasing, and for others, like *Gallinula chloropus*, *Aythya ferina*, *Aythya fuligula*, *Fulica atra*, *Anas platyrhynchos*, they decreasing (https://europe.wetlands.org/).

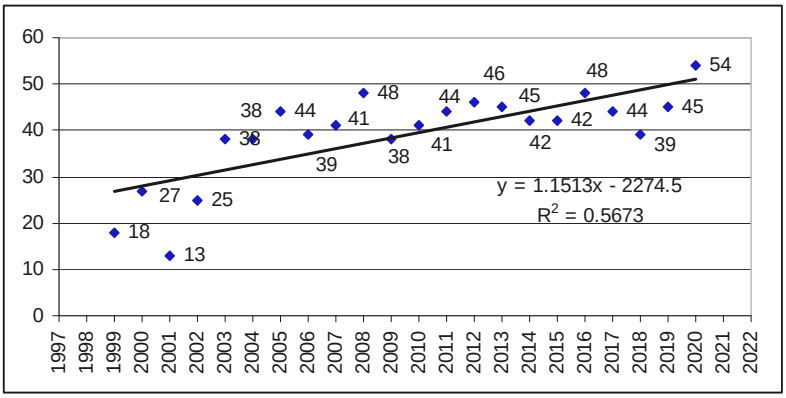

Figure 4. The variation of the number of species during 1999-2020.

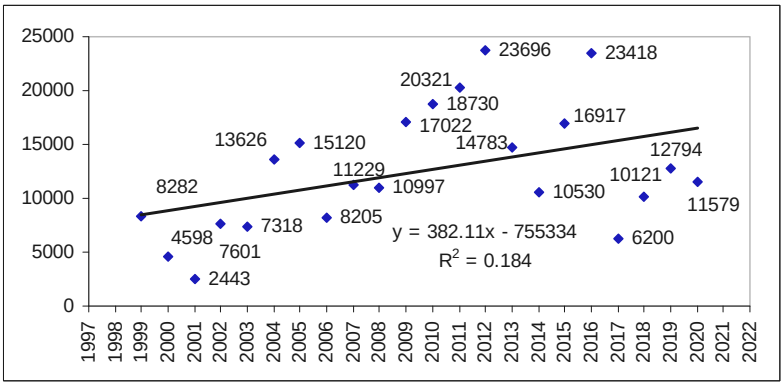

Figure 5. The variation of the number of individuals during 1999-2020.

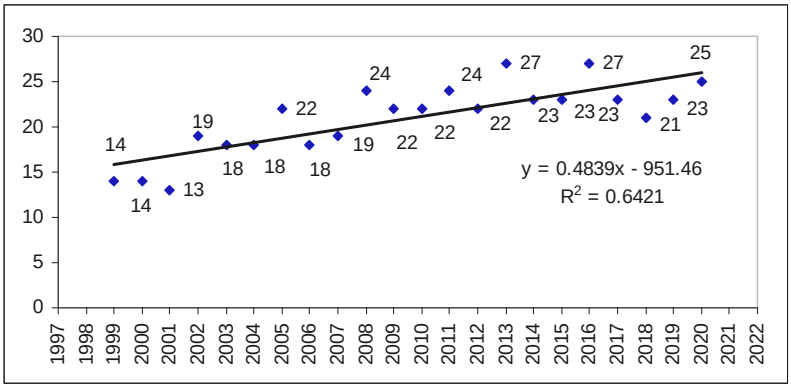

Figure 6. The variation of the number of species dependent on wetland during 1999-2020.

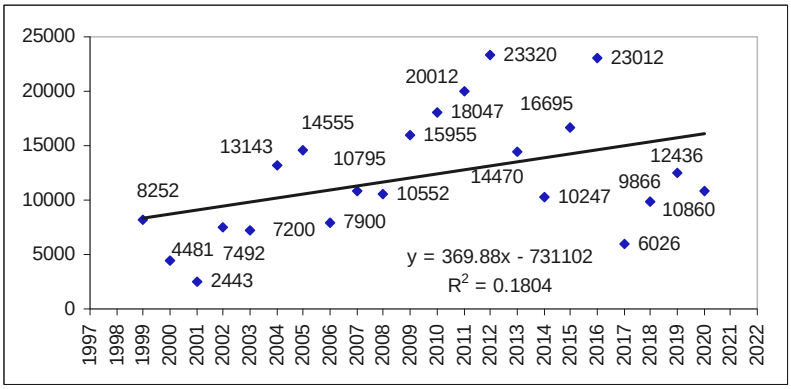

Figure 7. The variation of the number of individuals of species dependent on wetland during 1999-2020.

In our situation, during 1999-2020, *Mergus albellus* and *Gallinula chloropus* showed a stable trend, and *Aythya fuligula*, an increasing trend. For, *Anas platyrhynchos*, eudominant species, the trend was decreasing, while for the other dominant and eudominant species, the trend was increasing, except *Larus ridibundus*, for which it decreased in the last years. The variables are low correlated in the case of *Anas platyrhynchos*, *Fulica atra* and *Anas crecca*, while 30.48% of the variables are really correlated for *Aythya ferina*, 46.63% for *Larus canus*, and 51.23% for *Larus ridibundus*. The regressions lines are: y=-21.814x+49,082 for *Anas platyrhynchos* (R^2=0.0027), y=8.3252x-15,438 for *Fulica atra* (R^2=0.0059), y=117.6x-234,806 for *Larus ridibundus* (R^2=0.5123), y=25.382x-50,321 for *Anas crecca* (R^2=0.0817), y=93.257x-186,228 for *Aythya*

ferina (R^2=0.3048), and y=74.226x-148,508 for *Larus canus* (R^2=0.4663). If we compare the period 1999-2009 to 2010-2020, we see only two species (2.27% of all, *Gavia arctica* and *Picus canus*) with a stable trend, while 55 species (62.5% of all), respectively 24 species dependent on wetlands (27.27% of all, *Podiceps cristatus*, *Podiceps nigricollis*, *Pelecanus crispus*, *Phalacrocorax carbo*, *Phalacrocorax pygmeus*, *Egretta alba*, *Cygnus cygnus*, *Anser albifrons*, *Anas platyrhynchos*, *Anas strepera*, *Anas penelope*, *Anas crecca*, *Anas clypeata*, *Tadorna tadorna*, *Netta rufina*, *Aythya fuligula*, *Aythya ferina*, *Aythya nyroca*, *Bucephala clangula*, *Larus argentatus*, *Larus canus*, *Larus ridibundus*, *Alcedo atthis*, *Motacilla cinerea*) had increasing trends and 31 species (35.22% of all), respectively 13 species dependent on wetlands (14.77% of all, *Podiceps grisegena*, *Tachybaptus ruficollis*, *Ardea cinerea*, *Cygnus olor*, *Anas acuta*, *Aythya marila*, *Mergus albellus*, *Haliaeetus albicilla*, *Gallinula chloropus*, *Fulica atra*, *Gallinago gallinago*, *Tringa ochropus*, *Emberiza schoeniclus*) had decreasing trends. Consequently, 31 non-dependent on wetlands species (35.22% of all) had increasing trends and 18 non-dependent on wetlands species (20.45% of all) had decreasing trends (Tab. 1).

4. Was there a good correlation between the variation of the average temperature of the air and the dynamics of the avifauna?

Generally, the number of species varied inversely proportional with the average air temperature registered in the period of 20 days before the day of observations and directly proportional with the average air temperature registered in the periods that extend 30 days before the day of observations (Tab. 5). Broadly, that means that the lower the average temperature of the air in January before the day of observations, respectively the higher the average temperature of the air in December, the higher the number of species and inversely. Generally, the highest negative correlations were achieved for the first 7 days of January but they were very weak and weak (-0.11 for the species that not depend on wetlands, -0.26 for the dependent on wetlands species and -0.18 for all species). Very weak correlations, which are worth to be mentioned, were also found for the period of 3 days before the observations. The highest positive correlations were got for entire month of December before the observations (0.38 – weak correlation with the total number of species, 0.28 – weak correlation with the number of species dependent on wetlands, 0.40 – moderate correlation with the number of species that not depend on the wetlands). For the period of the first 16 days of December (30-45 days before the observations), the correlation between the average temperature of the air and the number of species was even better, but still moderate, 0.43.

Concerning the number of individuals (Tab. 5), normally, the correlations were positive, regardless the considered period. However, there were exceptions: the correlation for the first 7 days of January was always negative (-0.19 – very weak correlation both in the case of all number of individuals and of the individuals of species dependent on wetlands, -0.22 – weak correlation in the case of the individuals of species that not depend on wetlands). Also, the individuals of the species that not depend on wetlands were very weakly and negatively correlated with the average temperature of the air from other periods up to 20 days before the observations. Weak positive correlations, between 0.26 and 0.31, were obtained in the case of the census day, while, on long term, that exceeded 30 days, the correlations were always positive. The highest correlations were for December (0.40 – moderate correlation) in the case of the total number of individuals, for the last 15 days from December (0.39 – weak correlation) in the case of the individuals of species dependent on wetlands, and for the first 16 days from December (0.55 – moderate correlation) in the case of the individuals of species that not depend on wetlands. These mean that the strengths of the birds were significantly influenced by the average temperature of the days of observations and by the average temperature of the month of December before the time of observations (the higher the temperature, the higher the number of individuals) and by the average temperature of the air recorded in the first 7 days of January (the higher the temperature, the lower the number of individuals), and reversely. Noticeable also is that the individuals of species that not depend on wetlands, generally responded inversely proportionally to the average temperature of the air on short term, except the days of observations.

Table 5. The correlations (C) between the average air temperature (T) and the number of species (S), respectively individuals (I).

Period	Day of observations	1 day before the observations	3 days before the observations	7 days before the observations	First 7 days from January	10 days before the observations	14 days before the observations	20 days before the observations	30 days before the observations	45 days before the observations	Last 15 days from December	First 16 days from December	December before the observations
C. T. – S.	-0.04	-0.11	-0.19	-0.06	-0.18	-0.06	-0.12	-0.07	0.07	0.22	0.32	0.34	0.38
C. T. – I.	0.31	0.24	0.17	0.25	-0.19	0.25	0.07	0.07	0.23	0.30	0.39	0.19	0.40
C. T. – S.*	0.03	-0.04	-0.19	-0.06	-0.26	-0.07	-0.16	-0.12	0.01	0.08	0.26	0.13	0.28
C. T. – I.*	0.31	0.25	0.18	0.25	-0.19	0.25	0.08	0.08	0.23	0.29	0.39	0.18	0.38
C. T. – S.**	-0.08	-0.13	-0.17	-0.05	-0.11	-0.05	-0.09	-0.03	0.09	0.27	0.31	0.43	0.40
C. T. – I.**	0.26	-0.07	-0.12	0.05	-0.22	0.00	-0.07	-0.05	0.14	0.34	0.33	0.55	0.48

Legend: * - for the species dependent on wetlands; ** - for the species that not depend on wetlands.

It is interesting the way in which the eudominant species (*Anas platyrhynchos*, *Fulica atra* and *Larus ridibundus*) and the dominant species (*Anas crecca*, *Aythya ferina* and *Larus canus*) responded to the variation of the average air temperature (Tab. 6). For *Anas platyrhynchos*, usually, the correlations were positive, although the best were weak (maximum 0.28, for the period of 7 days before the observations). For *Fulica atra*, the correlations were sometimes positive, sometimes negative, the best of them being positive, but weak (0.29) and that in the case of the first 16 days of December. For *Larus ridibundus*, in majority, the correlations were positive, the best of them being moderate (0.42 for December, respectively 0.52 for the last 15 days of December). Also, for *Aythya ferina*, the correlations were positive, except for the first 7 days of January (-0.20, weak correlation), the highest of them being moderate (0.43), in the case of December. For *Anas crecca* and *Larus canus*, the correlations were always positive, the highest of them being moderate: 0.47, for the days of observations, respectively 0.46, for the last 15 days from December, and 0.47, for the entire month of December. Regarding the other constant or euconstant species, *Podiceps cristatus*, *Phalacrocorax carbo*, *Ardea cinerea*, *Anas penelope*, *Emberiza schoeniclus* (species dependent on wetlands) and *Parus caeruleus*, *Parus major*, *Passer montanus* (species independent on wetlands) responded in the same rhythm with the temperature variation. It seems they were advantaged by the mild winters, while *Cygnus olor* and *Cygnus cygnus* (species dependent on wetlands) and *Corvus corone cornix* (species independent on wetlands) inversely responded to the temperature variation and as the temperature decreased, the number of individuals increased and vice versa. *Cygnus olor* and *Cygnus cygnus* were constrained to gather here from the surroundings, where the waters were more inclined to freeze, and *Corvus corone cornix* was attracted on the dam basins by the dead birds. It is noticeable that, generally, the correlations were moderate for *Cygnus olor*, except the day of observations and the day before the observations, when they were very week, and the period of 45 days before the observations, when the correlation was strong (-0.71), that means the lower the local average air temperature of the last 45 days before the observations, the higher the number of individuals and inversely. Normally, the strengths of *Egretta alba*, *Bucephala clangula*, *Passer domesticus*, *Carduelis carduelis* and less of *Aythya fuligula*, *Streptopelia decaocto*, *Pica pica*, *Corvus frugilegus*, *Emberiza citrinella* positively correlated with the temperature, while the strengths of *Mergus albellus*, *Buteo buteo*, *Gallinula chloropus*, *Anthus spinoletta* negatively correlated. It should also be noted that, in general, the strengths of *Phalacrocorax pygmeus*, *Tadorna tadorna*, *Falco tinnunculus*, *Corvus monedula* and *Fringilla coelebs* negatively correlated with the temperature on short term and positively, on long term. Finally, about *Tachybaptus ruficollis*, because its

secretive life, a conclusion cannot be formulated (Tab. 6). As a result, we see that some species responded faster than others to the variation of temperature of the air, either positively or negatively, but their dynamics can be determined by both large and local movements.

Table 6. Correlations between the strengths of the constant and euconstant species and the average temperature of the air calculated for different periods from December and January before the time of observations.

Period	Day of observations	1 day before the observations	3 days before the observations	7 days before the observations	First 7 days from January	10 days before the observations	14 days before the observations	20 days before the observations	30 days before the observations	45 days before the observations	Last 15 days from December	First 16 days from December	December before the observations
Podiceps cristatus	0.32	0.20	0.20	0.25	0.25	0.27	0.28	0.24	0.27	0.33	0.16	0.27	0.23
Tachybaptus ruficollis	-0.03	-0.18	-0.16	-0.12	0.24	-0.04	0.03	0.03	-0.08	0.05	-0.18	0.28	0.01
Phalacrocorax carbo	0.18	0.20	0.08	0.20	0.15	0.27	0.20	0.22	0.35	0.34	0.41	0.12	0.42
Phalacrocorax pygmeus	0.00	-0.04	-0.27	-0.26	-0.43	-0.27	-0.37	-0.41	-0.20	-0.16	0.05	0.04	0.16
Egretta alba	0.44	0.32	0.24	0.34	-0.19	0.27	0.13	0.00	0.26	0.38	0.32	0.41	0.48
Ardea cinerea	0.45	0.26	0.28	0.32	0.33	0.35	0.37	0.29	0.35	0.34	0.21	0.11	0.21
Cygnus olor	-0.12	-0.14	-0.42	-0.47	-0.38	-0.47	-0.48	-0.56	-0.64	-0.71	-0.59	-0.44	-0.57
Cygnus cygnus	-0.20	-0.24	-0.36	-0.43	-0.26	-0.41	-0.40	-0.37	-0.33	-0.36	-0.10	-0.23	-0.13
Anas platyrhynchos	0.25	0.22	0.22	0.28	-0.18	0.27	0.10	0.11	0.14	0.15	0.23	-0.02	0.17
Anas penelope	0.22	0.16	0.12	0.31	0.22	0.34	0.30	0.34	0.24	0.29	0.14	0.16	0.20
Anas crecca	0.47	0.23	0.15	0.29	0.12	0.31	0.25	0.25	0.25	0.35	0.25	0.27	0.33
Tadorna tadorna	-0.13	-0.15	-0.08	-0.02	-0.06	0.02	-0.04	0.07	0.11	0.05	0.28	-0.14	0.16
Aythya fuligula	0.42	0.17	0.03	0.14	-0.26	0.11	-0.03	-0.06	0.05	0.15	0.19	0.21	0.28
Aythya ferina	0.29	0.24	0.17	0.21	-0.20	0.21	0.05	0.04	0.22	0.30	0.38	0.25	0.43
Bucephala clangula	0.19	0.24	0.19	0.26	-0.07	0.25	0.13	0.18	0.12	0.15	0.14	0.04	0.11
Mergus albellus	-0.22	-0.43	-0.47	-0.28	-0.38	-0.32	-0.36	-0.35	-0.37	-0.25	-0.28	0.16	-0.13
Buteo buteo	0.02	-0.18	-0.37	-0.20	-0.24	-0.26	-0.24	-0.32	-0.36	-0.40	-0.34	-0.28	-0.35
Falco tinnunculus	0.01	-0.32	-0.41	-0.33	-0.15	-0.29	-0.29	-0.24	-0.05	0.06	0.20	0.30	0.33
Gallinula chloropus	-0.50	-0.56	-0.40	-0.37	-0.02	-0.33	-0.25	-0.19	-0.34	-0.28	-0.31	0.01	-0.22
Fulica atra	0.07	-0.11	-0.18	0.01	-0.17	0.00	-0.07	-0.10	-0.06	0.09	0.03	0.29	0.17
Larus argentatus	-0.17	0.14	0.04	-0.06	-0.13	-0.01	-0.10	-0.09	0.04	0.06	0.21	0.04	0.19
Larus canus	0.15	0.32	0.22	0.22	0.02	0.24	0.15	0.16	0.36	0.38	0.46	0.23	0.47
Larus ridibundus	0.17	0.16	0.20	0.17	-0.11	0.15	0.06	0.06	0.33	0.34	0.52	0.18	0.42
Streptopelia decaocto	0.10	0.05	-0.18	0.01	0.06	0.09	0.03	0.03	0.01	-0.01	0.01	-0.10	0.09
Anthus spinoletta	-0.17	-0.45	-0.49	-0.32	-0.32	-0.36	-0.36	-0.37	-0.33	-0.17	-0.22	0.30	-0.02

Pica pica	0.02	-0.12	-0.06	0.10	-0.01	0.11	0.06	0.13	0.09	0.23	0.17	0.29	0.23
Corvus monedula	0.24	-0.06	-0.15	-0.07	-0.34	-0.10	-0.20	-0.23	-0.09	0.07	0.08	0.36	0.24
Corvus frugilegus	0.34	0.07	-0.02	0.03	-0.28	0.00	-0.11	-0.15	0.01	0.19	0.16	0.43	0.34
Corvus corone cornix	-0.07	-0.08	-0.19	-0.11	-0.13	-0.12	-0.13	-0.18	-0.20	-0.20	-0.23	-0.06	-0.18
Corvus corax	0.05	-0.01	0.02	0.21	0.31	0.27	0.28	0.36	0.20	0.18	0.12	-0.06	0.06
Parus caeruleus	0.51	0.27	0.23	0.20	0.05	0.14	0.15	0.13	0.33	0.38	0.41	0.28	0.39
Parus major	0.04	0.00	0.06	0.15	0.05	0.13	0.12	0.15	0.32	0.39	0.42	0.33	0.42
Passer domesticus	0.35	0.31	0.30	0.22	-0.25	0.15	0.03	0.00	0.05	0.09	0.15	0.04	0.13
Passer montanus	0.10	0.12	0.18	0.34	0.10	0.29	0.27	0.28	0.28	0.33	0.20	0.22	0.20
Fringilla coelebs	-0.16	-0.24	-0.21	-0.09	0.06	-0.08	-0.03	0.00	0.14	0.23	0.25	0.32	0.31
Carduelis carduelis	0.39	0.16	0.16	0.16	-0.17	0.06	0.03	0.03	0.19	0.34	0.30	0.45	0.39
Emberiza schoeniclus	0.24	0.05	0.03	0.29	0.25	0.27	0.30	0.23	0.20	0.29	0.06	0.23	0.15
Emberiza citrinella	-0.07	0.13	0.07	0.21	0.35	0.26	0.30	0.40	0.33	0.26	0.22	-0.01	0.18

The correlations between the temperature and the strengths explain only in small extent the dynamics of the birds and to accurately describe in what extent it influenced their presence, the other environmental conditions should remain constant. In reality, the things differ much and while the area of the basins virtually maintained the same over the years, the scale of silting, for example, strongly developed (Rădoane and Rădoane, 2005). Of course, the process was not linear, depending on the yearly precipitation amount and, primarily, on the human activities in the hydrographical basins of the rivers (and here we refer to the gravel exploitations and the cutting of trees, with results in the water turbidity). Consequently, the land area inside the reservoirs increased to the detriment of the water covered area, that unequally diminished on every dam basin depending on the source of silting, and the Piteşti and Goleşti dam basins seem to be the most affected in the last time. The water deep and the speed of flow changed on some portions and they influenced the time when the ice shell appeared and its size. The habitats evolved in their turn (the coppice vegetation appeared on the new created islets and mainly towards the mouths of the rivers) and the birds feeding or resting areas transformed, which was favourable for some species, whereas for others it was not. Also, the partial eutrophication led to an increase of biodiversity and food resources, beneficial or not to different species of birds. The penetration of some aquatic invasive species or some actions of populating with fish must be taken into consideration, too. Also, other forms of anthropogenic stress permanently manifested, especially through intrusive fishing, illegal hunting and sport competing, as we saw with other occasions (Mestecăneanu and Gava, 2014, 2016b, 2018, 2019b etc.). Although the dam basins constitute a protected area, the human pressure seems to grow with the development of some roads and commercial objectives in the immediate

surroundings. All these are factors more or less measurable, but other more or less known influences come from the northern breeding grounds or from the migratory ways where the status of the habitats of breeding, feeding or resting, the rate of breeding success, the mortality, etc., indirectly or directly caused by human or by the natural processes, also contribute to the yearly dynamics of the birds from the wintering or from the passage quarters. The non-climatic factors, like the urbanization, habitat loss, pollution, invasive species, diseases, loss of keystone species, overexploitations, were found as responsible in other studies on the avicoenoses changes, though the rising temperatures seem to have the major role (McLean *et al.*, 2022).

Conclusions

As a result of the data working collected during 1999-2020 about the species of birds that winter on a series of dam basins from the Argeş River situated between Vâlcele and Goleşti, when 88 species and 275,530 individuals were observed (43.18% of the species, respectively 97.17% of the individuals being dependent on wetlands) some conclusions can be draw:

1. A strong increasing trend of the average air temperature was observed in December-January.

2. Over time, the summer visitors, the resident species and the species of passage increased as number, in the detriment of the partially migratory species and of the winter visitors.

3. Except the partial migratory species, the other species increased as weight of individuals from 1999-2009 to 2010-2020 and, here, the weight of the winter visitors increased with not less than 299.52%, mainly due to *Larus canus*, this being the highest increase of all phenological categories.

3. A strong increasing trend of the number of species and of the number of individuals, quite similar to the trend of the average air temperature, both on the whole and regarding the species dependent on wetlands, was remarked, though decreasing tendences were observed in the last years.

4. Except *Anas platyrhynchos*, an eudominant species, *Fulica atra* and *Larus ridibundus* (eudominant species), respectively *Anas crecca*, *Aythya ferina* and *Larus canus* (dominant species) showed general increased trends that, apart from *Larus ridibundus*, suffered a decreasing in the last years. However, comparing 1999-2009 to 2010-2020, the trends are increasing for *Anas platyrhynchos*, *Anas crecca*, *Aythya ferina*, *Larus canus* and *Larus ridibundus* and decreasing for *Fulica atra*.

5. Generally, the lower was the temperature of the air in the first 7 days of January, the higher was the number of species and their strengths registered at the end of the second week of January and, more obviously, the warmer was

December, the higher was the recorded number of species and the number of the individuals.

6. In general, the correlations between the average temperature of the air from different periods of December and January and the strengths of the dominant or eudominant species, except *Fulica atra*, were positive, that means that the higher the average temperature of the air, the higher their strengths.

7. The absence of the strong or very strong correlations between the average temperature of the air and the number of species, respectively the number of individuals, as well as between the average temperature of the air and the strengths of the eudominant and dominant species, corroborated with the phenology of the species, show that **the winter dynamics of the avifauna from the considered dam basins from the Argeş River is a complex event that was partially influenced by the global warming**. The dynamics can indicate both long and short movements of the birds.

8. Other local and general elements, like the development of the habitats, the anthropogenic impact, as well as the breeding success, the mortality in migration, etc., more or less linked in their turn to the global warming, were implied in the dynamics of the avifauna, too. Among them, the silting process of the reservoirs, which led to the increase of the strengths of all species of gulls, for example, and the negative human pressure, mainly observable on the Bascov Dam Basin, arranged for nautical sports, and during the construction of the highroad and of the commercial complexes from the neighbourhood, when the number of birds diminished, were identified in the period of study.

Finally, we must admit that the global warming is a fact visible even without scientific methods and we don't have to be specialists to see some of its effects on the nature and people. Even if it is totally or partially caused by human, according to different opinions, it should be seriously combated since a few decades ago through gradual measures to avoid an inutile stress upon the life of Earth. It is important that the present transition toward a nature-friendly human society to be completed as soon possible, with minimum shocks on the people and without obscure interests.

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New records and check list of arthropods from two oasis ecosystems in Algeria

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Abstract. An arthropod sampling survey was performed in two palm groves from the wilaya of Biskra and Ouled Djellal. During 12 months (2020) of survey, the obtained results indicated the presence of 117 taxons divided into 2 classes (Insecta =103 species and Arachnids = 11 species). The species belonged to the orders: Coleoptera, Diptera, Orthoptera, and Hymenoptera. The Coleoptera order was the most represented in the two palm groves (32 from Ouled Djellal (OD) and 6 from Feliache (Fe)). The major trophic guild represented in the oasis ecosystems was the predator guild (OD= 39%, Fe=32%) in comparison to those of phytophagous (OD= 28%, Fe=12%) and pests (OD= 4%, Fe=15%). This indicates an ecological balance in the investigated oasis ecosystems between pests and their predators. The observation of two new species: *Scymnus frontalis* (Fabricius, 1787) and *Diomus zinon* (González and Honour, 2011), from the Coccinilidae Family increases the number of predator species of insect pests in the investigated oasis ecosystems.

Keywords: Arthropods, New record, Ziban oases, Coccinillidae, updated species list.

Introduction

With their presence on earth for more than 400 million years, insects constitute an unprecedented biological success and an essential component of life on our planet (Ring and Vincent, 2012; Lebreton *et al.,* 2013; Gilles, 2019).

They participate to natural processes essential for maintaining biological systems. Despite their ecological importance, they are still poorly understood and suffer from a lack of interest (Leraut, 1990; Leraut, 2003; Ramade, 2012; Coïc *et al.,* 2018). The known insect fraction, which is assumed to represents less than 1% of the world's species, is already in high danger of extinction (Gilles, 2019; U.I.C.N., 2019).

Insects form a cosmopolitan group, found in different ecosystems (Calatayud, 2011; Sauvion et al., 2013; Gilles, 2019). Each ecosystem gives, by its nature and its specificity, an opportunity for the appearance and maintenance of a variation of insects, which takes advantage of the typical conditions of the habitat. The structure of these habitats influences their diversity and abundance (Urban and Smith, 1989; Halaj *et al.,* 2000). Several groups of insects can be located in different biotopes and adapt to the local conditions of the region by building a mosaic of natural resources which remains favorable to the biodiversity of the entomofauna.

Climate change has effects at all levels of organization (animal or plant) (Chaupin *et al.,* 2000; Parmesan, 2006). In relation to the importance of the changes observed at the level of biodiversity, the IPBES experts gathered during the third session in January 2015, evoked its strong impact on ecosystems, their biodiversity and ecosystem services that they provide (IPBES, 2014; Belhamra *et al.,* 2014) and which are experiencing an accelerated decline (BNT, 2015). Due to the important role of biodiversity (insects) in maintaining the structure, the stability and the functioning of ecosystems and their productivity (Dajoz, 2008). The oases are ecosystems of ecological interest, for many faunal populations, due to the flora diversity that characterizes them (Belhamra *et al.,* 2014; Deghiche-Diab, 2020; Aouissi *et al.,* 2021). However, a lack of scientific knowledge on the modalities of adaptations and the evolution of the structure of insects under oasis ecosystems is induced by these disturbances (Aouissi *et al.,* 2021). This why, the legitimized and crucial question that arises; what is the state of play and the possible impact on biological diversity in our Ziban oasis ecosystems? An update list was performed at tow palm groves from Biskra and Ouled Djellal oasis ecosystem. This paper will review the list of arthropods present in oasis ecosystem especially those used in biological control or in an integrated pest management program for the maintain of its stability.

Materials and methods

Study area

In order to have an inventory of insect species, their distribution and community composition, we performed a monitoring study in two palm groves, located in the oasis of Biskra (Fig. 1) and that of Ouled Djellal (Fig. 2).

Covering an area of 2 035.978 km2, the Biskra region (34°, 48' N and 5°, 44' E) is one of the wilayats of southern Algeria. It is located in the south east of Algeria, and limited by the province of Batna in the north, to the North-West by M'Sila and Ouled Djellel, to the North east by Khenchela, to the south by El Oued and Ouargla. Its' vast expanse, combined with geological and geographic characteristics as well as climatic factors, show a series of ecosystems, sheltering a diversity of forests, mountains, plateau, steppes and oasis habitats, offering a diversity of animal and plant species (Fig. 1a).

The palm grove chosen for insect sampling is a living date palm collection created as part of the PNR1 project "Inventory, characterization and conservation of date palm cultivars in south-eastern Algeria", initiated to preserve genotypes of endangered date palms, their multiplication and the constitution of a genebank. It is located on an area of 4ha (46° 5'21.08''E 49° 34'35.24''N, 85m a.s.l) at Feliache (Fe) municipality on the national road N° 83, it include 88 cultivars from date palm trees spaced 9m between them (Fig. 1b).

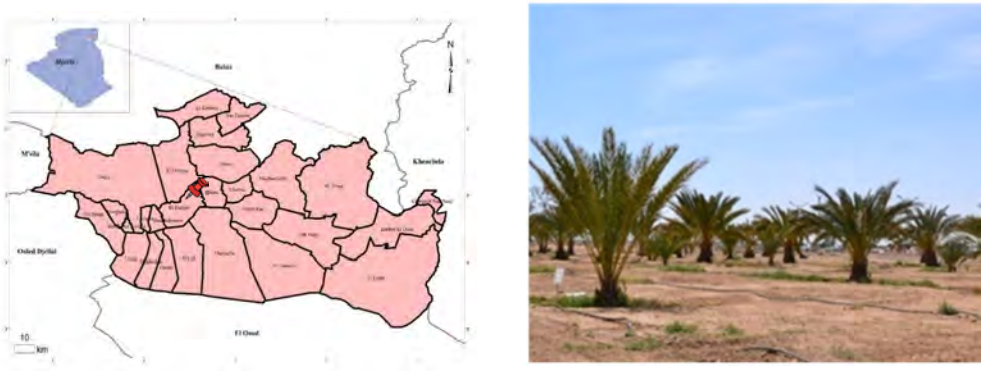

Figure1. Location of **a.** Biskra region and **b.** Chosen palm grove Feliache (Fe)

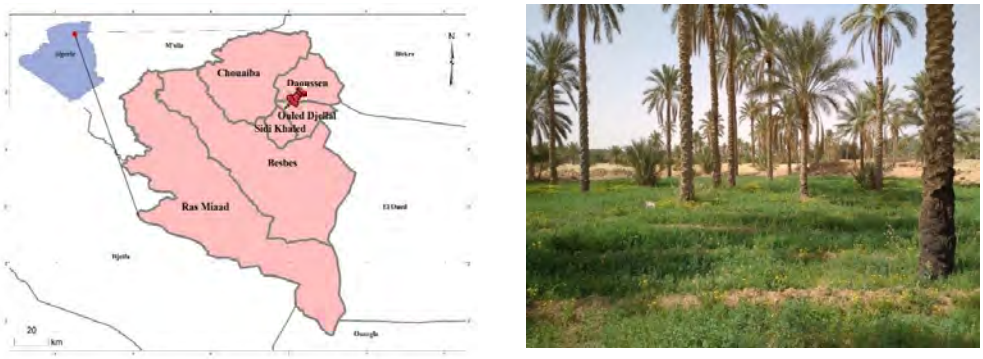

Figure 2. Location of the **a.** Wilaya of Ouled Djellal and **b.** sampling palm grove Ouled Djellal (OD)

Another palm grove (34°25'N 5°25'E, 195m a.s.l) was selected from the Oasis of the wilaya of Ouled Djellal (Figure 2b), the wilaya was created in 2019 and formalized in 2021. It is located in the Algerian Sahara with an area of 131, 220 km^2 and delimited to the north by the M'sila province, to the east by the Biskra province and El M'Ghair province, to the west by the Djelfa province and to the south by the Ouargla province (Fig. 2a).

Sampling arthropods

One of the most effective ways of collecting invertebrates during fauna surveys is by using pitfall traps (Laub *et al.,* 2008). It is designed as an inexpensive, easily made item and very effective (Deghiche-Diab, 2015), used in studies of seasonal occurrence of insects, to examine spatial distribution patterns, to compare relative abundance in different micro-habitats, to study daily activity rhythms and in community surveys.

Five pitfall traps were located in four opposite directions (Moulin *et al.,* 2007; Langor and Spence, 2006) in each palm grove and spaced 30m, where they were visited every week from january until décember 2020. In laboratory, the identification of species was done under binoculare magnifier and by using the references collection (Deghiche-Diab, 2009; Deghiche-Diab *et al.,* 2015a,b; Deghiche-Diab *et al.,* 2020 a,b,c) previously established. The identification of new species was done based on the articles of reviewed families, genus and species around the world (Pong and Slipinski, 2009; González and Honour, 2011; Abdolahi *et al*., 2018; Katayoun Pahlavan *et al.,* 2018; Albéryca *et al.,* 2020)

Data analysis

The collected observation and data were treated by using the following ecological indices, using PA.ST program (V. 2.17) software which is a data analysis tool that allows us to process statistical data, generate graphs and calculate different ecological and statistical indicators (Hammer, 2001; Dieumegard, 2010):

- The relative abundance AR or F = ni/ N x 100, that represents the percentage of individuals of a species (ni) compared to the total number of individuals (N) (Dajoz, 1971; Blondel, 1979).

- The constancy that is the ratio of the number of records containing the studied species to the total number of records C (%) = Pi / P x 100. (Dajoz, 1985). The species is constant if C ≥ 50%, accessory if 25% ≤ C ≤ 49%; accidental if 10% ≤ C ≤ 24% and very accidental qualified as sporadic if the C ≤ 10% (Bigot and Bodot, 1973).

Results and discussion

An arthropod inventory was assembled in the two investigated palm groves (Feliache and Ouled Djellal), during the study period. In total, 117 species and 11 orders were collected and identified. The Insecta class was the most represented with 86 species from Ouled Djellal and 31 species from Feliache palm grove. The Arachnida class was represented by 11 species where 7 species from Ouled Djellal and 5 from Feliache palm grove (Tab. 1).

Structural composition of arthropods in palm groves

In total eleven (11) orders of Arthropods were found in the two palm groves (Ouled Djellal and Feliache), eight (8) orders belonged to the class Insecta, from which Coleoptera and Diptera orders were the most represented, whereas the Arachnida class was represented by two (02) orders with seven families (Tab. 1).

Table 1. Total collected species and orders during the study period from Feliache and Ouled Djellal palm groves

		Palm groves			
		Ouled Djellal (OD)		Feliache (Fe)	
Class	Order	Family	Species	Family	Species
Insecta	Coleoptera	6	32	4	6
	Lepidoptera	4	8	2	2
	Hemiptera	4	8	2	2
	Hymenoptera	4	11	3	4
	Orthoptera	3	9	2	2
	Neuroptera	2	2	1	1
	Diptera	7	10	8	9
	Odonata	2	5	-	-
	Embiidae	1	1	1	1
Arachnida	Araneae	4	6	5	5
	Solpugida	1	1	-	-
Total	11	38	93	28	33

Total collected species by palm grove

Following the chosen method for trapping and collecting Arthropods from the chosen palm groves (Feliache and Ouled Djellal), it was noted that Ouled Djellal palm grove have the highest number of species (93 species) belonging to 38 families, in comparison to Feliache palm grove that groups only 33 species and 28 families. Fourteen species were observed in both palm groves.

In Ouled Djellal palm grove, we notice that the most represented species were from Coleoptera order with 32 species and 6 families. The second numerous orders were Hymenoptera and Diptera order, with 11 and 10 species, respectively.

Orthoptera, Lepidoptera and Hemiptera orders were represented by 9 and 8 species, respectively. Less species from Diptera order were collected from Feliache palm grove, where only 9 species belonging to 8 families were identified. The other orders were found in few numbers in our pitfall traps (Tab. 1).

The Arachnida class was well represented under Ouled Djellal palm grove conditions, where we counted 7 species, belonging to 5 families, in comparison to 5 species from the Feliache palm grove (Tab. 1).

The obtained results were analyzed using the constancy for each species (Tab. 2), under palm grove conditions we count 50 accidental species at Ouled Djellal palm grove in comparison to that of Feliache, whereas 17 accessory species were obtained from Ouled Djellal in comparison to Feliache, where no accessory species was identified. Only one constant species was obtained under Feliache palm grove in comparison to 5 from Ouled Djellal palm grove. The sporadic species were lower in Feliache palm grove (9 species) compared to those obtained from that of Ouled Djellal (14 species). The same observation was obtained for the rare species, where we counted 10 species from Feliache palm grove compared to 16 from that of Ouled Djellal.

Because insects have short generation times in comparison to plants and vertebrates, they are the most affected by climate change, it can have a direct influence on their development, reproduction, and survival (Bale *et al*., 2002). It is noted that different parameters from abiotic and biotic factors that characterize each palm grove, have an effect on the diversity on Arthropods. Different authors Armsworth *et al.* (2004) and Bonnemaison (1962), agree that the abiotic factors such as microclimate have a direct effect on the abundance of population and their distribution and fluctuation. In addition, Dajoz, (2008) and Nentwig (2007), reported that the variations in diversity of species varies with the climatic conditions (time and temperature). All these parameters can explain the difference distribution of species under each palm grove.

The effect of biotic factors on the distribution of species has been the subject of debate, as reported Barbault (1981) and Tilman (1997), the increase in plant diversity induces an increase in the diversity of phytophagous species. This can be an explanation of the presence of a large number of accidental species in the tow palm groves, where it is noted the presence of vegetative cover (weeds). The diversity of crops cultivated under oasis ecosystem can also represent a favorable microclimate (Honěk, 1998; Pan *et al.,* 2020) for the presence of prey (Bohan *et al.,* 2000) that provide food for the predator species that can explain the height number of accidental species under palm groves. As a palm grove plays a role of screen by protecting the species from desert influences and creates a microclimate favorable to their development following the various cultural practices applied (Deghiche-Diab, 2019). Human intervention under

oasis ecosystem through cultural practices can also have an effect on the diversity and number of species. It is noted that farmers at Ouled Djellal palm grove does not use pesticides or treatments. As reported Sauphanor *et al.* (1993), Bettiche, (2017), N'Goran *et al.* (2019); the massive use of pesticides can have a negative effect on several trophic levels of insects in the cultivated environment. The absence of treatments or chemicals can be another possible explanation of the high number of accidental species under Ouled Djellal palm grove in comparison to that of Feliache. The presence of constant species in the habitat maybe justified by the fact that they are favored by the presence of their host plants and their prey (Deghiche-Diab *et al.,* 2020a). The list of rare species includes species collected in the majority of cases at one time, their presence in few numbers is probably due to the probabilities of capture (type of traps used) or the ecological capacity of each species to populate biotopes (Blondel *et al.,* 1973; Deghiche-Diab, 2009; Deghiche-Diab, 2015). Another possible explanation that the species may be introduced from another ecosystem and did not yet adapt to the new conditions of the area (Deghiche-Diab, 2020; Deghiche-Diab *et al.,* 2021).

Table 2. Total number of species collected from Feliache (Fe) and Ouled Djellal (OD) palm groves

Orders	**Families**	**Species**	**C% Palm groves**		**in common**	**Trophic guild**
			Fe	**OD**		
Odonata	Libellulidae	*Sympetrum sanguineum* (Muller, 1764)	-	10.42		Pr
		Sympetrum vulgatum (Linné, 1758)	-	14.58		Pr
	Coenagrionidae	*Coenagrion* sp	-	6.25		Pr
		Ischnura pumilio (Charpentier, 1825)	-	6.25		Pr
		Enallagma cyathigerum (Charpentier, 1840)	-	4.17		Pr
Coleoptera	Tenebrionoidae	*Erodius emondi* spp. laevis (Solier, 1834)	-	20.83		Pr
		Akis lusitanica (Solier, 1836)	-	4.17		Pr
		Pimelia sp	-	22.92		Pr
		Pimelia payraudi (Latreille, 1829)	-	35.42		Pr
		Stenocara sp	-	31.25		Pr
	Cetoniidae	*Protaetia morio* (Fabricius, 1781)	-	41.67		Ph
		Tropinota squalida (Scopoli, 1783)	-	45.83		Ph
		Hoplia argentea (Poda-1761)	-	54.17		Ph
		Tropinota (epicometis) *hirta* (Poda, 1761)	-	72.92		Ph
	Meloidae	*Mylabris sp4*	2.08	2.08		Po
		Meloidae sp	-	6.25		Po
		Mylabris sp1	-	4.17		Po

Orders	Families	Species	C% Palm groves		in common	Trophic guild
			Fe	OD		
		Mylabris sp2	-	8.33		Po
		Mylabris sp3	-	4.17		Po
	Chrysomelidae.	*Clytra sp,*	-	2.08		Pr
	Coccinellidae	*Adalia bipunctata* (Linné, 1758)	-	4.17		Pr
		Oenopia conglobata (Linné, 1758)	-	10.42		Pr
		Exochomus nigripennis (Erichson, 1843)	-	31.25		Pr
		Psyllobara viaintiduopunctata (Linné, 1758)	-	8.33		Ps
		Hippodamia variegata (Goeze, 1777)	-	47.92		Pr
		Coccinella algerica (Kovàr,1977)	-	72.92		Pr
		Henosepilachna elaterii (rossi,1794)	-	25.00		Ps
		Coccinella septempunctata (Linné, 1758)	87.50	77.08	x	Pr
		Diomus zinon (González and Honour, 2011)	2.08	-		Pr
		Scymnus frontalis (Fabricius, 1787)	2.08	-		Pr
	Carabidae	*Brachinus explodens* (Duftschmid, 1812)	-	25.00		Pr
		Cicindela flexuosa (Fabricius, 1787)	-	25.00		Pr
		Bembidion sp	-	10.42		Pr
		Cicindela campestris (Linné, 1758)	-	16.67		Pr
		Calosoma inquisitor (Linné, 1758)	-	12.50		Pr
		Harpalus rufipes (De Geer, 1774)	-	16.67		Ph
		Calomera littoralis (Fabricius, 1787)	-	18.75		Pr
		Chlaenius decipiens (L.Dufour, 1820)	-	31.25		Po
		Licinus punctatulus (Fabricius, 1792)	6.25	4.17		/
	Scarabaeidae	*Amphimallon solsticialis* (Linné, 1758)	-	12.50		Ph
		Scarabaeus sacer. (Linné, 1758)	-	16.67		Co
	Curculionidae	*Cleonis pigra* (Scopoli, 1763)	4.17	27.08		Ph
Lepidoptera	Nymphalidae	*Venessa cardui* (Linné, 1758)	12.50	16.67	x	Ph
		Danaus chrysippus (Linné, 1758)	-	18.75		Ph
	Peridae	*Euchloe simplonia* (Freyer, 1829).	-	22.92		Ph
		Pieris rapae (Linné, 1758)	-	25.00		Ph
		Pieris-brassicae (Linné, 1758)	14.58	25.00	*x*	Ph
		Colias crocea (Fourcroy, 1785)	-	18.75		Ph
	Arctiidae	*Utetheisa pulchella* (Linnaeus, 1758)	-	4.17		Ph
	Sphingidae	*Hyles lineate* (Fabricius, 1775)	-	12.50		Ph
Hymenoptera	Chrysididae	*Omalus biaccinctus* (R. du Buysson, 1893)	10.42	6.25		Pa
	Ichneumonidae	*Ophion luteus* (Linné, 1758)	-	10.42		Pa
		Apechthis compunctor (Linné, 1758)	12.50	12.50		Pa
	Apidae	*Bombus terrestris* (Linné, 1758)	-	8.33		Po
		Apis mellifera (Linné, 1758)	-	25.00		Po

Orders	Families	Species	C% Palm groves		in common	Trophic guild
			Fe	OD		
	Formicidae	*Monomorium subopacum* (Smith, 1858)	14.58	54.17		Pl
		Pheidole pallidula (Nylander, 1849)	18.75	22.92		Pr
		Tetramorium biskrensis kahenae (Menozzi 1934)	-	35.42		Pl
		Tapinoma sp	-	16.67		Pl
		Messor capitatus (Latreille, 1798)	-	20.83		Pl
		Messor barbara (Linné, 1767)	6.25	22.92	x	Pl
		Tapinoma nigerrimum (Nylander, 1856)	-	31.25		Pl
		Cataglyphis bicolor (Fabricius, 1793)	-	6.25		Pl
		Cataglyphis bombycinus ((Roger, 1859)	10.42	4.17	x	Pl
	Vespidae	*Polistes dominula* (Christ, 1791)	-	29.17		Pl
		Polistes gallicus (Linné, 1767)	-	22.92		Pl
Orthoptera	Gryllidae	*Brachytrupes megacephalus* (Lefèvre, 1827)	-	12.50		Pl
		Acheta domesticus (Linné, 1758)	2.08	4.17	x	Pl
	Gryllotalpidae	*Gryllus campestris* (Linné, 1758)	-	2.08		Pl
		Gryllotalpa gryllotalpa (Linné, 1758)	-	2.08		Ph
	Acrididae	*Sphingonotus rubescens* (Walker, 1870)	-	14.58		Ph
		Chorthippus sp	-	18.75		Ph
		Chorthippus biguttulus (Linné, 1758).	-	22.92		Ph
		Melanoplus bivittatus (Say, 1825)	-	20.83		Pr
		Acrida pellucida algeriana (Dirsh, 1949)	-	10.42		Ph
		Aiolopus strepens (Latreille, 1804)	-	12.50		Ph
	Pyrgomorphidae	*Pyrgomorpha agarena (Bolívar, 1894)*	4.17	-		Ph
Hemiptera	Pantatomodae	*Eurydema dominullus* (Scopoli, 1763)	-	22.92		Ph
		Codophila varia (Fabricius, 1787)	-	20.83		Ph
		Graphosoma italicum (Müller,1766)	-	10.42		Ph
		Dolycoris baccarum (Linné, 1758)	-	10.42		Ph
	Miridae	*Stenotus binotatus* (Fabricius 1794)	8.33	12.50	x	Pr
		Lygus Lygocoris pabulinus (Linné, 1760)	-	8.33		Ph
	Anthocoridae	*Orius laevigatus* (Fieber, 1860)	-	10.42		Pr
	Cicadellidae)	*Psammotettix alienus* (Dahlbom 1850)	-	16.67		Ph
	Aphididae	*Myzus percecae (Sulzer, 1776)*	10.42	-		Ps
		Rhopalosiphum padi (Linnaeus, 1758)	12.50	-		Ps
		Aphis craccivora (Koch, 1854)	2.08	-		Ps
Diptera	Torymida	*Torymus flavipes* (Walker, 1833)	6.25	-		Pa
	Dictyopharidae	*Dictyophora europaea (Linné, 1767)*	-	-		Ph
	Drosophilidae	*Drosophila* sp	14.58	-		Ph
	Tachinidae	*Peleteria varia* (Fabricius, 1794)	10.42	-		Sa

Orders	Families	Species	C% Palm groves		in common	Trophic guild
			Fe	OD		
	Calliphoridae	Lucilia sp.	4.17	12.50	x	Sa
	Syrphidae	*Sphaerophoria scripta* (Linné 1758)	20.83	-		Pl
		Syrphus vitripennis (Meigen, 1822)	-	6.25		Pl
		Melanostoma mellinum (Linné, 1758).	-	10.42		Pl
	Muscidae	*Musca domestica* (Linné, 1758)	6.25	22.92	x	Sa
	Tephritidae	*Ceratitis capitata* (Wiedemann, 1824)	8.33	20.83	x	Ps
		Bactrocera oleae (Gmelin, 1788)	10.42	14.58	x	Ps
	Bombyliidae	*Systoechus vulgaris* (Loew, 1863)	4.17	12.50	x	Pa
	Asilidae	*Neoitamus* sp.	-	10.42		He
	Culicidae	*Culiseta* sp	-	4.17		He
		Anopheles sp	-	6.25		He
Embiidea	Oligotomidae	*Oligotoma nigra* (Hagen, 1885)	10.42	4.17		Pr
Neuroptera	Myrmeleontidae	*Myrmeleon formicarius* (Linné, 1767)	-	8.33		Pr
	Chrysopidae	*Chrysoperla* carnae (Stephens, 1836)	12.50	6.25	x	Pl
Solpugida	Daesiidae	*Syndaesia* sp	-	10.42		Pr
Araneae	Lycocidae	*Trochosa terricola* (Thorell, 1856)	-	12.50		Pr
	Dysderidae	*Dysdera westringi (*Cambridge, 1872)	8.33	14.58	x	Pr
	Thomisidae	*Tomisus onustus* (Walckenaer, 1805)	4.17	-		Pr
		Thomisus sp.	-	4.17		Pr
	Araneidae	*Agalenatea redii* (Scopoli, 1763)	-	6.25		Pr
		Argiope bruennichi (Scopoli, 1772)	-	10.42		Pr
		Argyope lobata (Pallas, 1772)	-	14.58		Pr
	Lycosidae	*Alopecosa pulverulenta (Clerck, 1757*	8.33	-		Pr
	Philodromidae	*Philodromus* sp	10.42	-		Pr
	Sicariidae	*Loxosceles* sp.	4.17	-		Pr
11	53	118	36	96	14	Pr

(x): Present; (-): not present: (/): unknown C%: Constancy; Ph: Phytophagous; Pr: Predator; Pl: Polyphagous; Po: Pollinator; Ps: Pest; Co: Coprophagous, Sa: Saprophagous; He: Hematophagous.

Trophic guilds

Under oasis ecosystem different species categories were identified where we counted, the most important category was represented by predators with 36% of the total collected species, the second numerous category was represented by the phytophagous species that represented 27% of the total collected species, the polyphagous were in the third position, with 15% of all collected species.

The specialization of species under Ouled Djellal palm grove indicated that the most important category was represented by predator species with 39% of all collected species. Phytophagous species were also well represented with 28% and 17% were polyphagous species (Fig. 3a).

From Feliache palm grove, that groups 33 species, the important category was represented by predators with 32% of all collected species, the category of polyphagous species were in the second position with 17% and 15% were pest species. 12% represent the phytophagous and parasitoids species (Fig. 3b). Each of these trophic groups includes insects that play various functional roles, thus constituting key organisms at different trophic levels (Koricheva *et al.,* 2000; Haddad *et al.,* 2009). This distribution takes into consideration the diet type of adult states although it is important to note that in nature there is no absolute trophic specialization (Beaumont and Cassier, 1983).

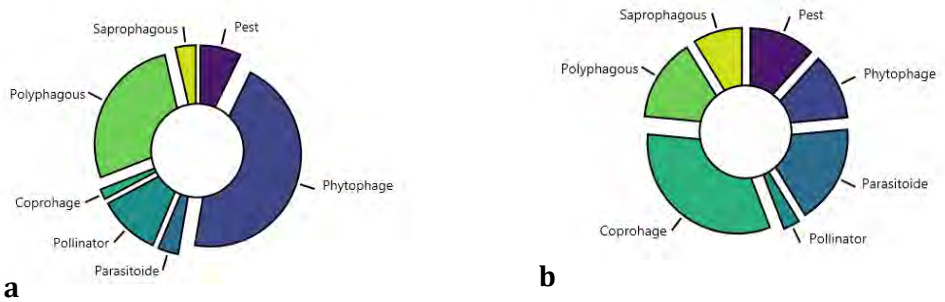

Figure 3. Trophic guild of collected species under **a.** Ouled Djellel and **b.** Feliache palm grove

We have to notice that the rate of predatory species exceeds that of pest and phytophagous species under palm groves (Ouled Djellal, Feliache), that shows a certain ecological balance and indicated that pest species are well controlled.

Contrary to the results obtained during our study, the importance of phytophagous group has always been pointed out by the authors who have worked under oasis ecosystem (Benameur-Saggou, 2009; Achoura and Belhamra, 2010; Deghiche Diab, 2015; Deghiche-Diab, 2020).

Indication of new recorded species

In general, because insect species have relatively short life cycles, high reproductive capacity and high degree of mobility, the physiological responses to warming temperatures can produce large and rapid effects on species population dynamics. We see a clear link between warm climate conditions and some recent large-scale insect outbreak events.

The Coleoptera order may contain the largest number of described species of any insect (Aberlenc, 2021). Their species play an important ecological role (Deghiche-Diab and Belhamra, 2019). They are characterized by a diversified way of life (Leraut, 2003; Leraut, 2008; Bardgett and Van Der Putten, 2014) and perform important functions in different ecosystem (Yamada *et al.,* 2007; Almeida and Louzada, 2009; Lee and Albajes, 2016). Species belonging to this order are well adapted to the conditions of oasis ecosystem (Deghiche-Diab, 2020). Ladybirds beetles belonging to the family Coccinellidae, Order Coleoptera, play an important role in pest control (Omkar and Pervez, 2016). In addition to the list of species established by authors working under oasis ecosystem (Saharaoui and Gourreau, 1998; Saharaoui *et al*., 2014; Saharaoui, 2017; Boukhlouf, 2018; Deghiche-Diab and Belhamra, 2019) we added two new records belonging to the Coccinellidae family.

Scymnus frontalis (Fabricius, 1787)

A single individual of beetle Scymnus frontalis (Fig. 4a) was collected from a trap set at Feliache palm grove (46 ° 5'21.08''E 49 ° 34'35.24''N, 85m a.s.l), a modern cultivation of date palm that groups over 80 cultivars of dattes planted sing 1995. It is a predatory species, that feeds on Aphids in oasis ecosystems. From Iran, the genus was reviewed by Katayoun Pahlavan *et al.* (2018) and indicated a description of new species that was also confirmed later by Abdolahi *et al*. (2018). In Algeria, the genus was listed by Saharaoui and Gourreau (1998); Saharaoui (2017) and Saharaoui *et al.* (2014) in their study on the Coccinillidae family, but without indication of the presence of the same species. It should be noted that this species does not appear also in the most complete recent list established for Algerian wild and domestic fauna (Belhamra *et al.,* 2020, Aouissi *et al.,* 2021).

Diomus zinon (González and Honour, 2011)

During winter season (February) another Coccinellidae, Diomus zinon (Fig. 4b) species was also observed at the Feliache palm grove (46 ° 5'21.08''E 49 ° 34'35.24''N, 85m a.s.l) where three specimens were collected from pitfall traps. The genus was first described as a subgenus of Scymnus (Mulsant, 1850) than Weise (1895) described it as separate genus. Their prey include aphids, mealybugs, scale insects, and whiteflies, with a distinct feeding preference for the family Pseudococcidae (Albéryca *et al.,* 2020).

From Austria, it was described for the first time by Pang and Slipinski (2009); from South America, later by González and Honour (2011) and recently from Spain by Albéryca *et al.* (2020). In Algeria, the species have not been listed before in any work from the same region (Saharaoui, 2017 and Saharaoui *et al.*, 2014) or from other ecosystems.

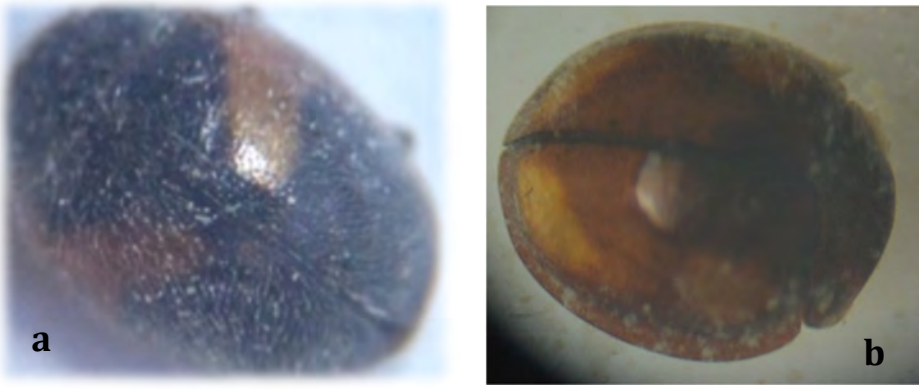

Figure 4. **a.** Adults of *Scymnus frontalis* and **b.** *Diomus zinon*

Conclusions

In order to have an updated state of arthropods species present in oasis ecosystems, an inventory was carried out at two palm groves. The first palm grove was chose in the wilaya of Biskra represented by the living collection at Feliache municipality. The second one is an old palm grove from the wilaya of Ouled Djellel at the Ouled Djellal oasis. The established list indicated the importance of insects' species mainly represented by the Coleoptera order that their species are well adapted to the arid areas conditions.

Two new species; *Scymnus frontalis* and *Diomus zenon* were observed for the first time in oasis ecosystems, by this observation the number of predatory species increase the list of biological agents that can help to maintain the stability of oasis ecosystem and the application of an Integrated Pest Managment program (IPM).

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Comparative assessment of indoor and outdoor air environment of poultry farms in Edo State, Nigeria

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Abstract. Intensive poultry farming creates the ideal environment for pathogen concentration and transmission. The presence of thousands of birds in an enclosed, warm, and dusty atmosphere is ideal for the transmission of infectious diseases from birds to humans. This study was conducted to assess the indoor and outdoor air quality of different poultry types in Edo State, Nigeria. The physicochemical conditions of the air around the poultry environments differed with location and poultry types. The concentrations of carbon dioxide (CO_2), nitrous oxide (N_2O), hydrogen sulphide (H_2S) as well as particulate matter (PM_{10}) were all within recommended limits established by the World Health Organization. However, significant elevations in Ammonia (NH_3) and sulphur dioxide (SO_2) levels were observed in substandard poultry farms across the locations. Total bacterial counts ranged from 1.38CFU/m^5 – 90.35 x 10^5CFU/m^3 irrespective of location and poultry type. Within the poultry types, bacteria count inside the poultry environment (3.11 x10^5CFU/m^3) significantly differed from concentrations outside the poultry environment (22.58 x10^5CFU/m^3, $p<0.05$). The Lowest microbial counts were obtained in the standard poultry farms. Molecular identifications revealed the presence of *Escherichia coli, Streptococcus pyogenes*, *Staphylococcus aureus*,

Pseudomonas aeruginosa, *Klebsiella pneumonia*, *Bacillus subtilis* as the bacterial isolates whereas *Fusarium oxysporum, Aspergillus niger*, *Rhizopus stolonifer, Trichoderma polysporum, Aspergillus fumigatus* were the fungal isolates. *Staphylococcusaureus* was the most predominant bacterial species (25%) while *Aspergillus niger* was the most predominant fungal species (30%).

Keywords: air quality, bacteria, chicken, fungi, microflora poultry.

Introduction

The importance of good air quality for health, quality of life, and the environment cannot be overstated. When pollutants that are hazardous to human, animal, and plant health are present in the air, it is considered contaminated. Air contaminants, both within and outside, are widely regarded as a major cause of medical problems in both urban and national settings (WHO, 2005). Poultry is a significant and rapidly growing source of meat on the planet today, accounting for a quarter of all meat produced in the year 2000. This is accomplished by genetic selection, modified feeding, and strict health-monitoring procedures that include the use of antibiotics as therapeutic agents for bacterial diseases in intensive farming systems (Apata, 2009).

The following components are common to all poultry systems: (a) an enclosed structure that can hold a large number of animals in a small space; (b) a ventilation system; (c) a system for watering the livestock; (d) a system for feeding the livestock; and (e) a system for handling animal waste (Aromolaran *et al*., 2013, Ohajianya *et al.,* 2013 and Cole *et al*., 2000). Nigeria has an estimated poultry population of over 140 million birds mostly concentrated in the south western part of the country. Approximately 60 percent of the poultry production takes place in small backyard farms distributed throughout the rural areas (FAO, 2006).

In the last few years, Nigerian poultry production has increased. In the years 2001, 2002, 2003, and 2004, the estimated number of fowls raised in millions was 117.3 126.7, 142.7, 143.1, and 144.3. Between 2001 and 2004, the production of poultry meat increased from 88,000 to 108,000 tonnes (CBN, 2004, Akinola *et al*., 2008). Nigeria's "140-160 million poultry account for 10% of its gross domestic product and a large proportion of the protein eaten by its 132 million citizens," according to the Food and Agricultural Organization, FAO. However, the country's H5NI avian influenza outbreaks, which began on February 8, 2006 and have resulted in the death of a large number of birds, might have caused a temporary setback (World Organization for Animal Health, 2006).

Although ambient air pollution is a major concern, indoor air can be more contaminated than outdoor air because indoor natural quality can be affected by a variety of factors, including organic compounds, and particulate pollutants (O'Connor *et al.*, 2004).

Infectious microbial spores, chemical pollutants, irritants and allergens may be found in both indoor and outside air which can reduce the quality of life and trigger diseases (Killebrew *et al*., 2010, Kusi *et al*., 2015), much like the inward breath of these airborne contaminants. The dangers of existing residue in enclosed environments acting as a source of synthetic concoctions and re-suspended elements, triggering inhabitant's presentation through both inward breath and incidental ingestion, are becoming more widely recognized. Increased exposure to indoor contaminants in residue may have negative consequences on children's health, including slowed growth and learning inabilities, hypersensitivities, malignancy, sensory system harm and different ailments (Oyeyinka *et al.,* 2011, Purnomo *et al*., 2014).

Gases in poultry confinements are a product of the degradation of droppings, animal respiration and building operations. Some of these gases include ammonia, carbon dioxide, carbon monoxide and hydrogen sulphide. Each of these gases may affect respiratory health (Cole *et al*., 2000, Pickrel, 1991, Okoli *et al.,*2006). They can be a respiratory toxicant or irritant such as ammonia (Smith *et al*., 2003, Wafi *et al*., 2011) which cause asphyxiation, blood poisoning, anoxia, pulmonary oedema or sudden death like carbon dioxide and carbon monoxide (Okoli *et al.*, 2006, Sainsbury, 1993) and pulmonary oedema or sudden death, like hydrogen sulphide poisoning (Donham and Rylander, 1986).

In communities surrounded by a high concentration of livestock, complaints of odour nuisance, environmental pollution, and ill health have become more common (Pinkerton *et al.*, 2000). Studies have shown that the physical and conceptual health of residents existing close to a high concentration of livestock is compromised (Cole *et al*., 2000, Kusi *et al*., 2015). Poultry and farm animals have also been reported to be a large reservoir of several bacteria species (Cole *et al*., 2000). Poultry birds have remained connected with higher airborne dust, microorganisms and endotoxin absorptions in confinements (Oyeyinka *et al*., 2011). Also, older litter, which is basically dried manure, is dustier owing to an escalation in friability when associated with fresher wood chip litter (Cole *et al.,* 2000). The aim of the study, therefore, was to evaluate the indoor as well as the outdoor air-borne quality and microflora assessment of standard, semi-standard and substandard poultry farms in Edo State, Nigeria.

Materials and methods

Study Area/ Study locations

The Data for this study were collected from 9 selected poultry farms in Edo State. The State is separated into three Agricultural regions, namely: Edo Central, Edo South and Edo North. The State lies between longitudes 05º 041 E and 06º 431E and latitudes 05º 441N and 07º 341N of the equator. The populace of the study consists of nine commercial poultry farms housing domestic chickens in confinements identified by the Poultry Association of Nigeria, Edo State Branch and the Ministry of Agriculture and Natural Resources, Benin City, Edo State (Figure 1). Three types of poultry systems (standard, semi-standard and substandard) were taken into consideration with respect to Mechanization and Automation. Commercial flocks of rearing and laying birds with a capacity of 2,000-6,500 were considered for this study; Standard Poultry farms with mechanical ventilation, automated feeding and drinking systems and Standard practices (Figure 2a). The semi-standard-Poultry farms with mechanical ventilation, automated drinking system and traditional feeding methods (Figure 2b). The third category was the sub-standard- Poultry farms without mechanical ventilation systems, only natural ventilation with traditional feeding and drinking methods (Figure 2c).

Sample Collection

Air sampling

For a period of 12 months, the air on all poultry farms was sampled at various points in the field between December 2017 and November 2018, the study was conducted in nine (9) poultry houses in Edo State's three senatorial districts (Edo Central, Edo North, and Edo South). The sampling was conducted at three different locations, Ekpoma (Edo Central), Auchi (Edo North), and Benin were the study sites (Edo South). The sampling times and dates were chosen based on high activity levels in the sampled poultry farms. Poultry air was sampled at 9 a.m. at each sampling period. Over 2,000 birds were housed in each of the poultry samples.Casella cell 712 air sampler (Casella incorporated) was used to collect air samples. (Casella incorporated, U.S.A). Depending on the predicted pollution level, measurements were made in triplicates, each time collecting 0.1m^3 of air in 1 minute. During the measurement, the sampler was placed 1.5 meters above the human inhalation zone. Each sampling site took 4 hours to complete. Throughout the fattening season, indoor and outdoor samples were collected in the poultry bio field. Airborne bacteria and fungi were used to continuously assess bio pollutants. In the poultry houses, samples of bacteria and fungi were taken at a central location. The discharge level outside the poultry farms was determined correspondingly.

Determination of Microclimatic Parameters.

The physicochemical parameters, and relative humidity in addition to temperature, were measured with Testo Device 400 (Testo GmbH & Co., Lenzkirch, Germany).

Media preparation

The media used in this study to isolate bacteria and fungi were Nutrient Agar (NA), MacConkay Agar, Potato Dextrose Agar (PDA), Sabouraud Dextrose Agar (SDA) and Blood Agar. All media preparations were carried out in accord with the Manufacturer's guidelines. (The composition and preparation of media are in Appendix). Antibiotics (Streptomycin and Chloramphenicol – 50mg/L each) were introduced into the dissolved media after sterilization was carried out for the inhibition of bacteria. Sterilization of media was by autoclaving for 15 mins at 121^{0}C and 15 pounds pressure.

Measurement of dust concentration

The concentration of dust of aerodynamic diameter of <10mn was determined electronically with the aid of a direct reading active personal sampler, Casella Cell Dust (Environmental Device co-operation, USA). An active sampler uses a pump as well as a power source to transport air over a collector (WHO, 2000). The sampler has a sampling flow rate of 1.0 l/minutes and the instrument software allows direct reading of dust concentration. The sampler was placed 1.5m above the floor, the device switched on and dust concentration was determined after 1 minute and measurements were taken on a monthly basis in each of the poultry houses investigated. The results were expressed in $mg/m^{3.}$

Enumeration and isolation of airborne bacterial and fungal isolates

Qualitative and quantitative bacterial and fungal investigations were carried out in the poultry buildings.

A filtration method was used in this study due to the predicted high concentration of microflora in poultry facilities. Indoor and outdoor air samples were obtained using measuring sets that included a Casella Personal Air Sampling Pump (Model: Apex2, IndiaMART, New Delhi) and mixed cellulose ester filter paper (ME Range ME 24, 3.1 mm white/black grid for membrane-butler, 0.2 µm pore size, 47 mm circle (400 pcs), manufactured by Cytiva, USA) to determine concentrations of airborne Microflora. The evaluating sets were calibrated with a Gillibrator 2 calibrator before each sampling process. After sampling, sterile tweezers were used to pick the filters containing the biological material into a tightly sealed container Stuart-Ringertz medium

(Sigma-Aldrich chemie GMLH Munich, Germany) was used, and the samples were transported to the lab for microbiological analysis. The filters in the transport medium containers were submerged in 5ml of phosphate buffer solution (BTL, Lodz, Poland) and the biological material on the filters was eluded by shaking on a shaker at 420 revolutions per minute for 50 minutes. From the elutes, a series of threefold dilutions were made.

Figure 1. Map of Edo State Displaying the Location of Study Sites Marked According to Poultry Standards

a b c

Figure 2. Different standards of poultry environment with birds in confinements (a) standard Poultry in Auchi, (b) semi-standard Poultry in Benin, and (c) substandard Poultry in Ekpoma

Isolation of airborne bacteria from the air:

All media were aseptically prepared according to the manufacturers' instructions. Thereafter 0.1ml of the 10^3 dilution were inoculated onto sterile plates of mannitol salt agar, nutrient agar, blood agar and MacConkay agar (Merck, Dermstadt, Germany). The plates, which were prepared in triplicates were covered, and incubated for 18-24 hours at 37^0C for the isolation of pathogenic bacteria. One set of plates was incubated under aerobic conditions, while the other set was incubated under anaerobic conditions. The airborne bacterial isolates were enumerated using the formula:

(Number of colonies x Dilution factor x Elute volume)/ (Serial dilution material plated x Volume of air sampled)

The resultant concentration was expressed in terms of the number of colony forming unit per cubic meter (CFU/m^3). Thereafter, discrete colonies were sub-cultured for preliminary identification subjected to biochemical tests and characterized on the basis of their cultural, morphological and biochemical characteristics, as described by Cheesebrough, 2006.

Isolation of airborne fungi from the air:

Sterile dishes of PDA and SDA (Oxoid Ltd., England) incorporated with penicillin and streptomycin were used for the enumeration and isolation of airborne fungal isolates. The plates were incubated for 3-5 days at room temperature (28 ± 2^0C), discrete colonies were sub cultured and the airborne fungal isolates were characterized based on their morphological appearances. The fungal colonies were counted using the same formula in the section above and subcultured to obtain pure cultures which were identified according to Barnett and Hunter (Barnett and Hunter, 1972).

Measurement of gases in poultry housings

The concentrations of ammonia (NH_3), hydrogen sulphide (H_2S), carbon dioxide (CO_2), methane (CH_4) and nitrous oxide (N_2O) in poultry houses were determined with the aid of a portable direct-reading instrument, the procedure involved taking a representable reading at different locations in the poultry farm. The measurements of gases were taken in triplicates inside the poultry houses at the three locations (near the entrance, at the center and the end wall). The representative evaluations from every confinement were assembled to obtain the mean for every poultry. The concentration of ammonia, hydrogen sulphide, carbon monoxide and nitrous oxide were measured in Parts Per Million (ppm) while methane was measured as Lower Emissible Limits (LEL) as a flammable gas with the aid of a Gasman Hand-held Personal Gas detector

(Crowcon Instruments Ltd., England), which employs electrochemical sensors and catalytic bead sensors for flammable gas measurements. All through the gas measurements, the handheld equipment was held at approximately one foot directly above the litter level and the evaluations were recorded within 20 secs. All analyses were calibrated for zero and span before and after reading.

Extraction and amplification of DNA

The isolated colonies were transferred from the surface of a single agar plate into a pre-cooled (20°C) sterile ceramic mortar, liquid nitrogen was added, and the mixture was ground into a fine powder with a sterile ceramic pestle. To suspend the powder, two ml of buffer G-2 (Genomic DNA buffer set; manufactured by Qiagen, Valencia, California, USA) with RNase (200 g/ml; manufactured by Sigma Aldrich Chemical Company, Germany) was applied, and the suspension was transferred to a clean test tube. A total of 45 microliters of proteinase K solution (20 mg/ml stock solution; Sigma Aldrich Chemical Company, Germany) was applied to the suspension, which was then incubated at 55°C for 3 hours with intermittent agitation. The suspension was centrifuged for 10 minutes at 21,500 g. The supernatant was transferred to a clean test tube, and DNA was extracted and purified using Qiagen Genomic-tip 20/G columns, as directed by the manufacturer. The eluted DNA was treated with two and a half microliters of glycogen solution (20 mg/ml; Gentra Systems, Minneapolis, USA), which was then precipitated using standard isopropanol and ethanol methods. DNA was resuspended in 60 l of DNA rehydration buffer (Gentra Systems' PureGene kit, QIAGEN, USA) and held at 20°C until required. After a pure PCR product of the 16S gene was obtained, sequenced, and aligned against bacterial or fungal DNA data base, the species were identified.

Statistical analyses

The Data obtained from this research were expressed as either mean ± SEM (standard error or mean) or percentages. The t-test statistics was used to test for statistical difference between the treatment and control groups studied. The statistical package used for data analyses was SPSS version 21.0. Values in triplicates were evaluated using measures of central tendency (mean ± standard deviation). One-Way ANOVA was used to compare multiple variables while Duncan's multiple range test was used to check for significant differences between means of values determined. *P*-values less than 0.05 were considered statistically significant. Diversity indices of microbial isolates were computed using PAST software (version 2. 17c).

Results

Mean indoor temperature levels ranged from 24.60°C - 32.21°C, 25.5°C - 32.20°C and 25.10°C - 32.40°C in Standard, Semi-standard and Sub-standard Poultry farms respectively. The highest temperature reading of 33.60°C was in the standard poultry farm in Auchi while the lowest temperature reading 24.60°C was recorded in the standard poultry farm in Ekpoma. The mean indoor relative humidity results ranged from 50 – 88%, 52- 93% and 51 - 91%in Standard, Semi-standard and Sub-standard poultry farms respectively with the highest reading of 95% recorded in Semi-standard poultry farm in Ekpoma while the lowest 50% was recorded in the standard poultry farm in Auchi (Table 1). The airborne monitoring of gaseous pollutants, fungi and bacteria was carried out in indoor and outdoor environments of the poultry farms. The idea of monitoring these variables in the outdoor environment was to examine for the existence of any significant variation exist in aerial pollutants levels between both extremes in the poultry houses.

Mean hydrogen sulphide concentrations in the environment were recorded between 0.02 ppm and 13.10 ppm (Table 1). There was a statistically significant difference in concentrations in indoor and outdoor environments in all poultry farms studied ($P<0.05$). The H_2S levels were generally higher in the indoor environment for the poultry houses studied. Hydrogen sulphide concentrations in Sub-standard poultry across all locations exceeded the WHO permissible limit (7.00ppm). A significant difference in H_2S concentrations between poultry types in all locations($P<0.05$) was also observed with the exception of Standard and Semi-standard poultry in Auchi and Ekpoma indoor and outdoor as well as semi-standard and sub-standard poultry outdoor in Benin City. The concentration of ammonia (NH_3) ranged from 0.004ppm to 9.14 ppm. The concentration was recorded to also be above the W.H.O set limit of 7ppm in Sub-standard poultry in all sampled locations. Indoor and outdoor air had significant differences in mean values, with outdoor air being generally higher.

While there was a statistically significant difference between semi-standard and sub-standard poultry in Auchi (indoor and outdoor) and Ekpoma, the levels between semi-standard and sub-standard poultry in Auchi (indoor and outdoor) and Ekpoma were statistically comparable ($P>0.05$). The recorded levels of methane were higher in the indoor air compared to the outdoor air with concentrations ranging from 0.22 LEL in Standard poultry (Auchi outdoor) to 7.54 LEL (Sub-standard Poultry Ekpoma indoor) (Table 1). Significant difference in methane concentrations was also observed among poultry types and was higher in semi-standard poultry than standard and sub-

standard poultry in the indoor and outdoor poultry farms in Auchi as well as the outdoor environment in the substandard poultry farms in all three locations.

PM_{10} levels in the indoor, as well as the outdoor environment of poultry houses, were similarly measured, concentrations were significantly different statistically up to 89% in all poultry types, and ranged between 0.01±0.00 to 1.75± 0.01 mg/m^3 (Table 1). The highest reading 1.75 mg/m^3 was recorded in the Semi-standard poultry farm in Auchi while the lowest reading of 0.01 mg/m^3 was recorded in the Standard poultry farm in Ekpoma. However, levels in Semi-standard poultry in Auchi were similar statistically, (P>0.05). Indoor CO_2 concentrations were significantly high in semi-standard and sub-standard poultry farms in Ekpoma and Benin, there was however no significant difference in CO_2 concentrations among poultry farms in Auchi. While outdoor concentrations of CO_2 were higher in semi-standard poultry than sub-standard in Auchi, there was however no difference in outdoor CO_2 levels in the three poultry farms in Ekpoma and Benin.

Table 1. Comparative mean annual physicochemical composition of air in and around poetry environments in Edo State

Poultry	**Temp.**	**Humidity**	**CO_2**	**NH_3**	**CH_4**	**N_2O**	**H_2S**	**SO_2**	**Dust**
A3I	29.28^{ab}	73.5^{a}	28.5^{de}	0.1^{a}	2.02^{bcd}	0.2^{bc}	1.34^{bc}	1.02^{de}	0.11^{ab}
A3O	30.79^{b}	77.92^{a}	13.1^{a}	0.04^{a}	0.84^{a}	0.09^{a}	0.56^{ab}	0.41^{ab}	0.03^{a}
A2I	29.09^{ab}	74.33^{a}	27.1^{d}	39.1^{d}	4.66^{h}	0.42^{e}	2.36^{de}	0.22^{ab}	0.87^{fg}
A2O	30.73^{b}	77.92^{a}	12.4^{a}	18.1^{b}	2.3^{de}	0.2^{bc}	1.18^{bc}	0.05^{a}	0.59^{de}
A1I	29.3^{ab}	73.33^{a}	30.5^{e}	40.5^{d}	6.12^{j}	0.49^{ef}	9.71^{h}	0.11^{a}	0.86^{fg}
A1O	30.73^{b}	76.33^{a}	16^{b}	18^{b}	2.81^{f}	0.22^{c}	4.1^{f}	0.04^{a}	0.44^{cd}
E3I	28.59^{a}	77.58^{a}	16.5^{b}	2.24^{a}	2.17^{cde}	0.32^{d}	1.65^{cd}	1.19^{de}	0.21^{ab}
E3O	29.23^{ab}	80.08^{a}	11.5^{a}	0.2^{a}	1.04^{a}	0.13^{ab}	0.64^{ab}	0.51^{bc}	0.05^{a}
E2I	29.18^{ab}	76.58^{a}	20.5^{c}	51.3^{e}	3.41^{g}	0.6^{g}	2.33^{de}	0.511^{bc}	0.99^{fg}
E2O	29.5^{ab}	77.75^{a}	10.5^{a}	22.8^{bc}	1.56^{b}	0.23^{c}	1.09^{bc}	0.19^{ab}	0.47^{cd}
E1I	28.73^{a}	77.92^{a}	21.1^{c}	61^{f}	6.15^{j}	0.45^{e}	11^{i}	1.61^{f}	1.06^{g}
E1O	29.76^{ab}	80.08^{a}	10.7^{a}	23.2^{bc}	2.93^{f}	0.2^{bc}	5.16^{g}	0.86^{cd}	0.47^{cd}
B3I	28.18^{a}	74.83^{a}	22.7^{c}	3.38^{a}	1.93^{bcd}	0.41^{e}	0.21^{a}	1.38^{ef}	0.29^{bc}
B3O	28.91^{a}	79.17^{a}	10.9^{a}	1.65^{a}	0.93^{a}	0.18^{bc}	0.05^{a}	0.5^{1bc}	0.09^{ab}
B2I	28.55^{a}	74.5^{a}	29.2^{de}	47.7^{e}	3.78^{g}	0.56^{fg}	2.49^{e}	1.21^{de}	0.64^{de}
B2O	29.57^{ab}	79.25^{a}	12.5^{a}	22.8^{bc}	1.75^{bc}	0.25^{cd}	1.23^{bc}	0.34^{ab}	0.17^{ab}
B1I	28.03^{a}	72.5^{a}	30.6^{e}	57.3^{f}	5.36^{i}	0.6^{g}	9.36^{h}	1.99^{g}	0.78^{ef}
B1O	28.69^{a}	76.58^{a}	12.2^{a}	25.7^{c}	2.56^{ef}	0.27^{cd}	4.3^{f}	1.03^{de}	0.16^{ab}
p-values	0.854	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	0.002

Means with similar alphabetic superscripts within the same columns do not differ from each other (p>0.05)

A is Auchi, B is Benin City, E is Ekpoma, 1 is substandard, 2 semi-standard, 3 standard poultry, I is inside and O is outside poultry environment

Table 2 shows the airborne fungal counts which range between 9.33 ± 0.78 CFU/m^3 to 285.62 ±16.76 CFU/m^3, with the highest count recorded in Semi-standard poultry in Ekpoma (indoor) in July 2017 and the lowest count at 9.33 ± 0.78 CFU/m^3 recorded in the standard poultry farm in Benin in February. Statistical significance in the difference between indoor and outdoor counts was only seen among Semi-standard and Sub-standard poultry farms in Auchi and Benin. Counts in indoor environments of poultry houses were significantly higher in standard poultry than semi-standard and sub-standard poultry in Auchi, on the contrary, fungal counts in Ekpoma were higher in sub-standard poultry than standard and semi-standard poultry while there were no significant differences in fungal counts among poultry houses in Benin City. The fungal counts in all poultry farms sampled were however below the 3000-5000 CFU/m^3 standard set by the Polish authority for occupational exposure to airborne fungi (Lonc and Plewa, 2010).

Table 2 also shows the indoor and outdoor airborne bacterial counts in Poultry houses which ranged between 90 ± 44.48 CFU/m^3 to 690.30±0.08 CFU/m^3. The highest counts of 690.30±0.08 CFU/m^3 was recorded in the Substandard poultry in Auchi indoor environment in September 2017 while the lowest count of 90 ± 44.48 CFU/m^3 was recorded in the Standard poultry farm in Ekpoma February. The bacterial counts between indoor and outdoor air varied significantly only in Standard and Sub-standard poultry farms in Benin as well as in Substandard poultry in Auchi. The bacterial counts were higher in all indoor environments than in the outdoor environment. A comparism between poultry types revealed that bacteria counts in indoor air varied significantly

Table 2. Mean airborne microbial counts Dec. 2016 - Nov. 2017.

	Bacterial			Fungal		
	Indoor	Outdoor	p-value	Indoor	Outdoor	p-value
Auchi						
1	296.65±48.38^{b}	266.80±58.92^{b}	0.184	42.98±9.11^{b}	45.24±8.71^{bc}	0.633
2	308.23±87.41^{d}	272.96±87.17^{d}	0.142	34.57±11.42^{ad}	25.82±8.74^{acd}	0.139
3	481.29±148.99^{abd}	351.75±69.28^{abd}	0.094	71.87±15.09^{abd}	65.98±9.97^{abd}	0.322
Ekpoma						
1	148.05±46.24	165.34±51.28^{b}	0.633	39.68±14.64^{b}	40.66±7.96$^{ab\,d}$	0.732
2	226.55±70.95	207.48±54.61^{d}	0.354	159.31±60.17^{d}	147.32±41.79^{c}	0.138
3	388.10±183.17	304.91±168.49^{bd}	0.535	150.32±39.89^{bd}	173.50±43.32^{b}	0.244
Benin city						
1	214.24±71.36^{ab}	165.13±45.02^{abc}	0.132	48.28±20.00	57.70±13.51^{c}	0.214
2	302.8967.80^{d}	277.00±75.46	0.325	50.99±8.49^{a}	42.27±8.23^{acd}	0.093
3	441.30±130.46^{abd}	332.86±108.01^{ab}	0.174	39.97±7.22^{a}	32.86±108.01^{ab}	0.112

Keys: 1-Standard Poultry, 2-Semi-standard Poultry, 3-Substandard Poultry

between standard and Substandard poultry farms as well as between Semi-standard and Sub-standard poultry farms in all three locations with Substandard having the higher count. The outdoor bacterial counts were different between standard and sub-standard poultry farms in all three locations as well as between semi-standard and sub-standard in Auchi with Substandard having highest counts. Counts in Benin were also significantly different between Standard and Semi-standard poultry with the Semi-standard poultry recording the highest concentration.

Results of diversity indices showed more individual bacterial and fungal isolates in Ekpoma than in Benin City (Table 3); however, no differences in dominance indices were obtained either for bacterial isolates or fungi irrespective of location or type of poultry. The diversity and statistical association between gaseous pollutants and microbial load showed a positive correlation between hydrogen sulphide and bacterial loads, an indication that an increase in hydrogen concentration in the Poultry farm could influence the increase in the bacterial loads (Table 4). The results of the high concentration of the hydrogen sulphide were traced to the anaerobic decomposition of the accumulated poultry litters in the environment. A regression model was projected to justify the relationship between the bacterial loads and the concentration of hydrogen sulphide. Figure 3 shows a significant positive relationship between bacterial loads and Hydrogen sulphide (Figure 3a) as well as between bacterial Loads and Ammonia (Figure 3b).

With repagard to the diversity indices of bacterial and fungal isolates presented on Table 3, there was a minimum of 4 bacterial taxa and 6 fungal taxa. All isolated organisms were present in Auchi. In terms of diversity, Auchi was also the highest. In terms of the surplus of the isolates, the Auchi location was also the highest. The individuals represent the species and the number of times they appeared. The highest individual species was recorded in the Ekpoma location. The Dominance index ranges from 0 to 1. The closer it is to 1, the more dominant it is. A location is considered to have a higher dominance over another if the isolates are not evenly distributed meaning that there is more of one type of organism in a particular location than others. The dominance index indicated that no single organism showed dominance over the others. The evenness index also ranges from 0 to 1 indicating the even distribution of the individual species. The lowest evenness ratio was in Auchi locations but there was a general indication that the isolates were evenly distributed. The Menhinick or Margalef indices are called specie richness indices indicating which location had more specie richness. The highest Margalef indices was in Auchi location (Table 3).

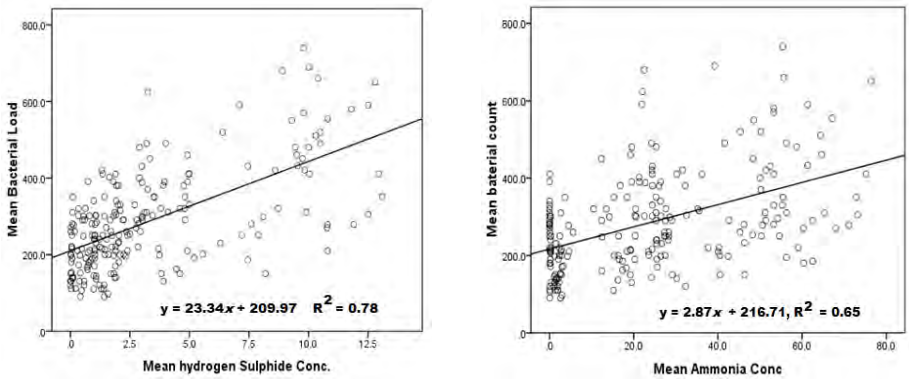


Figure 3. Regression Model of Bivariate relationship between (a) Bacterial loads and Hydrogen sulphide and between (b) bacterial Loads and Ammonia

Table 3. Diversity indices of (a) fungal (b) bacterial isolates obtained from the designated sampling areas

	A3I	A3O	A2I	A2O	A1I	A1O	E3I	E3O	E2I	E2O	E1I	E1O	B3I	B3O	B2I	B2O	B1I	B1O
Bacteria																		
Taxa_S	5	4	5	5	5	4	5	4	5	5	5	5	5	5	4	4	5	4
Individuals	26	26	28	22	26	22	45	38	47	42	46	41	39	38	39	40	43	36
Dominance	0.3	0.3	0.3	0.3	0.3	0.3	0.2	0.3	0.2	0.2	0.2	0.2	0.2	0.2	0.3	0.3	0.2	0.3
Simpson	0.8	0.7	0.8	0.7	0.7	0.7	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.7	0.8	0.8	0.7
Shannon	1.5	1.3	1.5	1.4	1.4	1.3	1.5	1.4	1.5	1.5	1.5	1.5	1.5	1.5	1.4	1.4	1.5	1.4
Evenness	0.9	0.9	0.9	0.8	0.8	1	0.9	1	0.9	0.9	0.9	0.9	0.9	0.9	1	1	0.9	1
Menhinick	1	0.8	0.9	1.1	1	0.9	0.8	0.7	0.7	0.8	0.7	0.8	0.8	0.8	0.6	0.6	0.8	0.7
Margalef	1.2	0.9	1.2	1.3	1.2	1	1.1	0.8	1	1.1	1.1	1.1	1.1	1.1	0.8	0.8	1.1	0.8
Fungi																		
Taxa_S	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6
Individuals	27	34	27	29	32	33	61	58	53	57	60	57	51	49	50	50	44	47
Dominance	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Simpson	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8
Shannon	1.6	1.7	1.6	1.7	1.7	1.7	1.8	1.8	1.8	1.8	1.8	1.8	1.8	1.8	1.7	1.8	1.7	1.7
Evenness	0.9	0.9	0.8	0.9	0.9	0.9	1	1	1	1	1	1	1	1	1	1	1	0.9
Menhinick	1.2	1	1.2	1.1	1.1	1	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.9	0.9	0.9	0.9	0.9
Margalef	1.5	1.4	1.5	1.5	1.4	1.4	1.2	1.2	1.3	1.2	1.2	1.2	1.3	1.3	1.3	1.3	1.3	1.3

Keys: *A is Auchi, B is Benin City, E is Ekpoma, 1 is substandard, 2 semi-standard, 3 standard poultry, I is inside and O is outside poultry environment*

Table 4. Bivariate correlation between Selected Gases and Microbial Load

Correlations		
	Bacterial count	Fungal count
CO_2	0.442**	-0.098
NH_3	0.476**	0.264**
CH_4	0.576**	0.155*
N2O	0.391**	0.211**
H_2S	0.621**	0.219**
SO_2	0.239**	0.135*
Dust	0.219**	0.230**
Temp	-0.253**	-0.124
Humidity	0.107	0.059

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

*. Correlation is significant at the 0.05 level (2-tailed).

Keys; CO_2 - Carbon dioxide, NH_3 – Ammonia,CH_4 – Methane, N_2O - Nitrous oxide, H_2S – Hydrogen sulphide, SO_2 – Sulphur dioxide.

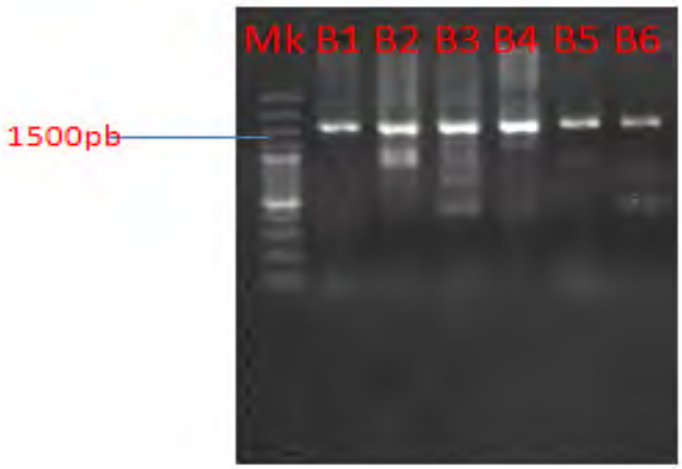


Figure 4. Agarose gel electrophoresis of the extracted DNA from six bacteria samples isolated from poultry houses. Gel labelled B1-B6 correspond to *Escherichia coli, Enterococcus faecalis, Staphylococcus aureus, Pseudomonas aeruginosa, Klebsiella pneumonia, Bacillus subtilis* respectively while Mk represents the molecular marker.

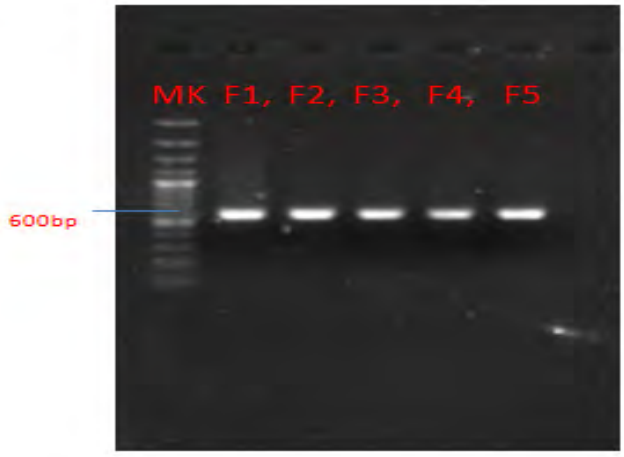


Figure 5. Agarose gel electrophoresis of the PCR products of five fungi samples isolated from poultry houses. Band size of approximately 600bp confirms positive amplification. Samples isolated from, Ekpoma (F1, F2) and Benin (F3, F4, F5,) were identified to *be Fusarium oxysporum, Aspergillus niger, Rhizopusstolonifer Trichoderma polysporum and Aspergillus fumigatus* respectively.

Table 5. Molecular identification of bacterial and fungal isolates

Code	Organism	NCBI accession number	Strain
Bacteria			
B1	*Escherichia coli*	MK271753	strain RB1
B2	*Streptococcus pyogenes*	MK271754	Strain RB2
B3	*Staphylococcus aureus*	MK271755	Strain RB3
B4	*Pseudomonas aeruginosa*	MK271756	Strain RB4
B5	*Klebsiella pneumoniae*	MK271757	Strain RB5
B6	*Bacillus subtilis*	MK271758	Strain RB6
Fungi			
F1	Fusa*rium oxysporum*	MK271759	strain RF1
F2	*Aspergillus niger*	MK271760	strain RF2
F3	*Rhizopusstolonifer*	MK271761	strain RF3
F4	*Trichoderma polysporum*	MK271762	strain RF4
F5	*Aspergillus fumigatus*	MK2717639	strain RF5

Table 6. Frequency of Occurrence of Airborne Bacterial and Fungal Isolates in Poultry Farms

Airborne Bacterial Isolates	Percentage frequency (%)
Bacteria	
Staphylococcus aureus	40 (25.3)
Enterococcus faecalis	20 (12.5)
Bacillus subtilis	22(13.8)
Escherichia coli	24 (15.0)
Klebsiella pneumonia	30(19.0)
Pseudomonas aeruginosa	22 (14.4)
Total	***158 (100)***
Fungi	
Fusarium oxysporium	8(19.6)
Aspergillus niger	13 (29.8)
Rhizopusstolonifer	9 (21.1)
Trichoderma polysporium	1 (2.6)
Aspergillus fumigatus	11 (26.9)
Total	***42 (100)***

Phenotypically, six airborne bacterial and five airborne fungal isolates were isolated and characterized. The airborne bacterial and fungal isolates were further characterized using culture-dependent molecular characterization and identification technique to reveal the presence of the following; *Escherichia coli*, *Streptococcus pyogenes*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Enterococcus faecalis*, *Klebsiella pneumonia* and *Bacillus subtilis*, *F*usa*rium oxysporum*, *Aspergillus niger*, *Rhizopusstolonifer*, *Trichoderma polysporum* and *Aspergillus fumigatus* (Table 5, Figures 4 and 5). The highest frequency of occurrence of the airborne bacterial isolates was recorded for *Staphylococcus aureus* (25.34%) while the least was recorded for *Enterococcus faecalis* (13.25%) (Table 6) Among the airborne fungal isolates, *Aspergillus niger* (30.21%) recorded the highest frequency of occurrence while the least was recorded for *Trichoderma polysporum* (2.34%)(Table 6). A comparison of types of poultry with respect to bacterial and fungal loads has been presented (Table 7). Bacterial and fungal loads were significantly higher in the substandard poultry compared to the standard ones. Similarly, with respect to weather, bacterial and fungal loads were higher throughout the rainy period collections as likened to dry season collections (Table 8). The dendrogram from hierarchical cluster analysis showing a bivariate association between any two poultry environments on the basis of bacterial (Figure 6a) and fungal (Figure 6b) composition revealed that the Auchi groups were statistically separated from the others and this can be attributed to the weather condition peculiar to that environment.

Table 7. Comparison of types of Poultry with respect to Bacterial and Fungal loads.

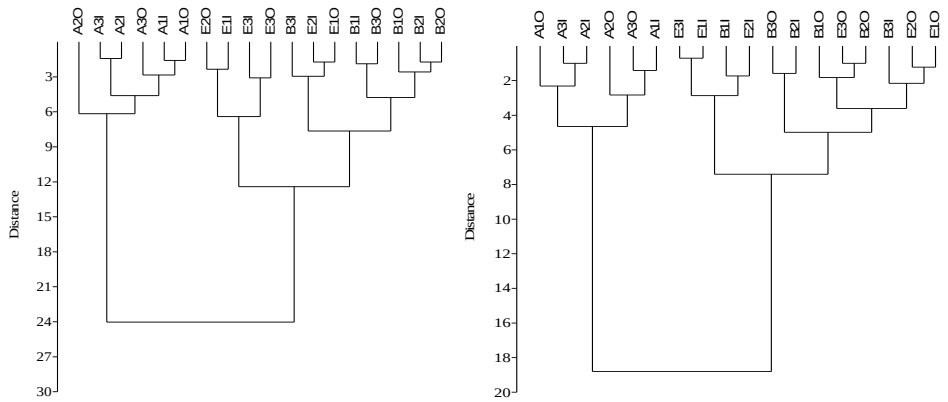
Poultry types	Bacterial load	Fungal load
Sub-standard Poultry	383.22a	93.94a
Semi-standard Poultry	266.01b	76.71b
Standard Poultry	209.02c	59.09c
P-value	<0.001	<0.001

Values within similar columns with the same alphabets do not differ from each other (p>0.05)

Table 8. Differences in Bacterial and Fungal composition in the Sampled Season.

	2017 Season	Mean counts (x 10^2CFU/m^3)	SD	SEM	t-value	p-value
Bacterial loads	Rainy	3.62	92.07	27.76	2.103	0.049*
	Dry	2.72	104.47	33.03		
Fungal loads	Rainy	0.68	21.88	6.59	-1.148	0.265
	Dry	0.8	23.71	7.49		

Mean difference is significant, p<0.05



(a) (b)

Figure 6. Dendrogram from hierarchical cluster analysis showing a bivariate association between any two poultry environments based on (a) bacterial composition and (b) fungal composition.

Keys: *A is Auchi, B is Benin City, E is Ekpoma, 1 is substandard, 2 semi-standard, 3 standard poultry, I is inside and O is outside poultry environment*

Discussion

This research showed that gaseous pollutants, as well as airborne fungi and bacteria, were relatively higher in the indoor environment than in outdoor areas, this is similar to findings of other authors (Lonc and Plewa 2010), the sources of these variations may be farm objects. There were however contrary findings in all poultry farms in Ekpoma and Benin as well as standard poultries in Auchi and Benin, where the fungi load in indoor and outdoor areas were not significantly different, this can be attributed to a lack of good ventilation system. A Similar trend was also observed for bacterial load in all poultry types in Ekpoma and standard poultry in Auchi as well as semi-standard poultry in Auchi and Benin, a key reason for this uncommon occurrence in standard and semi-standard poultry farms may be a result of non or improperly cleaned ventilation system, mechanical ventilation systems not properly cleaned can be a source of microbial proliferation and spending of microorganisms as reported by Collins (Collins, 2007).

Results from this study indicate that hydrogen sulphide concentrations were relatively higher in sub-standard poultry farms across all locations, however, the high concentrations recorded in some standard poultries are an indication of an unhygienic state and hydrogen sulphide is released from manure decay. The Highest indoor level in this study recorded in sub-standard poultry was far above the > 1 ppm concentration in poultry confinement (Jones *et al*., 2000).

Nitrous oxide concentrations in the indoor areas of poultry houses were generally higher in sub-standard poultry farms, however, concentrations in semi-standard and sub-standard poultry farms in Auchi and Benin were similar statistically, this may be a result of improper heating systems coupled with the relatively higher levels recorded in these areas during May through July which are the peak wet seasons in Nigeria, as higher concentrations of N_2O were recorded by Calvet *et al.*, 2011 during winter periods when compared with values record in summer. Another possible reason may be the bird feed compositions in the poultries as well as the stage of maturity of the birds.

Indoor levels of methane in this study though below the 25 LEL permissible limit set by the World Health Organization similar to findings of previous authors (Calvet *et al.*, 2011) who also stated that the amount of methane emitted from poultry houses depends on management and condition of the poultry. Concentrations were, however, higher in sub-standard poultry farms in all locations except that levels in standard and semi-standard poultry farms in Benin were not significantly different, this may be attributed to the number of birds in the poultry (Calvet *et al.*, 2011).

Sulphur dioxide concentrations inside poultry facilities were higher in sub-standard poultry in Benin City, relatively higher concentrations were recorded in standard poultry farms in Ekpoma and Benin than in semi-standard and sub-standard poultry farms. This result thus gives a clue into the fact that the ventilation system has little role to play in the amount of SO_2 in poultry confinement.

Ammonia concentrations in semi-standard poultry in Ekpoma were above the W.H.O permissible limit, this may be attributed to the feed sources as previously reported by (Nahm, 2000), undigested proteins in poultry manure are potential sources of ammonia polarization.

Considering poultry types, the concentrations of ammonia were significantly higher in sub-standard poultry farms than in other types of poultry farms, there was, however, no significant difference between concentrations in semi-standard and sub-standard poultries in Auchi and Ekpoma. The feed types used in these poultry farms maybe a probable explanation for this.

Carbon dioxide levels observed indoors during this research were higher in semi and sub-standard poultry with no difference between concentrations in all three poultry farms in Auchi. As reported in earlier studies high CO_2 levels may be a result of type of heating system used (Knížatová *et al*., 2010, reported that the use of natural gas as source of heating system could contribute to the amount of CO_2 emitted in an animal farm. These authors also suggested that the CO_2 amount in the indoor air of poultry should be considered in the operation of ventilation systems.

Dust levels in the poultry houses were the highest in semi and sub-standard poultries with similar levels statistically recorded. Dust in the range of PM_{10} was, however, the only form sampled in this study, as this is the maximum level that is respirable and is capable of lodging in the lungs. Statistically similar levels were recorded in the indoor and outdoor areas of the semi-standard poultry in Auchi, this can be attributed to the presence of several quarries around the sampling areas and thus the poultry facility may not be the only contributor to the outdoor levels of the PM_{10} observed.

The Highest PM_{10} level recorded in this study (1.75mg/m^3 in sub-standard poultry) was higher than the 0.02 mg/m^3 level recorded by Jones *et al*. (2000), though the poultry they studied was not defined. In poultry facilities PM_{10} originate from feed particles, bedding material, manure particles and feather particles blown from poultry fans (Nahm, 2000), thus ventilation system may be a major factor in the distribution of PM_{10} inside a poultry building.

Fungal loads in Ekpoma were not significantly different in the indoor and outdoor environments, this was contrary to the studies by Lonc and Plewa 2010, the indoor fungal load was higher than the outdoor fungal load, this may

be a result of the relatively high humidity in the outdoor environment of these poultry farms, which is capable of supporting the proliferation of fungi (Lonc and Plewa, 2010 and Knížatová *et al*., 2010). Indoor fungi load was the highest in substandard poultry farms, this may be a confirmation of the assumption that poultry types play vital roles in determining fungal count as previous studies by Sowiak *et al*., showed that mechanical ventilation systems coupled with increase air flow rate contribute significantly to reducing fungal loads inside poultry facilities, however, no significant difference was recorded among poultry types in Benin-City.

High fungal loads recorded in standard and semi-standard poultry farms in Benin- City may be as a result of improper cleaning of the ventilation systems in these facilities, as suggested by Collins, 2007, who reported high fungal counts in poultry with mechanical ventilators when compared to those adopting gravity ventilation.

Performed analysis of variance demonstrated a significant impact of poultry types on indoor airborne bacteria loads, owing to the fact that high bacterial loads were recorded in all sub-standard poultry farms sampled, but similar to fungal, there was no significant difference between indoor and outdoor bacterial loads in all poultry farms in Ekpoma as well as in standard and semi-standard poultry farms in Auchi and Benin City. This result did, however, not agree with the findings by Lonc and Plewa, 2010, and may be an outcome of improper hygiene and practices that encourage the growth and abundance of bacterial outside poultry environment.

The isolated bacteria in this study are of both veterinary and public health importance. *Staphylococcus aureus, Streptococcus pyogenes, Bacillus subtilis, Escherichia coli, Klebsiella pnemoniae* and *Pseudomonas aeruginosa* were identified culturally and biochemically, with *S. aureus* being the most frequently isolated. *E. coli* and other enteric bacteria such as *Klebsiella* and *Pseudomonas* species isolated from the sampled poultry houses in this study are members of normal intestinal flora. These bacteria become pathogenic when they reach tissues outside their normal intestinal or other normal flora sites. The anatomic sites of clinical importance in humans are urinary tracts, biliary tract, lung, bone, meninges, prostate gland and blood (bacteraemia). The presence of these bacteria in poultry facilities is in conformity with previous studies (Collins, 2007, Jones *et al*., 2000). Two of the bacteria, *E. coli* and *K. pnuemoniae* isolated during this study belong to the risk group 2 bacterial according to the Polish Ordinance, which is a risk classification for occupational exposure to bioaerosols (Lonc and Plewa, 2010). *E. coli* is an opportunistic pathogen, which can cause urinary tract infections*, K. pnuemoniae* on the other hand is also an opportunistic pathogen capable of causing respiratory tract infections.

S. aureus, while not being a spore-producing bacteria, has been shown to live longer in the air than any other bacteria, implying that it has a high capacity for airborne dissemination and infection. Its high frequency in aerosols, combined with this, makes it a likely candidate for bioaerosol airborne emissions. Given its high pathogenicity and virulence, the high prevalence of *Staphylococcus aureus* in the sampled poultry farms may be cause for concern. Several human diseases have been linked to the organism, including cellulitis, local abscess formation (furuncles and carbuncles), and lymphadenitis. Primary osteomyelitis and septic arthritis may occur when the infection spreads to the bones and joints (Brodka *et al*., 2012) Inhalation of *Pseudomonas aeruginosa* may cause necrotizing pneumonia and the involvement of the ear and eye may result in otitis externa and rapid destruction of the eye respectively (Jones *et al.*, 2000).

All fungi isolated in this study were in the mould group and include *Fusarium oxysporium, Trichoderma polysporum, Aspergillus niger, Aspergillus fumigatus* and *Rhizopus stolonifer.* They are referred to as opportunistic fungi. They do not usually induce diseases, but do so when the body's host defense is compromised (Brooks *et al*., 2007). Similar results were obtained in the study by Sowiak *et al.,* 2012 who isolated moulds as the major group of fungi from poultry facilities. Moulds are associated with humid environments and are capable of causing respiratory tract infections, as well as allergic effects, more worrisome, is that *A. fumigatus* which among moulds isolated during this study is classified as a risk group two biological agent. *A. fumigatus* is closely associated with humid environment and is frequently isolated from the surface of ventilators and settled dust (Sowiak *et al*., 2012).

Conclusions

Poor standardization of poultry farming processes implied poor air quality as well as the worsened microbial quality of the air within and around the poultry farm. As evident from this research, poultry farms are substantial reservoirs and emitters of microbiological and gaseous contaminants into the environment. The growth of the Nigerian poultry industry needs a holistic approach that provides the best environment, nutrition and health for birds as well as minimizes occupational and environmental health risks. Strict biosecurity will reduce bacteria and other infectious microbes in the poultry environment.

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Revealing the CRISPR array in bacteria living in our organism

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Abstract. CRISPR (clustered regularly interspaced short palindromic repeats) is an immune system used by bacteria to defend themselves from different types of pathogens. It was discovered that this immune system can modify itself in specific regions called spacers due to previous interaction with foreign genetic material from phages and plasmids. Through our research, we have identified in different bacterial isolates CRISPR arrays belonging to the subtypes I-E (present in 42 samples) and I-F (present in 9 samples). The number of spacers in CRISPR arrays was also estimated based on the array length as a possible connection with the systems activity. Our results yielded arrays as small as 200 bp and as large as 1400 bp.

Keywords: CRISPR array; clinical isolates; pathogens; bacteria

Introduction

The end of the XXth century represented a reference point regarding genetics and molecular biology. The discovery of the CRISPR-Cas system (Clustered Regularly Interspaced Short Palindromic Repeats) meant a new opportunity for research, an area never explored before, that can bring new therapies in the medical domain, as well in associated sciences and areas.

CRISPR-Cas system consists of an immune system used by bacteria to protect themselves from pathogens. Over the years, scientists have discovered the inner workings of this immune system and harvested the power it holds: targeted genetic manipulation. The CRISPR-Cas system has been discovered in around 90% of Archaea and 60% of Bacteria (Palmer and Gilmore, 2010).

CRISPR arrays are a family of diverse DNA sequences with very similar structures. Each locus comprises repeated sequences of 20–40 bp, called repeats, and sequences containing the genetic information, called spacers, ranging between 25–70 bp (Kunin *et al.,* 2007). Besides the CRISPR array, there are many associated proteins. These proteins have been named Cas proteins (CRISPR Associated Proteins). In the beginning, only 4 Cas proteins have been identified (Jansen *et al.,* 2002). However, over the years, at least 45 families of Cas proteins have been discovered. Out of these proteins, Cas 1 and Cas 2 are universally conserved (Haft *et al.,* 2005).

There are different classes of CRISPR loci. The main method for classifying them is by the Cas cluster (Makarova *et al.,* 2011a). Thus, there is CRISPR Class I, defined by the use of multiproteic effectors. In this CRISPR class, there is a multitude of different proteins involved in the immune process (Barrangou, 2015). The second class is defined by the now-famous protein Cas 9. The Cas 9 protein can cut DNA strands, resulting in DNA sequences with straight ends and aids in the insertion of new spacers (Garneau *et al.,* 2010).

The immune response mediated by CRISPR-Cas works in three different stages. The first stage is adaptation, which consists of acquiring new spacer sequences, creating a genetic memory that will be used in further stages (Devashish *et al.,* 2015). In this stage, the Cas 1 and Cas 2 proteins play a crucial role in the integration of new genetic data (Yosef *et al.,* 2012). These proteins create a complex that will bind to the CRISPR DNA due to the Cas 1 protein (Nuñez *et al.,* 2014). Vital to this stage is the existence of PAM (Protospacer Adjacent Motif), which helps differentiation between self and non-self sequences (Mojica *et al.,* 2009). After the non-self sequence has been identified, the protein cascade or Csy complex in junction with host cell proteins (polymerases, ligases) will begin working towards incorporating the new spacer sequence (Devashish *et al*., 2015).

The second stage is the expression. The result of this stage is the formation of an RNA molecule called pre-crRNA (Devashish *et al*., 2015). This RNA molecule needs to mature; thus, maturation is essential to ensure the proper functionality of the CRISPR-Cas system. This stage requires the synthesis of specific proteins that modify the pre-crRNA molecule in the final crRNA (Gesner *et al.,* 2015). This initial transcript will be processed by Cas proteins (Carte *et al*., 2014), Cas 6 being the main protein realizing the transcript

processing (Nam *et al.,* 2014). This protein recognizes stem-loops present in pre-crRNA repeat sequences. The cutting takes place at the base of these loops, ensuring that cleaving only occurs in repeat sequences, not affecting the spacers sequences. After this process is finished, the mature crRNA is obtained (Xue and Sashital, 2019).

The last stage is interference. The PAM sequence plays a significant role in the identification of pathogens. After the pathogen is identified, the mature crRNA is guided with the help of the protein cascade or Csy complex. The interaction between these complexes and the foreign DNA destabilizes double-stranded DNA (dsDNA) (Xue and Sashital, 2019). This destabilization enables the crRNA to invade, leading to the formation of crRNA-DNA heteroduplex and the creation of the R-loop. The creation of the R-loop will then trigger the recruitment of the Cas 3 protein that will degrade the target DNA (Guo *et al*., 2017).

The Enterobacteriaceae family contains a high diversity of bacteria, responsible for a variety of diseases in humans. (Fritz *et al.,* 2005). In a previous study, 228 genomes from 38 species of bacteria were analysed. Out of these genomes, 38,6% (*Escherichia coli, Cronobacter, Citrobacter, Erwinia, Klebsiella, Proteus, Salmonella, Shigella*) contain the I-E CRISPR subtype, 14% have the I-F subtype (*Enterobacter, Erwinia, E. coli, Klebsiella, Pectobacterium, Providencia, Rahnella, Serratia, Yersinia*) and 4% have subtypes I-E and I-F (*Dickeya, Erwinia, Pectobacterium*). 0.44% of the genomes, the equivalent of one isolate, identified as *Serratia*, contains three different subtypes (Medina-Aparicio *et al*., 2018).

The study of the CRISPR-Cas system in these bacteria is essential for genotyping and developing of possible therapies for hard-to-treat infectious diseases in humans. This study investigated the presence of CRISPR loci in clinical isolates of *Pseudomonas aeruginosa* and *Escherichia coli*. The subtype of the CRISPR array and the number of spacers offer an integrative view of the activity of the CRISPR-Cas system in a particular environment exposed to permanent selective pressure exerted by antibiotics and bacteriophages.

Materials and methods

Bacterial isolates

The bacterial isolates have been collected in hospital units from patients with: urinary infections, infections resulting from wounds or burns, pneumonia, ocular infections and circulatory system infections. Patients were not involved in this study. Different bacteria have been isolated and identified in the hospital laboratory according to specific protocols, including *Citrobacter, Enterobacter,*

Hafnia, Klebsiella, Proteus, Providencia, Salmonella, Yersinia and *E. coli*. (Farkas *et al.,* 2019). Thus, 80 different isolates were analyzed for the presence of CRISPR arrays.

Molecular identification of CRISPR array

CRISPR arrays were identified by PCR amplification with different primer pairs designed in our laboratory. PCR amplification was performed with bacterial suspensions as templates. Bacterial pure cultures were suspended in sterile water to a concentration of approximately 106 cells/ml (Crăciunaș *et al,* 2010). PCR reaction mix contained in 25 µL total volume: 12.5 µL DreamTaq Green PCR master mix (2x) (Thermo Fisher Scientific, USA), 10.25 µL nuclease-free water (Lonza, Switzerland), 25 pmol each primer, and 2 µL bacterial suspension. As a negative control, we used 2 µL of sterile water in the PCR mixture. The PCR programs are shown in Table 1. PCRs were performed using a thermocycler TProfessional Trio (Analytik Jena, Germany), Mastercycler Nexus (Eppendorf AG, Germany) or Gradient Palm-Cycler (Corbett Life Science, Australia). The amplicons were separated in 1.5% w/v agarose (Cleaver Scientific, United Kingdom) gel in 1×TBE buffer (Lonza, Switzerland) and stained with 0.5 µg/ml ethidium bromide (Thermo Fisher Scientific, USA). Data acquisition was performed using the BDA Digital Compact System and BioDocAnalyze Software (Analytik Jena, Germany).

Table 1. PCR (Polymerase Chain Reaction) program used for the identification of CRISPR in clinical samples. EC represents the primers used to amplify CRISPR structures of Type I-E. PA represents the primers used to amplify CRISPR structures of Type I-F.

Step	Temperature	Time	Number of cycles
Initial denaturation	94ºC	4 min	
Denaturation	94ºC	40 s	x 35
Annealing	EC: 55ºC PA: 57ºC	20 sec	
Elongation	72ºC	2 min	
Final Elongation	72ºC	7 min	

Data analysis

Gel analysis and estimation of the number of spacers was performed further. Several numbers of base pairs were removed from the length of bands, flanking the CRISPR structure. These flanking elements are usually the genes encoding Cas proteins. In Table 2, the number of bp removed for each

primer pairs are shown. After the subtractions, the resulted number is divided to 58, the equivalent of the average size of the spacer-repeat pair, to obtain the number of spacers in CRISPR arrays in each bacterial isolate. Bands having ≤ 100 bp cannot be considered because they are too small to contain CRISPR arrays.

Table 2. Number of base pairs subtracted in the case of each primer pairs. Only primer pairs that give PCR amplification of CRISPR arrays are shown.

Primer	Size of flanking regions in bp	Size of a spacer/repeat/spacer+repeat
EC2-A	100 bp	30bp /28bp /58bp
EC2-B	94 bp	
EC3	71 bp	
PA1	138 bp	
PA2	138 bp	

Results

The amplification using the primer EC1 resulted in bands being 100 bp or smaller than that. Thus, these results were excluded when calculating the number of CRISPR spacers. Two primer pairs EC2-A and EC2-B were used to target different variants of the same CRISPR locus and to identify as many CRISPR arrays as possible. The amplifications using EC3 primers yielded a high amount of amplicons that could be used further in the study.

Regarding the PA primers, we used two sets of primers: PA1 and PA2. Amplification with the primer pair PA1 showed 1400 bp fragments, meaning CRISPR arrays with high number of spacers. CRISPR arrays are identified in 34 bacterial isolates by PCR amplification with different primer pairs (Table 3). In Table 4, the estimated number of spacers per CRISPR array is shown.

The highest number of spacers was found in samples EM1, EM3, EM13, EM14 and EM26, having 1400 bp, 23 spacers in average. These spacer sequences were found by using the PA1 primer pairs. The lowest number of spacer sequences were identified in the following samples: EM2, EM3, EM7, EM13, EM14, EM42, EM44, EM45, EM47, EM48, EM56, EM61, EM64, EM65, EM67 and EM81. The spacer sequences with 200 bp, the equivalent of 1 spacer, were identified using the EC3 primer pairs.

Figure 1 shows the distribution of isolates identified by each set of primers. By using the primer pair EC3, CRISPR arrays were identified in 22 isolates. The lowest yield can be observed in the case of EC2-B, two isolates that contain CRISPR array. This was expected because this set of primers was

designed to identify extra spacers that EC2-A set of primers could not amplify. The EC2-A primer pair showed positive results in 18 isolates, and the PA-C primer pair showed positive results in 9 isolates.

The most common length is 200 bp being the equivalent of 1.2 spacers. The downfall of these samples is that there might be a possibility that the CRISPR loci in this bacteria might be inactive. It is worth mentioning that bands over 800 bp have a high number of spacers which is correlated with an intense activity of the system, and they were identified in 11 isolates.

Table 3. CRISPR array identified in different bacterial isolates.

Sample	Size in bp			
	EC2-A	EC2-B	EC3	PA1
EM1				1400
EM2			200	
EM3			200	1400
EM4		600		
EM7			200	900
EM8				900
EM13			200	1400
EM14			200	1400
EM18	800			
EM19	800			
EM25	400		250	900
EM26	400		250	1400
EM28	500			900
EM37			400	
EM38	400	400	250	
EM39			250	
EM42	450		200	
EM44	500		200	
EM45	450		200	
EM47	450		200	
EM48			200	
EM49	450			

Sample	Size in bp			
	EC2-A	EC2-B	EC3	PA1
EM53	400			
EM56	400		200	
EM59	400			
EM61			200	
EM64			200	
EM65			200	
EM67	400		200	
EM81	400		200	

Table 4. Number of CRISPR spacer sequences identified in bacterial isolates.

Sample	Number of spacers			
	EC2-A	EC2-B	EC3	PA1
EM1				23
EM2			1	
EM3			1	23
EM4		8		
EM7			1	14
EM8				14
EM13			1	23
EM14			1	23
EM18	11			
EM19	11			
EM25	4		2	14
EM26	4		2	23
EM28	6			14
EM37			5	
EM38	4	4	2	
EM39			2	
EM42	5		1	
EM44	6		1	
EM45	5		1	
EM47	5		1	

Sample	Number of spacers			
	EC2-A	EC2-B	EC3	PA1
EM48			1	
EM49	5			
EM53	4			
EM56	4		1	
EM59	4			
EM61			1	
EM64			1	
EM65			1	
EM67	4		1	
EM81	4		1	

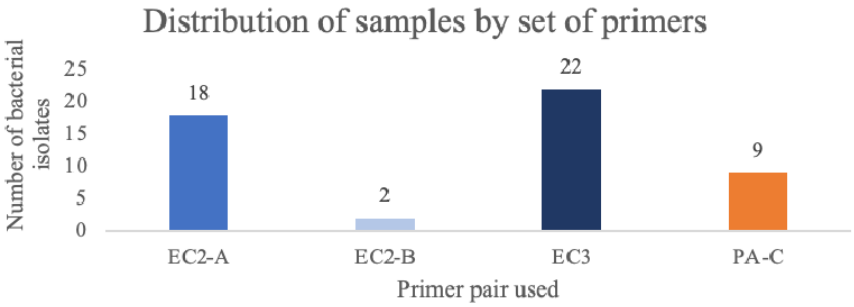

Figure 1. Bacterial isolates bearing CRISPR arrays revealed by different primer pairs.

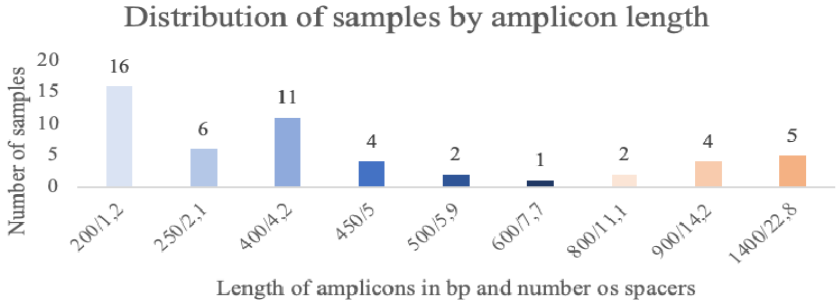

Figure 2. Distribution of samples by amplicon length.

The distribution of CRISPR subsystems between the bacteria in the clinical samples is shown in Table 5.

Table 5. Distribution of CRISPR subtypes between bacteria.

Genus	CRISPR subtype
Citrobacter	I-E
Enterobacter	I-F
Klebsiella	I-E and I-F
Proteus	I-E
Providencia	I-F
Salmonella	I-E
Yersinia	I-F
E. coli	I-E and I-F

Discussion

The CRISPR-Cas system is divided into many types. This classification has been made based on the Cas proteins that take part in the CRISPR machinery (Makarova et al., 2011b). One of the most frequent subtypes of CRISPR is the I-E subtype. This subtype was first discovered in *E. coli* and it was shown that the spacers contained are derived from bacteriophages, mobile genetic elements and plasmids (Kiro et al., 2013).

The I-F subtype was discovered in *P. aeruginosa*. An interesting discovery regarding this subtype is that most of the spacer sequences are 100% identical to mobile genetic elements that can insert themselves in the bacterial chromosome (Cady et al., 2012). Another significant discovery regarding the I-F subtype is that a slight mismatch of nucleotides between the PAM sequence and the protospacer of this subtype can lead to the inactivation of the whole system, while in I-E subtype, only the CRISPR loci would be inactive, the Cas proteins were still active and transcribed (Semenova et al., 2011).

Type I of CRISPR arrays is characterized by the presence of a protein cascade that fulfils the role of the CRISPR-Cas system. At the core of this protein cascade is the Cas 3 protein that acts as a helicase and has DNA-ase activity (Sinkunas *et al.,* 2011). Besides the Cas 3 protein, many other proteins create the protein cascade in the case of subtype I-E (Brouns et al., 2008). Finally, the protein cascade is replaced by the Csy surveillance complex in the case of subtype I-F (Xue and Sashital, 2019).

Previous studies (Mlaga *et al*., 2021; Horvath *et al*., 2008) have identified a positive correlation between the number and diversity of spacers and the activity of the CRISPR-Cas system. Thus, looking at our results, we can

identify that most CRISPR arrays in isolates amplified with the PA-1 primer set have an intense activity. The isolates having CRISPR arrays identified with EC2-A, EC2-B and EC3 primer sets have, on average, reduced activity, compared to isolates with CRISPR arrays identified with primer set PA-1.

Most bacteria contain a single subsystem of CRISPR, as identified by Medina-Aparicio et al., 2018. However, it can be noticed that some genus can have more than one subtype. This is possible because of the ability of bacteria to transfer genes horizontally.

Horizontal gene transfer (HGT) represents one of the most important tool used by bacteria to enrich their genome, especially when talking about antibiotic resistance genes (ARGs). However, active CRISPR arrays may inhibit HGT (Wheatley and MacLean, 2021). When looking at the isolates having a high number of spacers in CRISPR array we can hypothesize that this system is active. A previous study (Farkas *et al.,* 2019) showed that these isolates bear a high number of ARGs encoding the resistance to different antibiotics. Assuming that CRISPR-Cas system inhibits the HGT of ARGs, most probably in these isolates, the CRISPR-Cas system is not active at present, ensuring the accumulation of ARGs. This inactivation is most probably recent; the unuseful spacers sequences were not deleted yet. If the selection pressure of antibiotics persists (as happens in the clinical environment), some of the spacers will probably be lost. Another hypothesis is that the system is active, but the spacers sequences are accumulated by previous bacteriophage infection (Wheatley and MacLean, 2021).

Conclusions

In the Enterobacteriaceae family, CRISPR is present under two subtypes: I-E and I-F.

When looking at the length of CRISPR array and number of spacers, we identified the largest CRISPR array in isolates containing I-F subtype. We have identified spacer sequences having 1400 bp, equivalent to 23 spacers. This indicates that the I-F subsystem is active.

The I-E subtype has smaller spacer sequences. However, there are some isolates that presented larger CRISPR arrays with higher number of spacers (600 bp – 8 spacers). This might indicate that the I-E subtype might not be as active as the isolates containing the I-F subtype.

The subtype of the CRISPR array and the number of spacers offer an integrative view of the activity of the CRISPR-Cas system in a particular environment exposed to permanent selective pressure exerted by antibiotics and bacteriophages.

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Leaf heteroblasty and morphotypes of *Acer monspessulanum* (Djurdjura, Algeria) as revealed by traditional and geometric morphometrics

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Abstract. The genus *Acer* which is largely distributed in the Northern hemisphere is represented in Algeria by four species of which *Acer monspessulanum* is the most common. These four species and their putative hybrids are coexisting in many parts of their Algerian distribution range but their leaf morphology has not been the subject of quantitative analyses despite the interest of such kind of data in taxon delineation particularly in the case of interspecific hybridization. The present work is the first step towards a quantitative analysis of leaf morphology in *Acer* species in Algeria. We presently relied on traditional and geometric morphometrics methods in the study of *Acer monspessulanum* subsp. *monspessulanum* leaf morphology in two sites of the Djurdjura Mountain with consideration of tree and within-shoot effects. The results showed congruence between the two methods which both highlighted the presence of a marked heteroblasty. Basal leaves are twice as large as apical ones for all measured leaf features. The petiole is longer than the blade in basal leaves and inversely in apical ones. The median leaves have intermediate values. The results revealed also the coexistence of two contrasted leaf morphotypes on distinct trees of both sites.

Keywords: *Acer monspessulanum,* Djurdjura, heteroblasty, leaf, morphotypes.

Introduction

The Maples (*Acer* L.; *Sapindaceae*) consist of over 150 species with a large distribution in the Northern hemisphere (Van Gelderen *et al.*, 1994). Numerous authors identified Maple species based on foliar and reproductive traits (De Jong, 1976), bud morphology (Ogata, 1967), vein architecture (Tanai, 1978) and more recently with molecular data and phylogeny (Ackerly and Donoghue, 1998; Grimm *et al.*, 2007).

The genus *Acer* is well-known for its polymorphic leaves rendering the taxonomical identification difficult and controversial even actually (De Jong, 2019). In addition to the variability among species, leaf morphology may change under the influence of environmental factors (Gratani, 2014). For example, colder temperatures induced an increasing leaf dissection and tooth number in *A. rubrum* (Royer *et al.*, 2008; Royer *et al*., 2009). In *Ziziphus jujuba*, the density of leaf venation increased while leaf area and leaf perimeters decreased in arid areas inversely in humid areas (Li *et al.*, 2015). Furthermore, leaf morphology may also vary within the same individual. This trend is studied in many woody genera such as *Acer* (Critchfield, 1971; Powell *et al*., 1982; Steingraeber, 1982), *Crataegus* (Dickinson and Phipps, 1985), *Eucalyptus* (Vlasveld *et al.*, 2018), *Populus* (Eckenwalder, 1980; Eckenwalder, 1996; Slavov and Zhelev, 2011), *Quercus* (Blue and Jensen, 1988; Bruschi *et al.*, 2003; Kusi and Karsai, 2020). Kusi and Karsai (2020) demonstrated that the branch position was the main source of variation in leaf morphology of *Quercus* species. Indeed, outermost leaves of the canopy are smaller, more lobed and have higher LMA contrary to innermost leaves. Leaf form may vary even from node to node during the growing season of a given individual. This pattern is called seasonal heteroblasty (Herrera, 2009). It is present in Maples (Critchfield, 1971) whose shoots bear leaves of different shapes depending on their position on shoot nodes (either proximal or distal). The leaves formed on the basal nodes are supposed to have initiated their development in early spring and rest in the overwinter buds as leaf primordia, while the leaves of the distal nodes are formed in the same season in which they are initiated (Critchfield, 1971; Eckenwalder, 1980; Herrera, 2009). However, the intensity of seasonal heteroblasty expression differs in Maple species. It is marked on some species such *Acer pensylvanucum* and *A. rubrum* and uncommon or poorly expressed on others such as *A. spicatum* (Critchfield, 1971).

Leaf morphology may be analyzed by traditional and by geometric morphometrics methods. Traditional morphometrics consists of analysing different morphological variables (such as linear distance measurement, counts, ratio, angles, etc.) with the uni- or multivariate statistical analyses (Rohlf, 1990; Rohlf and Marcus, 1993; Adams *et al.*, 2004). It is an interesting method which

is used until recently in plant systematic studies (Marcysiak, 2012; Morel *et al.*, 2021). The second used method is the geometric morphometrics comprising the landmark approach, among others, and which has been increasingly used since thirty years (Viscosi and Cardini, 2011). It consists of collecting 2 or 3-dimensional coordinates of biologically definable landmarks (Adams *et al.*, 2004). These points must be homologous because landmark-based methods operate only with the coordinates of these reference points, so-that the objects studied should be directly comparable (Pavlinov, 2001). This method has proved its effectiveness in several studies and fields (Klingenberg, 2010; Viscosi and Cardini, 2011). In botany, Jensen *et al.* (2002) were the first authors who used geometric morphometrics in analysis of leaf shape variability. Since then numerous studies were performed (Viscosi *et al.,* 2009; Klingenberg *et al.,* 2012; Chitwood and Otoni, 2017). There are also authors who combined traditional and geometric morphometric methods in their studies of leaf shape variation (Viscosi *et al.,* 2009; Proietti *et al.,* 2021).

The genus *Acer* is represented in North Africa by 5 taxa including species and subspecies. Algeria records the greatest diversity of this genus in this area. According to flora books (Battandier and Trabbut, 1888; Lapie and Maige, 1916; Quezel and Santa, 1963) four species are naturally occurring in Algeria: *Acer monspessulanum* L. subsp. *monspessulanum, A. opalus* subsp*. opalus, A. opalus* subsp*. obtusatum* (ex. *A. obtusatum* K. & W.)*,* and *A. campestre*. Quezel and Santa (1963) mentioned also the presence of *A. monspessulanum* subsp. *martinii* and *A. x hyrcanum* a putative hybrid between *A. opalus* and *A. monspessulanum*, and they mentionned the commonness of the later in Chelia forest (Aurès Mountain). All these *Acer* species occur either sparsely or as clumps of variable size and occupy the understory of oak and Atlas cedar forests in Algeria (Mediouni and Azira, 1992; Yahi *et al.,* 2008). In Morocco, two species are present; *A. monspessulanum* L. subsp. *monspessulanum* and *A. opalus* subsp. *granatense* (Boiss.) Font Quer & Rothm (Fennane *et al.,* 2014), mainly as understory species of Pinapo fir, Atlas cedar and oak forests (Benabid, 2000; Navarro-Cerrillo *et al.*, 2013; Navarro-Cerrillo *et al.*, 2020). While in Tunisia, only *A. monspessulanum* L. subsp. *monspessulanum* is found in a single site i.e. at Jebel Serej National Park in an Aleppo pine matorral (Le Floc'h *et al.,* 2010; Mechergui *et al.,* 2018; Jaouadi *et al.,* 2020).

The range of these Maples is variable: Mediterranean and Euro-Sibirian (*A. campestre*), North african and Southern West European (*A. opalus* subspecies), or Mediterranean (*A. monspessulanum*). Nord Africa is therefore the southernmost or the westernmost distribution limits of some of these maples. Hybrids could occur between species of the same section, especially between *A. monspessulanum* and *A. opalus* (Van Gelderen *et al.,* 1994; Grimm *et al.,* 2007) increasing the complexity of species identification.

A. monspessulanum is the most common Maple in Algeria. It has also a wide distribution throughout the Mediterranean area until Iran and occurs in the central to the northern latitude of Europe. This large distribution allows the presence of many geographical variants (Van Gelderen *et al.,* 1994). However, their classification is controversial. In Turkey and Iran for example, 5 and 8 subspecies are identified respectively (Amini et al. 2016; Seki 2019). In Algeria, Quezel and Santa (1963) mentioned two subspecies of A. *monspessulanum*: subsp. *monspessulanum* and subsp. *martinii.* However, A. *monspessulanum* subsp. *martinii* Jordan is recognized as a hybrid between *A. monspessulanum* and *A. opalus* (Van Gelderen *et al.,* 1994; Bottacci, 2014), while in Europe only the typical subspecies is recognized (Van Gelderen *et al.,* 1994). Nonetheless, these taxa have not been the subject of comprehensive studies in North Africa and more specifically in Algeria where the species reaches the southernmost limit of their range. Indeed, if we except flora books (authors op cit.) mentioning these taxa, quantitative data on morphological traits of Algerian Maples are lacking.

In this study, we are focusing on variability of leaf morphology in *A. monspessulanum* subsp. *monspessulanum* in the Djurdjura Mountains (northern Algeria) using traditional and geometric morphometric methods as a first step towards a quantitative analysis of the whole co-existing *Acer* taxa in this Mountain including specimens of intermediate leaf morphology between typical Maples. And because lack of knowledge on heteroblasty pattern could lead to taxonomical confusions (Steingraeber, 1982; Eckenwalder, 1996), we sought to analyze the contribution of this variable to leaf morphology variation. As a whole, we addressed the following questions: which amount of leaf morphological variation may be explained by factors such as site, tree within-site and leaf position within-shoot? Is the trend of variation revealed by the traditional morphometric method comparable to that revealed by the geometric- morphometric method?

Materials and methods

Study species

Acer monspessulanum L. is a deciduous tree shrub up to 12 m tall, usually bush (Lapie and Maige, 1916), occasionally taller with a rounded crown (Van Gelderen et al. 1994), characterized by a grey-yellow smooth barks (Lapie and Maige, 1916). The leaves are extremely variable (Van Gelderen *et al*., 1994), lobed to three short and subequal lobes 3-6 cm, coriaceous, green on abaxial and glaucuous on adaxial side. The flowers are greenish-yellow, in corymbs, erect at first and pendent later, with long and slender pedicel (Tutin *et al.,* 1978). The samara is glabrous, with dressed and subparallel wings, shrinks at the base and a very convex dorsal margin (Quezel and Santa, 1963).

Study site

The study was done on samples collected in two sites of the Djurjdura Mountain which comprises both pure and mixed patches of holm-oak and Atlas cedar and tree species such as Maples participate to the forest composition. Such forests patches have been the subject of floristic studies mentioning the existence of *Acer* species (Mediouni and Azira, 1992; Yahi *et al.,* 2008). *A. monspessulanum* is actually the most present Maple on this mountain occupying both open and relatively dense stands either as scarce individuals or as more or less dense clumps.

The two investigated sites are located at the most distant parts of the Djurdjura Atlas cedar-holm-oak forests in order to maximize expression of the potential leaf shape variability (Fig. 1). The first one, i.e. Tala Guilef (hereafter referred to as TG) is located in the Djurdjura National Park (36°28'14.4"N, 03°59'55.48"E) and the second one, i.e. Azro n'Thor (hereafter referred to as AT) is located in its periphery (36°29'35.42"N, 04°23'44.15" E).

Figure 1. Location of two harvested *Acer monspessulanum* sites in the Djurdjura (Google Earth, 2022).

Both sites occupy the same altitudinal range (1290 m a.s.l.) on North-eastern exposures and are characterized by a fresh perhumid bioclimate. They correspond to the open parts of the forest stands with the presence of sparse *Cedrus atlantica* and *Quercus ilex* trees as well as *Crataegus monogyna* and some shrubs such as *Prunus prostrata*, *Juniperus oxycedrus* and *Rubus ulmifolius*.

Sampling and measurements

We harvested 15 mature trees in each site and collected several shoots per tree. The collected shoots have an average of 3 leaf nodes and are 4 cm long. They may reach a maximum of 8 leaf nodes (unpublished data). For the homogenization purpose, we only retained shoots with 3 nodes (Fig. 2). Leaves were removed from the shoots with respect of their insertion level. They were dried using a standard method and scanned on the abaxial surface using a scanner Epson stylus SX100 at 300 dpi resolution. Hereafter we refer to the leaves of the first node as basal leaves (BL); to those of the second node as median leaves (ML) and to those of the third node as apical leaves (AL).

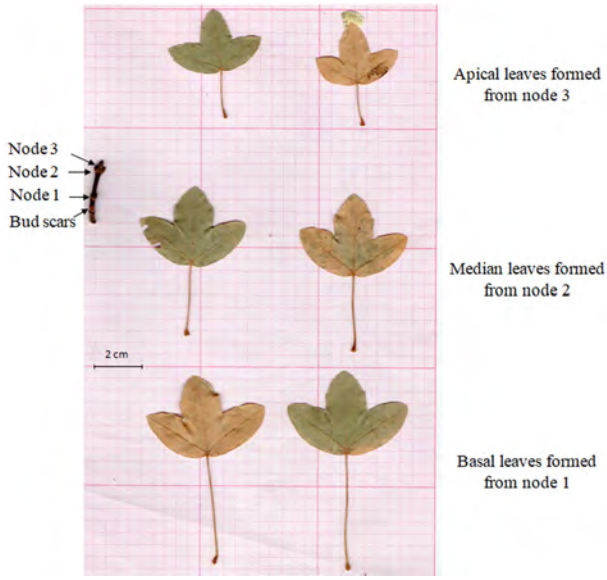

Figure 2. Leaves of *A. monspessulanum* and their position on the shoot.

Measurements with the traditional morphometrics method

Ten parameters were measured on a total of 597 leaves representing 30 trees and two sites (Fig. 3) with the Digimizer software (version 3.7.). Eight quantitative variables were directly measured in addition to two synthetic ones (i.e. ratios between variables). Then all measurements were analyzed with R statistical software v. 4.0.3. First, we performed a descriptive analysis, then we computed a Principal Component Analysis on average values of leaf traits according to their insertion within shoots.Values obtained on median and apical leaves were divided on those of basal leaves and expressed in percent of basal leaf values.

N°	Morphological characters	Units
	Parameters measured:	
1	Leaf length	
2	Blade width	cm
3	Blade length	cm
4	Petiole length	cm
5	Apical lobe width	cm
6	Apical lobe length	cm
7	Angle between nervures of the lateral lobes	cm
8	Leaf area	°
	Ratios :	cm^2
9	Blade / petiole length ratio	
10	Apical lobe length/ blade length ratio	

Figure 3. Morphological leaf traits computed by traditional morphometrics on *A. monpessulanum* leaves.

Measurements with the geometric morphometrics method

For this method we retained a total of 251 leaves with intact margins to avoid measurement errors during landmarks digitalization. Based on several authors (Jensen *et al.,* 2002; Viscosi *et al.,* 2009; Viscosi and Cardini, 2011), we recorded seven landmarks on each leaf surface with the TPS package (Rohlf, 2015) (Fig. 4). We repeated the procedure twice in order to estimate measurement error as mentioned by Viscosi and Cardini (2011). We performed a geometric analysis with MorphoJ 1.06d software (Klingenberg, 2011) available on the website (MorphoJ (morphometrics.uk)).

Landmark	Description
1	Beginning of the petiole
2	Junction of the blade and petiole
3	Tip of the right lateral lobe nerv
4	Right base of the apical lobe sinus
5	Tip of the apical lobe
6	Left base of the apical lobe sinus
7	Tip of the left lateral lobe nerv

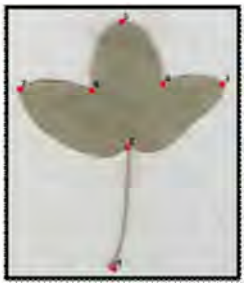

Figure 4. Landmarks configuration recorded on the entire leaf of *A. monspessulanum*

We carried out Procrustes Anova analysis of leaf shape variation at the following hierarchical levels: Between sites, between trees within site, between nodes within shoot, then we calculated an average of the effect that induces the largest share of the total variance of the leaf shape and performed a Principal Component Analysis (PCA) and a Discriminate Analysis (DA). The shape variation along each axis was visualized using wireframe diagrams.

Results

Traditional morphometrics

Descriptive statistics

The mean and coefficient of variation of measured traits are showed in Tab. 1. The basal leaves revealed the greatest values compared to median and apical ones for all measured leaf traits. The total leaf length, the blade length, the blade width, the petiole length, and leaf area were found as 6.56 cm, 3.19 cm, 4.42 cm, 3.99 cm, and 7.14 cm^2, respectively. These values were twice as long as those of apical leaves which correspond to 3.65 cm, 2.33 cm, 2.96 cm and 1.82 cm and 3.29 cm^2 for the same leaf traits, respectively. In contrast, the *blade to petiole length ratio* showed lower values in basal leaves (0.83), comparatively to apical ones (1.82).

Table 1. Overall mean and coefficient of variation of *Acer monspessulanum* leaf morphological traits according to within-shoot leaf insertion level.

Leaf characteristics		Basal leaf	Median leaf	Apical leaf
Total leaf length (cm)	Mean	6.56 (22.6)	5.31 (23.0)	3.65 (33.9)
	Relative growth (%)	100%	80.9%	55.6%
Blade width (cm)	Mean	4.42 (17.2)	3.98 (20.3)	2.96 (30.1)
	Relative growth (%)	100%	90.0%	66.9%
Blade length (cm)	Mean	3.19 (19.4)	2.97 (23.2)	2.33 (31.6)
	Relative growth (%)	100%	93.1%	73.0%
Petiole length (cm)	Mean	3.99 (29.7)	2.89 (32.9)	1.82 (47.2)
	Relative growth (%)	100%	72.4%	45.6%
Apical lobe width (cm)	Mean	1.79 (32.0)	1.68 (36.8)	1.30 (42.9)
	Relative growth (%)	100%	93.8%	72.6%
Apical lobe length (cm)	Mean	1.65 (35.1)	1.60 (37.4)	1.32 (40.6)
	Relative growth (%)	100%	96.9%	80%
Angle between lateral lobes (°)	Mean	117 (13.9)	117 (12.6)	125 (12.4)
	Relative growth (%)	100%	100%	106.8%
Area (cm^2)	Mean	7.14 (25.2)	5.80(30.8)	3.29 (50.1)
	Relative growth (%)	100%	80.8%	46.0%
Blade length/petiole length	Mean	0.83 (20.1)	1.08 (20.6)	1.39 (21.8)
Apical lobe length/blade length	Mean	0.50 (19.1)	0.52 (18.5)	0.56 (15.7)

The numbers in the parentheses are coefficients of variation (CV %).

The angle between lateral lobes was similar in basal and median leaves (i.e. 117°) while it was slightly wider in apical ones (i.e. 125°). Overall, the *apical lobe length to blade length ratio* seemed not to vary along the shoot with mean values ranging between 0.50 and 0.56 respectively on basal and apical leaves.

As reported in Tab. 1, there is a gradual decrease in values of leaf traits along the shoot from the basal to the apical node. Values of apical leaves represented between 46 and 80% of those of the basal leaves depending on traits while those of the median leaves represented between 72 and 96.9% of those of the basal ones. This indicates more expressed differences between the basal and apical leaves than between the basal and median ones. Mean values of some leaf traits were slightly higher at Tala Guilef site comparatively to Azro n'thor one (Tab. 2) with leaf area as the most variable feature between these two sites (i.e. 7.64 cm^2 at TG versus 6.49 cm^2 at AT).

Table 2. Mean and coefficient of variation of *Acer monspessulanum* leaf morphological traits according to site and within-shoot leaf insertion level.

Leaf characteristics	TG			AT		
	BL	ML	AL	BL	ML	AL
Total leaf length (cm)	6.80	5.51	3.72	6.25	5.09	3.55
	(20.5)	(20.5)	(31.6)	(24.6)	(25.5)	(37.0)
Blade width (cm)	4.57	4.16	3.10	4.24	3.78	2.77
	(16.8)	(19.7)	(29.7)	(17.0)	(19.9)	(29.4)
Blade length (cm)	3.26	3.07	2.42	3.11	2.87	2.21
	(21.6)	(23.7)	(33.1)	(15.7)	(21.9)	(28.2)
Petiole length (cm)	4.13	2.96	1.79	3.82	2.80	1.86
	(28.3)	(31.2)	(45.5)	(31.2)	(34.8)	(49.4)
Blade length/petiole length	0.81	1.08	1.45	0.86	1.08	1.30
	(17.1)	(20.3)	(20.8)	(22.6)	(21.1)	(21.9)
Apical lobe length (cm)	1.61	1.60	1.37	1.69	1.59	1.27
	(41.0)	(42.4)	(44.6)	(26.8)	(30.9)	(33.2)
Apical lobe width (cm)	1.90	1.80	1.37	1.66	1.54	1.21
	(32.3)	(36.8)	(44.7)	(29.5)	(34.5)	(38.6)
Apical lobe length/blade length	0.47	0.50	0.55	0.53	0.54	0.56
	(19.1)	(20.3)	(16.0)	(17.3)	(15.5)	(15.1)
Angle (°)	118.	119.	127.	115.	115.	121.
	(13.1)	(12.7)	(11.8)	(14.9)	(12.3)	(12.8)
Area (cm^2)	7.64	6.44	3.57	6.49	5.08	2.92
	(21.4)	(24.7)	(44.8)	(27.8)	(34.0)	(56.5)

The numbers in the parentheses are coefficients of variation (CV %)

Principal component analysis (PCA)

The two first axes of the PCA explained 82.8% of the total variance of which 65.1% captured by the first principal component (PC1) which clearly separates leaves according to their position on the shoot (Fig. 5). The positive

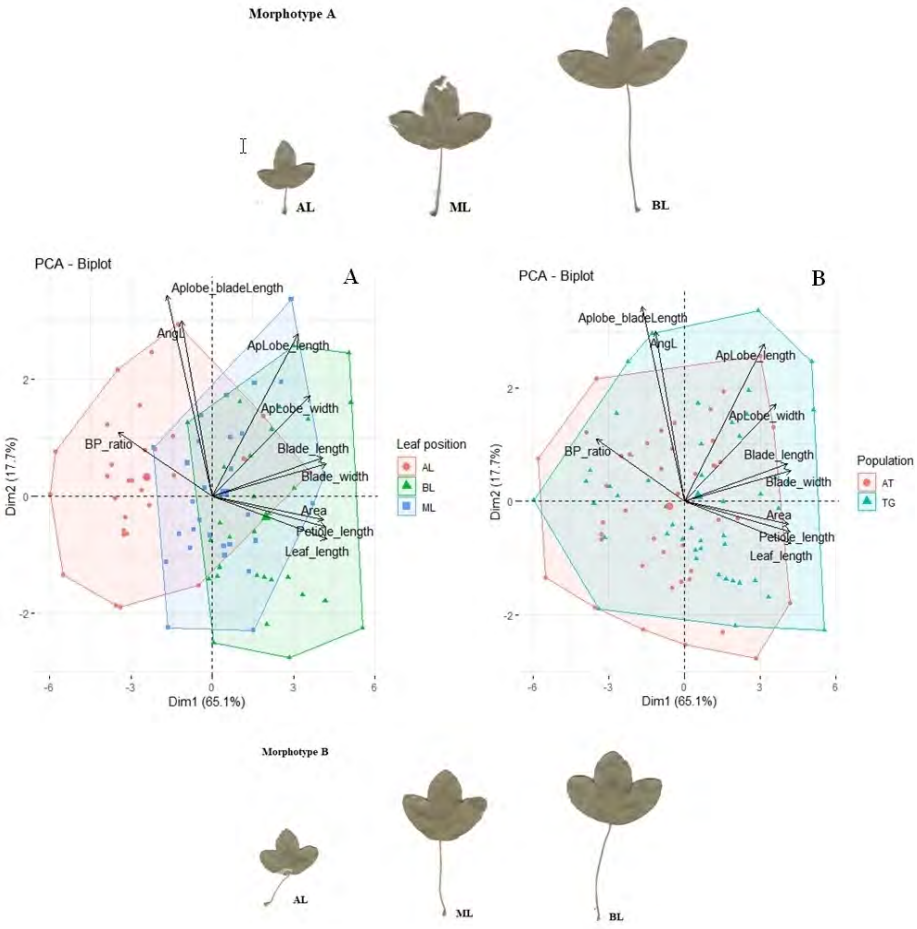

Figure 5. PCA Bitplot showing the distribution of leaf individuals and a vector plot of 10 variables. **A**: Leaf mean values are coloured according to their position on the shoot: Red circles (apical leaves); green triangles (basal leaves); blue squares (median leaves). **B:** leaf mean values are coloured according to their site: red circles at Azro n'Thor (A) and blue triangles at Tala Guilef (TG). The representative leaf images corresponding to the traits illustrated by the PC1: from right to left: basal (BL), median (ML) and apical leaves (AL); and PC2 from the top to bottom: morphotype A and morphotype B.

side of the PC1 is characterized by leaves which are longer, with a bigger area but with a blade relatively shorter than the petiole. These features are associated to basal leaves (BL). Conversely, the negative side of the PC1 is characterized by leaves which are shorter, with a smaller area but with a blade

longer than the petiole. These characteristics are associated to apical leaves (AL). The median leaves (ML) are located in the centre of the graph and are overlapping with the previous groups. The PC2 is related to the angle between lateral lobes from a hand and apical lobe to blade length ratio from another hand. This axis is related to the variability between trees as shown on Fig. 5. The positive side of the PC2 is characterized by a wide angle between lateral lobes (AngL) and high apical lobe to blade length ratio (ApLobe_bladeLength), which corresponds to the morphotype "A". In the opposite side of the PC2, the leaves have a narrower angle between lateral lobes and a small apical to blade length ratio which corresponds to the morphotype "B".

Geometric morphometrics method

Procrustes ANOVA

The analysis of variance (Tab. 3) showed that leaf size did not vary significantly with site and tree but varied significantly with leaf position within shoot. Indeed, leaf position explained 70.1% of the total variance. Leaf effect was highly significant ($p<0.001$), accounting for about 14% of the total variance.

Table 3. Results of the Procruste ANOVA computed on the whole sample of leaves.

Centroide size variation						
Effect	Explained SS (%)	SS	MS	Df	F	P
Site	0.07	1,058	1,058	1	0.15	0.70136
Trees	14.93	203,897	7,031	29	0.645	0.909
Leaf position	70.15	958,144	10,888	88	7,09	<.0001
Leaves	14.83	202,629	1,535	132	8519,46	<.0001
Measurement error	0.003	0,0452	0,0001	251		
Total	100	1365.773				
Shape variation						
Site	2.39	0,255	0,0255264797	10	2.72	0.00329
Trees	25.46	2,713	0,0093553007	290	1.54	<.0001
Leaf position	49.68	5,293	0,0060156850	880	3,35	<.0001
Leaves	22.23	2,369	0,0017953602	1320	188,32	<.0001
Measurement error	0.22	0,024	0,0000095335	2510		
Total	100	10.654				

SS: sum of squares; MS: mean sum of squares; df: degree of freedom.

The Procrustes shape showed that the factors taken into account (i.e. site, tree, leaf position and leaves) contributed significantly to leaf shape variability of Montpellier Maple (Tab. 3). Variation driven by site effect was slight

(explaining only 2.4% of the total variance) but statistically significant. Trees and insertion level explained about 25.4 and 49.6 % of the total variance, respectively. Leaves for their part, explained 22.2% of the total variance.

These results indicate that leaf position (i.e. insertion level) is the main source of variation in leaf size and shape. Therefore we conducted a subsequent Principal Component Analysis (PCA) using data pooled by leaf position (i.e. 3 means per tree and a total of 90 on the whole sample of trees).

Principal component analysis (PCA)

To study leaf shape variability, we conducted a PCA on data averaged by insertion level. The results showed that 91.9% of the total variance was explained by the three first PCs. In detail, the main part of total variance was explained by the PC1 (76.26%), followed by the PC2 and PC3 which explained respectively 9.12 and 6.16% of the total variance.

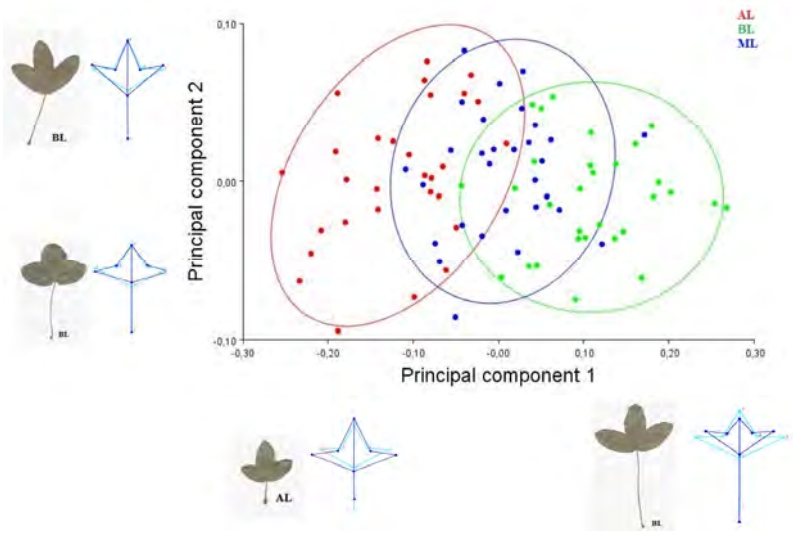

Figure 6. Scatter plot of the PC1 and PC2 scores derived from landmarks data. Wireframe graph and its corresponding leaf images showing major features correlated with the extremes of variation along the axis.

The scatter plot of PC1 and PC2 scores (Fig. 6) showed that the three leaf positions were separated with a partial overlap along the PC1, which was mainly related to change in leaf shape along the shoot. The positive values were characterized by a contracted apical lobe (lm5), a narrow angle between lateral

lobes (lm3, lm2, lm7), a narrower blade (lm3, lm4, lm6, lm7) and a long petiole (lm1). The negative values were characterized by an elongated apical lobe, a wider angle between lateral lobes, an expanded blade and a short petiole.

The PC2 discriminated the trees according to the angle between lateral lobes (lm3, lm2, lm7), lateral lobe tips (lm3, lm7), and to a lesser extent, according to apical lobe width (lm4, lm6). The angle between lateral lobes, lateral lobe tips and consequently the blade width and the apical lobe base were narrower in positive values and wider in negative ones.

The PC3 explained only 6.16% of the total variance (Fig. 7), which was related to leaf base (lm2), apical lobe width (lm4 and lm6) and the importance of apical lobe to blade ratio. Leaves characterized by a wide apical lobe base, a short apical lobe compared to blade length and a tronquate leaf base were found on the positive side of the PC3, while those with a narrow apical lobe, a long apical lobe compared to blade length and a cordate leaf base, were localised in the negative side of the PC3.

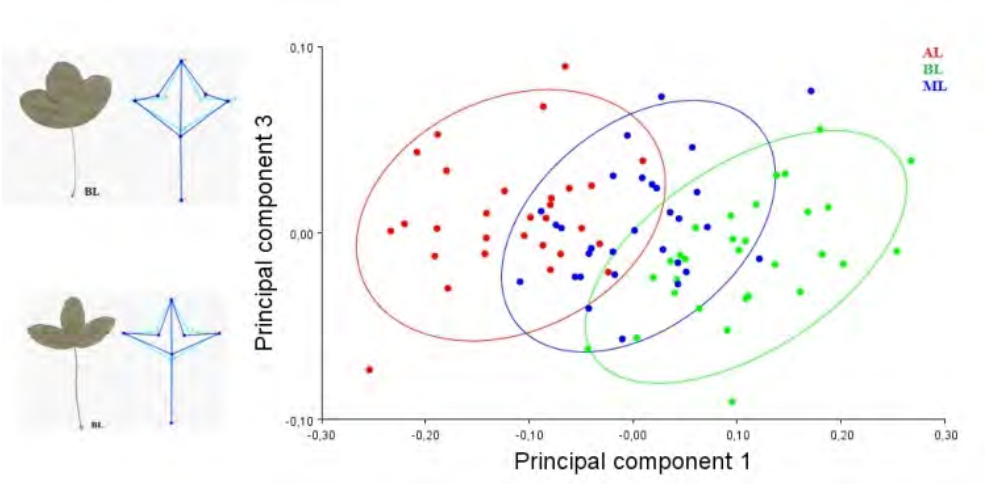

Figure 7. Scatterplot of the PC1 and PC3 scores derived from landmarks data. Deformation grids and wireframe graph showing major features correlated with the extremes of variation along the axes.

Discriminant analysis (DA)

The cross-validation DA showed the important differences in leaf shape between basal and apical position (Fig. 8a), and a small overlap in shapes of basal vs median leaves (Fig. 8b), and median vs apical ones (Fig. 8c). The pairwise comparison showed T^2 constantly significant ($P<0.0001$); the values were 324.64, 160.79 and 107.70 for basal vs apical, basal vs median and median

vs apical leaves, respectively. In addition to these comparisons, we obtained a correct classification for 100 and 93.10% of the basal and apical leaves, 93.33 and 90% of the basal and median leaves and 90 and 82.75% of the median and apical leaves.

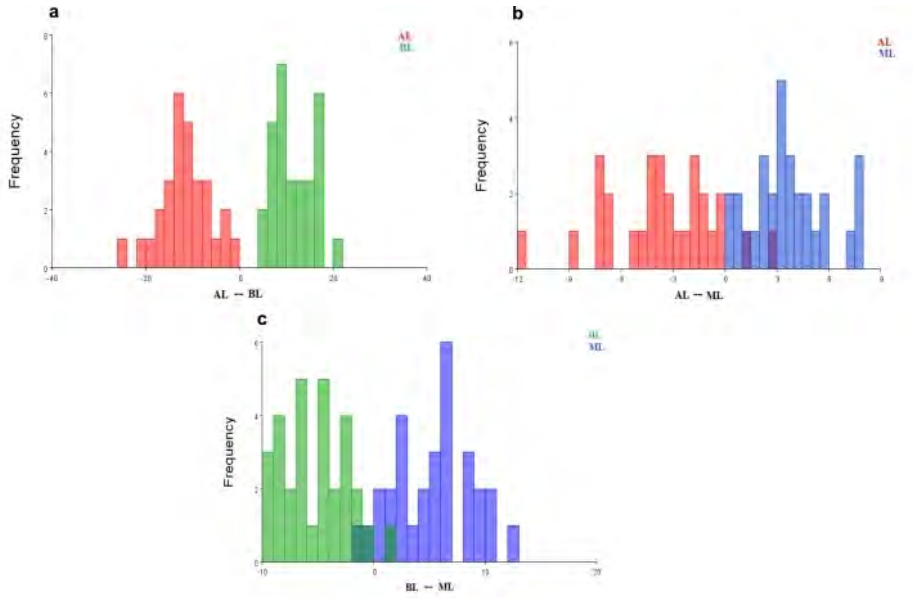

Figure 8. Discriminate analysis of the leaf shape of the three positions on shoot of *A. monspessulanum*. Green bars: Basal leaves (BL); Blue bars: Median leaves (ML); Red bars: Apical leaves (AL).

Discussion

This paper represents the first contribution to the quantitative study of foliar morphological traits of Montpellier Maple at its southernmost limit in North Africa, and especially in Algeria. Here, we used both traditional and geometric morphometrics methods in order to analyze leaf morphology of *Acer monspessulanum* subsp. *monspessulanum* harvested in the Djurdjura Mountain (Algeria north-centre) at three hierarchical levels: sites, trees within site and nodes within shoot.

Both traditional and geometric morphometrics revealed significant differences in leaf morphology according to its position on the shoot despite the smaller sample size retained for the geometric morphometrics analysis (251 leaves compared to 597 for traditional morphometrics). The basal leaves

showed higher values for all measured morphological traits, followed by median leaves with intermediate attributes while apical leaves displayed the smallest values. Although leaf position on tree may cause variation of some leaf traits (Blue and Jensen, 1988; Bruschi *et al.,* 2003), we consider leaf variation recorded in this study as rather driven by heteroblasty. Firstly, because we sampled only small shoots (average of 4 cm long) discarding the bias which may result from shoot length variation, secondly, because we collected the shoots from small and well spaced trees with a full access to sunlight discarding the bias which may result from a differential access to sunlight.

Heteroblasty is previously reported on some *Acer* species such as *A. pensylvanicum* and *A. rubrum* (Critchfield, 1971), *A. saccharum* (Powell *et al.,* 1982; Steingraeber, 1982) and other genera such as *Berberis* (Pabón-Mora and González, 2012), *Populus* (Eckenwalder, 1996; Slavov and Zhelev, 2011), with within-shoot nodes bearing leaves of different morphologies, a trend linked to leaf ontogeny, since basal leaves of a given shoot initiate their development in the previous season, overwinter as embryonic leaves and leaf primordia, and reach their maturity rapidly after leaf unfolding in the following spring, while distal leaves initiate their development during the growing season (Steingraeber, 1982). Furthermore, Spriggs *et al.,* (2018) suggested that heteroblasty in *Viburnum* is closely related to growth architecture and is linked to leaf position along the shoot rather than to the exact timing of leaf emergence.

This node to node variability is not limited to leaf shape but concerns also leaf nitrogen content in *Olea* europaea which declines from basal to apical leaves, monoterpene composition of essential oil in *Mentha piperita* which increases from the base to the tip and is also found in other organs like flowers and fruits (Herrera, 2009).

Critchfield (1971) stated that heterophylly is marked in *A. campestre*, *A. orientale* and *A. monspessulanum* (ex. *Campestria* Pax. section) and these species have 1 to 3 pairs of leaves formed completely in buds and may be reinforced by additional pairs of leaves (i.e. late leaves) on longer shoots. He reported that the most obvious distinction between the two kinds of leaves (early vs. late) in *A. monspessulanum* is the presence of more lobes and blunt teeth in late leaves. However, data of the present study and additional observations (data not shown) don't corroborate this author's statement about the distinctive trait between leaves of the basal and distal nodes. Indeed, we observed that shoots of *A. monspessulanum* bear an average of 3 pairs of leaves regardless of shoot types (long or short) and may reach rarely a maximum of 8 pairs of leaves. Although we considered only those shoots bearing three pairs of leaves in this study, the results showed evident differences in leaf morphology from the base to the tip of the shoot. We didn't study the morphology of leaf

margins in this paper, though, based on our observations, the apical leaves did not have systematically more lobes and teeth and some of them have an entire margin like basal leaves.

The Procrustes ANOVA results (Tab. 3) showed measurement errors which are lower to all main effects (0.003% and 0.2% for centroide size and shape respectively), therefore we may consider the error as negligible and the digitalization well carried out as stated by Viscosi and Cardini (2011). The main source of variation (70 and 49 % for centroide size and shape respectively) was induced by leaf position on the shoot. The first principal component explained 65 and 71.6 % of the total variance in traditional and geometric morphometrics respectively, and separated clearly the basal and the apical leaves into two groups, while the median leaves overlapped with apical ones from a hand and basal ones from another hand. The basal leaves may be characterized by a long petiole compared to blade length, and a contracted apical lob while apical leaves seem to have a short petiole, a large blade and an elongated apical lobe. The median leaves, for their part, have intermediate attributes.

The traditional morphometrics (Tab. 1) showed that the relative development of median leaves reached 72 to 96% of that of basal ones. The contrast was more evident for the apical leaves which showed a development representing between 45.6 and 80% of that of basal leaves depending on the trait. This trend was evidenced by the discriminate analysis (*DA*) which showed that 100 % of basal and 93% of apical leaves were well classified. This is similar to results of Spriggs et al. (2018) on *Viburnum sp.* who found that 87 to 97% of early and late leaves were well distinguished on the basis of shape only.

Variation of the apical lobe to blade length ratio and of the angle between lateral lobes along the shoot was more clearly evidenced by the geometric morphometrics method than by the traditional one. And such trend of variation is in concordance with Critchfield (1971), who reported, on other *Acer* species, a larger apical lobe and a wider angle in apical leaves, comparatively to basal ones.

According to our results, this variability of lateral lobes' angle is more related to variability among trees as revealed in the PC2 of both traditional and geometric morphometrics. Therefore, we suggest, that mean values calculated on the whole sample of leaves (Tab. 1) are hiding a certain amount of among-tree variability. Indeed, as depicted in Figure 5 and 6 both the traditional and the geometric morphometrics methods showed that apical lobe to blade length ratio and the angle between lateral lobes are rather related to variability among trees. Despite the major part of variation being due to leaf position on the shoot, the second principal component of the PCA with the classic morphometrics method, and the second and the third principal components of the PCA with the

geometric morphometrics, revealed the presence of two leaf morphotypes regardless of leaf node position. This discrimination was related to lateral lobes' angle and the apical lobe to blade length ratio according to the traditional morphometrics, and to the apical lobe features according to the geometric morphometrics.

Among the variety of leaf morphotypes observed in this study, we retained the two most contrasted ones named as morphotypes A and B. The morphotype A is characterized by elongated and triangular lobes with a long apical lobe, reaching 2/3 of the blade length and a wide angle between lateral lobes (≈ 120°). The morphotype B is characterized by obtuse lobes, with a short apical lobe to blade length ratio of about 1/3 and a narrow angle between lateral lobes (≈ 90°). The great variability of *Acer monspessulanum* leaf shape is reported in the literature, the lobes can be rounded, cordate, triangulate, ovoid, oblong or toothed. Indeed, the apical lobes may represent ½ to 2/3 of the blade length (Pignatti, 1982; Van Gelderen *et al.,* 1994; Seki, 2019). However, to our knowledge, there are not published works about the coexistence of these morphotypes as observed in the present study.

The PCA computed with both traditional and geometric morphometrics data didn't discriminate between sites regarding leaf morphotype and didn't support the relatively greater values revealed by the descriptive statistics for Tala-Guilef site. The coexistence of the two leaf morphotypes in the two investigated sites may be due to their location within the same latitudinal and altitudinal range (i.e. distant by only 35 kms from east to west). Investigating a larger area of Montpellier Maple in Algeria could reveal additional morphotypes and trends of variation since Nikzat-Siahkolaee *et al.,* (2021), for example, found a relationship between geographical gradients and the three leaf morphotypes recognized in *A. cappadociccum* of Iran.

Flora books suggested studying only mature leaves in order to avoid the leaf heteroblasty, which there are sometimes referred as "typical" or "adult" leaves (Critchfield, 1971). However, basing on the precedent studies on leaf maple heteroblasty such as Critchfield (1971), Steingraeber (1982) and Powell *et al.* (1982), the leaves were categorized into two kinds according to their morphology: the preformed (early) and neoformed (late) ones. Nevertheless, in this study we showed that basal leaves are bigger than median ones, particularly in leaf area and petiole length, eventhough those latter were fully expanded and considered as mature. On the other hand, Fennane *et al.,* (2014) suggested considering only mature leaves of fructified shoots. However, there may be an inconvenient in this case due to the complexity of *Acer* reproductive system (De Jong, 1976; Van Gelderen *et al.,* 1994). In addition, a large interannual fluctuation in seed production was recorded in *Acer* species (Houle, 1999), with zero production

in some years for *A. pseudoplatanus* (Wesołowski *et al.,* 2015), *A. monspessulanum* and *A. opalus* subsp. *obtusatum* (personal observations). Consequently, we suggest taking into account only leaves of the first node within a given shoot.

Conclusions

The present morphological study of *Acer monspessulanum* subsp. *monspessulanum* leaves, using a classic method and a geometric-morphometrics one, revealed concordant trends. Among the following factors: site, tree, and leaf insertion within shoot; the latter revealed to be the main driver of leaf shape and size variation. And since leaf shape is used as a principal characteristic in species identification, we suggest considering only the basal leaves in future morphological investigations in order to ovoid confusions. In addition to the heteroblasty, the study revealed also the coexistence of two leaf morphotypes on distinct trees of both sites. To complete this first quantitative study of *A. monspessulanum* leaf morphology, it would be interesting to include samples harvested on a larger range of the species in Algeria.

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Habitat preferences of European green lizard *Lacerta viridis* (Laurenti 1768) in a protected area, Romania

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Abstract. Anthropic induced changes in land use modify the habitat and microhabitat conditions for many species. Afforestation and grazing abandonment in steppe-like grasslands alters the characteristics of open natural areas. We aim to understand the habitat preferences of the European Green lizard in a nature reserve affected by both processes, using CORINE land cover and data recorded in the field. The results show that the species prefers sparsely shrubbed areas and edge habitat but avoids the interior of the pine plantation and totally open grassland. On microhabitat scale, most preferred structures were shrubs and logs. CORINE Landover data yields statistically less robust information than the microhabitat features recorded in the field Management measures should aim to increase habitat heterogeneity and to decrease compact afforested areas.

Keywords: afforestation, nature protected areas, habitat alteration

Introduction

Habitat destruction, modification and alteration are among the main threats to reptile species around the globe (Doherty *et al*., 2020; Fitzgerald *et al*., 2017; Gibbons *et al.,* 2000) and particularly to habitat alteration that results in modification of thermal regime (Nowakowski *et al*., 2018). Afforestation of

grasslands with high natural values, often with non-native trees, represents a major threat for the native biodiversity of the grassland ecosystem (Cao *et al*., 2010; Vassallo *et al*., 2013). The steppe-like grasslands of Eastern Europe have an enormous role as biodiversity reservoirs at local and landscape scales (Ruprecht *et al*., 2009; Rákosy and Kovacs, 2001; Cremene *et al.,* 2005). These ecosystems are under threat not only because of changes in agricultural practices (land conversion in croplands, overgrazing with sheep), but also from alteration due to afforestation e.g. with Austrian pine *Pinus nigra* (Cremene *et al*., 2005). In Romania, and especially in Transylvania, this practice was regarded in the last 50 years as an anti-erosion and equilibration of slopes action (Baciu *et al*., 2010), and even as 'ecological reconstruction' of degraded terrains (Oprea *et al*., 2009), despite the risk of losing plant and invertebrate unique diversity (Cremene *et al*., 2005). Some local populations of European Green lizard *Lacerta viridis* are also locally threatened by such plantations, but also by shrub encroachment resulting from a complete abandonment of farming (Rehák, 2015).

Lacerta viridis (Laurenti, 1768) is a large, thermophile lacertid lizard inhabiting a wide range of habitats: sand dunes, dry areas with shrubs and bushes, steep slopes with rocks and bushes, forest edges and clearings with a certain degree of humidity, road verges or deep river valleys (Covaciu-Marcov *et al*., 2006; Covaciu-Marcov *et al*., 2009; Fuhn and Vancea, 1961; Heltai *et al*., 2015; Rehák, 2015). The green lizard *Lacerta viridis* is widespread in Romania (Cogălniceanu *et al*., 2013) and it is listed as a species of community interest in need of strict protection, with a decreasing population trend (O.U.G. 57/2007; Crnobrnja-Isailović *et al.,* 2009). To ensure a favourable conservation status, active habitat management measures might be needed, which should rely on accurate knowledge of habitat and microhabitat requirements and preferences of the species.

The aim of this study was to understand the habitat use of the green lizard in an arid, steppe-like nature reserve that had been dramatically altered by afforestation and farming abandonment (and subsequent scrub encroachment). We used two approaches to explore the habitat preferences of this lizard: using CORINE land cover data for each transect and by recording the microhabitat features in the field. Our objectives were to identify the habitat and microhabitat preferences of the species and to provide recommendations for research and conservation practice based on our results.

Materials and methods

Study area

Our study area was the Butterfly Hill (Dealul cu fluturi) Nature Reserve (46.527117°N, 23.941970°E), located in the Transylvanian Plain, Romania, 30 km South-East from Cluj-Napoca. It consisted of 20 ha of steep slopes facing

south-west, originally covered in dry steppe-like grasslands maintained by extensive grazing. The majority of herbaceous steppic species originated from East European and Southern Russian steppes, but there were several endemic plant species and at least one butterfly species that evolved locally (Rákosy and Kovacs, 2001; Cremene *et al.*, 2005). Over the last 40 years, the area has been afforested with the non-native tree species Austrian pine (*Pinus nigra*) (Cremene *et al.*, 2005). As a result, the nature reserve lost most of its steppe-like character and consisted of a mixture of steppe-like grasslands (*Stipa* sp., *Carex humilis, Festuca rupicola*), pine plantation with snags and logs, and scrubland compose of native (*Prunus spinosa, Crataegus monogyna, Cornus sanguinea*) and introduced (*Hippophae rhamnoides, Eleagnus angustifolia*) species (Rákosy and Kovacs, 2001).

Field survey and data collection

The field survey took place over a period of three months, starting with the onset of the reproductive season, between April and June 2019.

Data was gathered from 20 sampling units (5 x 100 m), randomly selected from a total of 64 units, separated by 20 m distance, covering the entire area of the nature reserve. The sampling area covered 10.000 m^2, representing 5% of the total area. All selected sampling units were surveyed monthly, during a single day, resulting in three visits per unit. The surveys were done by line transects of 100 m length and a 2.5 m width on each side to be visually inspected for the presence of the species at walking speed in a constant pace. Surveys were carried out in good, sunny weather, without rain or wind, between 07.00 a.m. and 14 p.m. We recorded the gender and age of every observed individual, as well as the microhabitat feature where each animal was spotted. Each sampling unit was assessed regarding habitat characteristics. We used two types of habitat variables to classify the sampling units. First, transect variables based on Corinne Land Cover system (area of shrubland, grassland, (pine) forest plantation and habitat diversity based on Shannon entropy). Variables like insolation and slope were initially also considered but later removed from modelling to keep the explanatory variables low related to the number of transects (20). Univariate analysis showed no influence of insolation and slope on lizard abundance. Second, microhabitat variables were collected in the field while walking along each transect, in order to characterize the surroundings of each observed lizard. Variables recorded represented the full spectra of microhabitats available in the points where lizard occurred, such as: the presence of living shrubs, dead shrubs, pine plantation edge, fallen trees, and tall grass. The representation of habitats and microhabitats captured by our variables is visible in Figure 1.

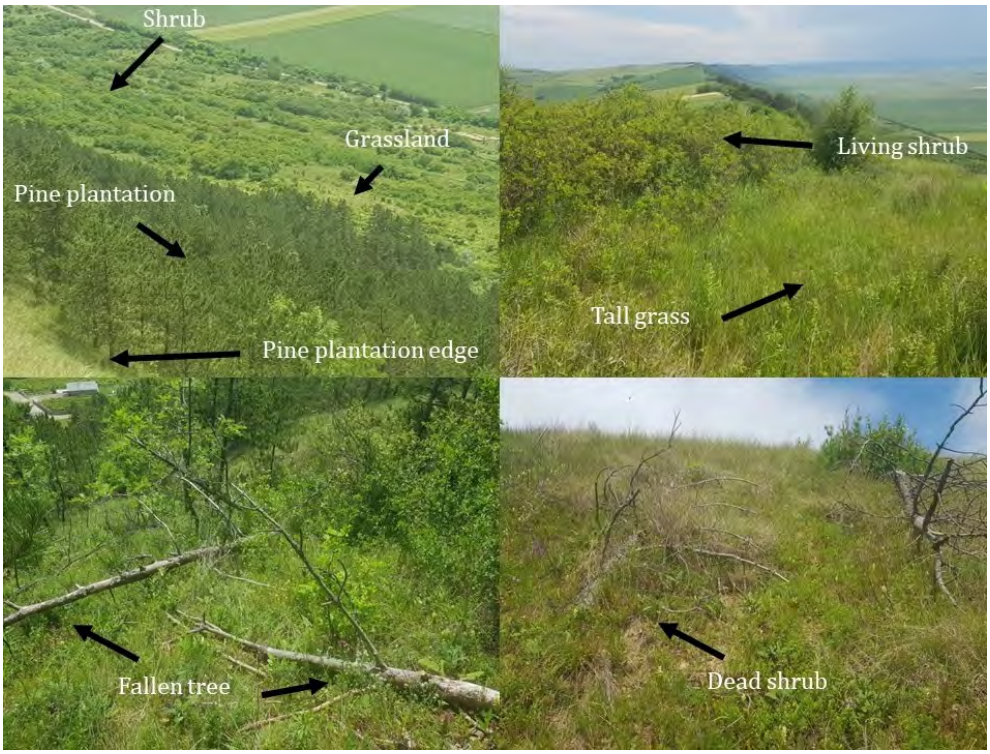

Figure 1. Diversity of habitats and microhabitats analysed in "Dealul cu fluturi" Nature Reserve

Data analysis

The statistical analysis was performed at two levels, according to the two approaches (see above) by implementing habitat use models and descriptive statistics. Before applying the habitat use models and presenting the descriptive statistics, we explored the detection probability of the lizards using occupancy models. The detection probability (p) shows the probability of detecting the organism in a site given that the organism is present. The detection probability can also be used to assess the number of site visits to infer the species absence (with 95% confidence) through the formula:

N_{min}=log(0.05) / log(1-p), where N_{min} is the minimum number of surveys per site and p is the detection probability (Pellet & Schmidt, 2005).

To model the relationship between the number of lizards and the transect characteristics, we used the following variables: grassland, shrub, pine plantation, slope and isolation. We excluded the survey month from the analysis because and initial check showed that it has no effect on the number of lizards detected (Kruskal-Wallis test, P = 0.22). The values of the transect variables were *log* transformed and standardised (average of 0, SD of 1). We tested for the collinearity between the transect variables and found no correlation between the independent variables.

To model the abundance *L. viridis* we used the information theoretic model selection approach based on Akaike information criterion (AIC) (Tab. 1).

Table 1. Model description

Variable (s)	**Hypothesis**
Grassland+Shrub+Pine forest+Habitat diversity	Green lizard abundance is determined by multiple transect variables
Grassland	Green lizard abundance along transects is determined by the proportion of grassland along the transect. Open areas are feeding habitats.
Shrub	The proportion of shrub along the transect is important. Shrubby areas are shelters against predators and excessive sun.
Pine plantation	The proportion of pine plantation along the transect negatively affects lizard abundance. Pine is a suboptimal habitat for *L. viridis* because it creates moist and shady microhabitat.
Habitat diversity (Shannon)	The habitat diversity along the transect positively influences green lizard abundance.
Null model	None of the recorded variables is important for green lizard abundance along the transects.

We used percentage values to visualize the distribution of individuals detected in the field in different types of microhabitats (this being the descriptive part of our analysis).

Statistical analyses were performed in R (with bbmle and AICcmodavg packages) and Excel.

Results

Detection probability

During the 3 surveys we observed the species 66 times. We detected 28 European Green Lizard individuals in April, 14 in May and 24 in June. The naïve estimate of the occurrence in the transects was 0.85 while the estimate

based on occupancy model was 0.87 (SE=0.08). The detection probability was high (p = 0.70). The number of visits to infer absence with 95% confidence was 2.48, showing that three surveys per transect were enough for this species in the study area. We did not find any relationship between the CORINE variables (Tab. 1) and the detection probability of this lizard (data not showed).

Abundance modelling according to transect variables (CORINE data)

The habitat modelling shows that the best models explaining the green lizard abundance along the transects were the 'Shrub' and 'Grassland' models followed by the 'Pine plantation' model (Tab. 2). The model coefficients show positive relationship between lizard abundance and shrub proportion (estimate ± SE = 0.23 ± 0.16) and grassland proportion (estimate ± SE = 0.25 ± 0.19) along the transect and a negative relationship with pine plantation (estimate ± SE = -0.14 ± 0.16). While the model results have sense based on the expert knowledge related to the ecology of lizards (see below), from a statistical perspective the habitat models cannot be distinguished from the null model.

Table 2. Results of the model selection for the abundance of *L. viridis*. The model coefficients (estimate and SE) for the first three variables are presented in the text.

Model variables	**AICc**	**Δ AICc**	**AIC*wt***	**Cum.*wt***
Null model	88.33	0.00	0.28	0.28
Shrub	88.65	0.31	0.24	0.53
Grassland	88.98	0.64	0.20	0.74
Pine plantation	89.98	1.64	0.12	0.87
Habitat diversity	90.16	1.82	0.11	0.98
Full model	94.64	6.30	0.01	1.00

Microhabitat preference based on field data

Out of 66 detections of *L. viridis*, the vast majority (70%) of observations were related to living shrubs (38%), the pine plantation's edge (32%), fallen pine trees (14%), dead shrub (12%) and tall grassland (4%) (Fig. 2).

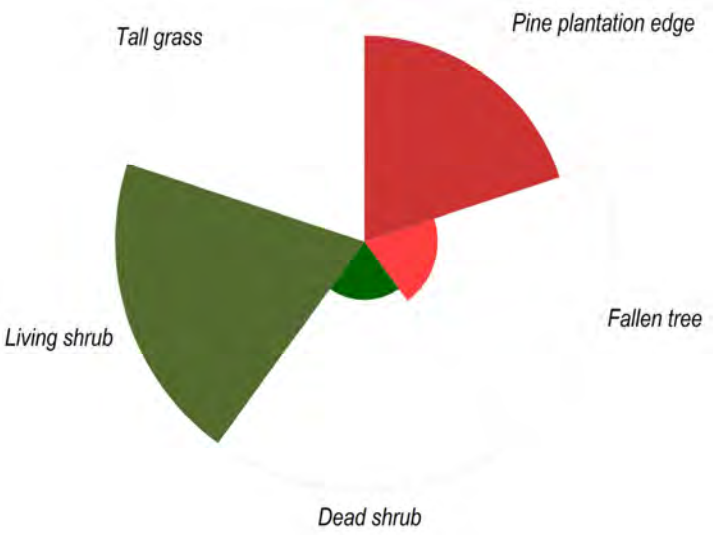

Figure 2. The proportion of green lizards recorded in habitat types. Min-max normalized values are presented, where the habitat with lowest proportion of lizards (Tall grass) is 0 and the habitat with the highest proportion of lizards (Living shrub) is 1. See text for proportion values.

Discussions

Our results show that the detectability of the green lizards in the studied area is high, and the sampling effort was adequate to detect these organisms. We showed that the abundance of the green lizards was highest in locations with living shrubs and pine plantation edge, and it was lowest in the tall grass. Furthermore, we show that the transect method as employed in this study combined with CORINE Landover data yields statistically less robust information (albeit ecologically interpretable) than the microhabitat features recorded in the field. The explanation may be that in very small scale the CORINE data have limited precision and/or the transect method is not suitable for the available CORINE landcover types of data at small scales.

The availability of thermally suitable microhabitats is one of the key parameters characterizing the habitat quality for lizards, but not the only one, food availability and predation pressure being also important, perhaps even more than the structural features of the habitat (Díaz, 1997). All three parameters might be reduced to habitat features on which they depend, like shelter and increased invertebrate diversity in shrubland (Walker *et al.*, 2014) that serve as food source. Thermoregulation implies moving between sun and

shade (Huey, 1974), and our results show that the two most important habitat variables are the presence of shrubs and trees in combination with grassland on a small scale, thus providing a mosaic of different ecological conditions. Our results confirm other findings that heterogeneous areas with bushy parts are most suitable for the species (Heltai *et al*., 2015; Prieto-Ramirez *et al*., 2018, 2020a). The results regarding microhabitat use show that shrubs and logs the most used features by lizards for basking and as shelter and/or hiding place, confirming other observations for the European Green lizard (Heltai *et al*., 2015), but also for related species *L. bilineata* (Luppi *et al*., 2020). Although statistically not robust result, the modelling approach resulted in negative estimate regarding the proportion of pine plantation along the transect and lizard abundance. The pine plantation interior is compact, shaded, and cold, and these conditions are far from optimal for the green lizard. However, the edge of the plantation is almost as important as the presence of shrubs. Linear structures and edges availability in the patches are very important conservation measures for the species (Prieto-Ramirez *et al*., 2020). Our results show that within the nature reserve, areas with compact pine plantation does not constitute favourable habitat for the European Green Lizard. Plantations of exotic tree species have a negative effect, resulting in increased rarity of other poikilotherms species like *Salamandra salamandra* or *Rana dalmatina,* coniferous plantations being the least important areas for herpetofauna (Covaciu-Marcov *et al*., 2009). Coniferous plantations are utilised by reptile species in early successional stages, but not in mature plantations, with well-developed canopy cover (Jofré *et al*., 2016). Strong shading, as in pine plantations, has a negative effect on invertebrates' diversity and abundance (Cremene *et al.,* 2005; Corfey *et al.,* 2018; Cifuentes-Croquevielle *et al*., 2020) and invertebrates represents the food of this species (Mollov *et al*., 2012; Maier *et al.,* 2020). We show that the green lizard abundance is low in tall grass without any structural elements (trees, shrubs). Structural features such as the woody vegetation benefits the green lizard in several ways (see above) while the compact tall grass may limit its movement and habitat options, highlighting the importance of extensive management.

In the successional stages which are characterized by shrub and tree dominance resulting from the abandonment of the traditional management only few species of butterflies reach their maximum abundance (Cremene *et al.*, 2005), and the distribution of lizard species is restricted by the lack of appropriate sun basking sites (Huey, 1974). Pine plantations in former natural oak forests are also associated with modification in lizard species assembly, with the loss of *Timon lepidus* (Amo *et al*., 2007)*,* a species that prefers open and dry areas of woodland and scrubland.

Despite their longevity and exceptional biodiversity (Feurdean *et al*., 2015), in the last decades, steppe-like grasslands were considered either unproductive or less profitable than other types of land use, so afforestation was practiced on a large scale, including in protected areas (Resmeriţă *et al.,* 1968; Cremene *et al*., 2005), profoundly altering the habitat of many species. Management actions should be guided by habitat and microhabitat preferences of the species, and it is also important to protect the habitat surrounding the patches where the species is present, at a scale of 250m (Prieto-Ramirez *et al*., 2020).

Conclusions

Anthropic induced changes in land use in nature protected areas modify the habitat and microhabitat features that are essential for the persistence of European Green lizard. The short-term abandonment of grazing benefits the species by increasing the habitat heterogeneity through the installation of native shrub species that provide appropriate basking and shelter conditions. Afforestation also creates favourable habitat by providing ecotonal condition on the edge of the plantation, but the interior of the plantation is avoided by the species. In areas already afforested, the elimination of some pine trees to create patchy areas with grassland and shrub would greatly improve the habitat quality.

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Breathing chemicals: a review of air pollution over the years

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Abstract: Air is necessary for human survival and the preservation of the environment. The scientific community is concerned about the ongoing rapid expansion of the population, which uses resources faster, and thus the accumulation of an enormous amount of waste will gradually worsen the air quality. The change in the pollutants released in the atmosphere became more complex throughout human history, and they were released in huge quantities. The sources of air pollution vary greatly – from burning fuel, the household, agricultural or mining activities to natural disasters or significant industrial accidents. New techniques that monitor the air composition are being developed to ensure air quality control. The population exposed to these harmful compounds is predisposed to various health concerns, including skin, cardiovascular, brain, blood, and lung illnesses. The substances also contribute to global warming, acid rains and ozone depletion. During the COVID-19 pandemic, it was noticed that reducing human activities causing pollution leads to improved air quality, which shows that long-term solutions can also be found. This paper aims to offer an overview of the air pollution problems persisting around the globe and present the current state, causes and evolution of air pollution. Some of the solutions we propose in this article include energy-saving, public transportation and material recycling. We also emphasize the need to develop new technologies to control the air quality and implement a sustainable approach.

Keywords: air pollution, biosensors, greenhouse gases, human health.

Introduction

The air quality and the view of the scientific community

In recent years the topic of air pollution has been brought more into discussion by scientific community. It becomes evident that the ability of the environment to purify itself does not cope with the amount of pollution caused by human activity (Tan *et al.,* 2021). The poor quality of air is felt in most cities, where there are multiple sources of pollution (Fig. 1), and the Global Burden of Death claimed in 2017 that air pollution caused 4.9 million deaths (Tan *et al.*, 2021).

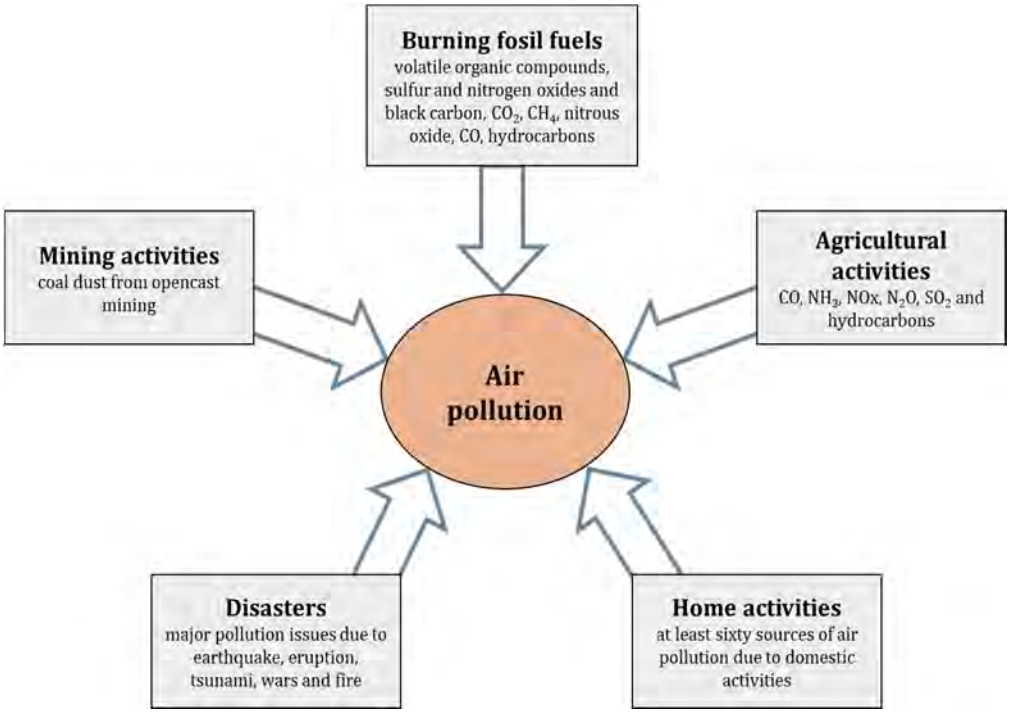

Figure 1. Sources of air pollution

Energy consumption has grown significantly due to urbanization and intense human activity in cities (Lin and Zhu, 2018). Overpopulation has led to a high concentration of pollutants (Hood *et al.,* 2018). Rapid population expansion, car ownership, solid fuels, and inadequate waste management practices have contributed to the degradation of air quality in cities, especially in developing countries (Amegah and Agyei-Mensah, 2016). In addition to the

increase in global temperature, there are other existing problems due to air pollution, such as changes in precipitation (Stohl *et al.,* 2015), increased rate of acid rains (Grennfelt *et al.,* 2020), and ozone depletion (Barnes *et al.,* 2019). Because of the energy structure stored in coal and fossil fuels, pollutants such as carbon dioxide, sulfur dioxide, particulate matter, nitrogen oxide, and dioxide are produced (Stohl *et al.,* 2015; Lin and Zhu, 2018). Air quality monitoring can help provide the data to develop a response to the problem of air pollution and reduce the disease rate attributable to air pollution (Amegah and Agyei-Mensah, 2016). Society has begun to be aware of the risks of air pollution, and research is promoted to develop methods of quantifying different pollutants (Bai *et al.*, 2018). To reduce and fight global air pollution in recent years several measures have been initiated, such as accelerating the deployment of low-carbon, climate-resilient infrastructure that is critical not only for achieving climate targets but also for guaranteeing long-term development and equitable economic growth (Afrifa *et al.,* 2020). Studies to estimate the level of pollution and examine its impacts on the humans would help scientists to develop techniques for improving and maintaining air quality (Bai *et al.*, 2018).

Air pollution over time

Air pollution is closely tied to citizens' daily lives, habits, and behaviours. History has demonstrated that the public's ability to tolerate air pollution in urban environments results from a complex and changing inter-relationship between political, financial and social decisions (Charlesworth, 2019). Air pollution occurred long before the industrial revolution, approximately 1760, but it started having a significant impact on the atmosphere, population and environment once the industrial revolution began in the 19th century (Meetham *et al.,* 2016). Some claim that the process of air pollution started once humans started burning fuels. This single act alone would lead to emissions of different chemical gases in the atmosphere that would alter its composition (Daly and Zannetti, 2007). The evolution of pollution over time can be separated into three periods: The Pre Industrial Era, The Age of Smoke, and The Era of Invisible Threats. In the Pre Industrial Era, smoke generated in households and small manufacturing works (potteries, smelting furnaces, wood and charcoal burning) and the use of coal had a considerable impact on the air quality in the cities (Mosley, 2014). The Age of Smoke represented the period between the years 1780-1950. The rapid increase of coal usage has generated an increase in air pollutants that considerably damaged the air quality in urban areas in different parts of the world (Germany, The United Kingdom, and The United States). Smokestacks generated by the furnaces of the factories in important industrial cities substantially helped the spread of pollutants in the

air in the 19th and early 20th centuries (Mosley, 2014). The Era of Invisible Threats started in the 1950s and is ongoing. While the coal-based pollution decreased, new concerns arose from the Alkali industry and the accelerating use of cars which generated emissions of SO_2, NO, CO_2 (Mosley, 2014). These gases are responsible for acid rains that negatively impact agriculture, interact with the sunlight, and form a photochemical ozone smog that can seriously affect the inhabitants (Rawate, 1980; Dickerson *et al.,* 1997; Tiwary *et al.,* 2018).

Sources of air pollution

Burning fossil fuels

Globally, the primary source of air pollution is burning fossil fuels such as coal, oil, gasoline, diesel fuel, and natural gas, which has major importance in several industries, such as transportation, heating, and electricity production (Perera, 2018). Around 80% of the energy generated by humans stems from fossil fuels, which shows that humankind highly depends on them. The combustion of non-renewable fossil fuels generates air pollutants such as volatile organic compounds, sulfur and nitrogen oxides and black carbon, CO_2, CH_4, nitrous oxide, CO and hydrocarbons. These gases and compounds alone can cause severe air pollution, but they can also transform into secondary air pollutants such as ozone and airborne particulate matter that form the photochemical smog (Armaroli and Balzani, 2011). Global warming and the greenhouse effect are the most critical consequences caused by these compounds in the atmosphere. Ever since humans started burning fossil fuels, the concentration of CO_2 in the atmosphere has increased by 25%, leading to the combustion of fossil fuels and being responsible for almost 65% of the global greenhouse gas emissions, which has become a severe and alarming issue that our planet is dealing with (Casper, 2010; Covert *et al.*, 2016).

Agricultural activities

Agriculture is a substantial source of greenhouse gases (GHG), contributing to poor air quality and causing climate change (Wollenberg *et al.,* 2016). Agriculture is estimated to be responsible for 30% of anthropogenic GHG emissions (Tubiello *et al.*, 2013). Agriculture-related emissions are expected to rise in the coming years as the world's population grows, posing a threat to the environment (Wollenberg *et al.*, 2016). Ruminant animals are the largest source of agricultural emissions, producing CO_2, methane, N_20 through enteric fermentation (Nayak *et al.*, 2015). Cattle, in particular, are responsible for more than 60% of farm animal emissions (McAllister *et al.*, 2011; Tubiello *et al.*, 2013). Manure from farm animals is a significant contributor to GHG

emissions (McAllister *et al.*, 2011; Tubiello *et al.,* 2013). Another significant source of agricultural air pollution is synthetic fertilizer, which emits harmful substances into the atmosphere, and rice cultivation, which produces a significant amount of CO_2 during the growth process (Tubiello *et al.*, 2013). Farmers have a limited time between harvests to remove straws and agricultural waste. Burning is a popular method for crop residue removal because it cleans the field faster and is less expensive than other methods. These practices are responsible for emitting up to 50% of total PM10 (the amount of particles that are smaller than 10 μm in diameter) concentrations in China's agricultural regions (Shi *et al.,* 2014). Agricultural fires emit various hazardous gases, including CO, NH_3, NOx, N_2O, SO_2, and hydrocarbons (Bray *et al.*, 2019). Only in Romania this year, in March 2022, it was estimated that 20.000 hectares of land were set on fire (Fig. 2), of which 1.000 were forested, where the fire got out of control (Europa Liberă România, 2022).

Mining activities

Opencast mining is the most common type of mining operation, and it is known to contribute to air pollution due to dust from haul and transport routes, which are regarded as an essential source of air pollution (Ghose and Majee, 2000; Mandal *et al.,* 2012). Furthermore, because they rely on fossil fuels and coal to generate electricity, several countries use opencast surface mining,

Figure 2. Agricultural fires out of control, Romania, 2022

which adds the benefit of increasing coal output (Ghose and Majee, 2001). As a result of these circumstances, opencast mining is being used more frequently, resulting in the release of a significant amount of dust (suspended and respirable particles) as well as other gaseous pollutants into the atmosphere (emissions from vehicles) (Mandal *et al.,* 2012). As a result, the collected coal dust from opencast mining would pollute the air, affecting the flora and wildlife in and around the mining sites and posing serious health risks (Pandey *et al.*, 2014; Nayak and Chowdhury, 2018). For example, several studies have shown that mining activities (particularly those involving the extraction of minerals such as coal) negatively influence people in southern Brazil (Honscha *et al.,* 2022). Another example is copper mining in Iran (specifically, the Arasbaran forest region), which was discovered to negatively influence the region's wildlife after extensive research (Khazini *et al.,* 2022).

Home activities

Domestic activities are responsible for at least sixty sources of air pollution worldwide (Pluschke and Schleibinger, 2018). Among them, indoor tobacco smoking counts along with construction materials and the fuel used for cooking, heating, and lighting (Hu *et al.*, 2014). Different methods of cooking: stir-frying, deep or shallow frying and grilling have different emission levels of particulate matter (Pluschke and Schleibinger, 2018). The control of the temperature may require heating and cooling of the house. Poor ventilation caused by preventing the air that is temperature-controlled from escaping the closed environment inside the house is the main cause that leads to the accumulation of pollutants inside it (Hu *et al.,* 2014). Chemical repellents are used for mosquito control, which has become a burning need because of the high mortality rates caused by mosquito-borne illnesses (Hu *et al.,* 2014). The most commonly used repellent is the mosquito coil. Other examples include vaporizers, sprays and ointments that can irritate the airway mucosa due to the production of gaseous air pollutants. A study identified the volatile organic compounds from air fresheners, laundry and personal care products, and cleaning agents, and it identified 156 volatile organic compounds, of which at least 42 are toxic or hazardous to human health (Apte and Salvia, 2016). The main volatile organic compounds found indoors are benzene, formaldehyde, dichloromethane, styrene, acrolein, naphthalene and d-limonene. It has been found that some of them (benzene, formaldehyde, dichloromethane) may represent a real threat to human health due to their carcinogenicity, as they can contribute to several types of cancer, such as leukaemia, lymphoma, kidney and liver cancer (Dimitroulopoulou *et al.,* 2015; Tsai, 2018). Also, paints and varnishes used in household activities spread considerable amounts of volatile

organic compounds (Apte and Salvia, 2016). Carcinogens such as benzopyrene and particulate matter, carbon monoxide, nitrogen dioxide, sulfur oxides are emanated by biomass and coal smoke, which constitute hazards for human health (Ezzati and Kammen, 2002). Among some human activities that cause air pollution, burning wood also counts because people in semi-rural and urban areas burn wood during winters to keep their households warm. The gas kitchen stoves release nitrogen dioxide, a highly reactive gas. A potent oxidizing agent by nature, nitrogen dioxide reacts with the oxygen and moisture in the air to form toxic nitrates and nitric acid. The refrigerators use ozone-depleting aerosol sprays to condition and modulate room temperature (Apte and Salvia, 2016).

Disasters

A disaster may be a hazard leading to a significant impact on the environment as well as physical damage and loss of life. Many disasters can cause air pollution and the other way around. Here it is elaborated on major pollution caused by earthquakes, eruptions, tsunamis, wars and fire accidents that occur worldwide at different magnitudes (Chandrappa and Kulshrestha, 2015; Knap and Rusyn, 2016). Disasters like earthquakes, wars and volcanic eruptions disturb the air quality within airsheds for a long or short period. Therefore, releasing a large concentration of pollutants may result in an unpredicted impact, as witnessed in the release of toxic gases in industrial accidents (Chandrappa and Kulshrestha, 2015). The most noteworthy air pollution episodes are: in the Meuse Valley, Belgium episode in 1930, where, due to temperature inversion in the valley, the concentration of pollutants emitted from industries along the narrow valley increased considerable and led to the death of about 60 and sickness of 6.000 people in valley (Nemery *et al.,* 2001). Another episode occurred in London, UK in 1952, where the widespread burning of high-sulfur coal caused a dense smog. This event is known in history as The Great Smog of London (Polivka, 2018). Extreme air pollution occurred in 1997 in Sumatra and Indonesian Borneo due to the massive burning of vegetation and peat for soil cleaning, and it was intensified by an El Nino climate event (McDonald and Horwell, 2020). Bhopal, India in 1984, Chernobyl, Ukraine in 1986 and the Fukushima Daiichi, Japan nuclear disaster in 2011 are other significative events witch led to air pollution. (McDonald and Horwell, 2020). Wildfires can affect the air quality for thousands of kilometres. Aside from the smoke, which contains unburned charcoal particles, the emission of carbon monoxide, ash participles, methyl chloride, methyl bromide, polynuclear aromatic hydrocarbons, aldehydes, and volatile organic compounds (VOCs) also affect the air quality (Sapkota *et al.,* 2005). Tsunamis are a sequence of water waves caused by the movement of a significant amount of water in a body

of water, and the effect on air pollution depends on the activities in the tsunami-affected area. In 2011, the nuclear accident at the Fukushima Nuclear Power Plant resulted in the collapse of three of the plant's nuclear reactors after the plant was struck by a tsunami triggered by the Tohoku earthquake. The disaster resulted in the release of large amounts of radioactive materials, making it the second-largest nuclear disaster after Chernobyl (Chino *et al.,* 2011). Human errors, negligence and even intentions could cause hazards named anthropogenic disasters, such as the industrial disaster at Bhopal in 1984 at the Union Carbide India Limited, where more than 500.000 people were exposed to methyl isocyanate (MIC). Many major wars in history had severe effects on air quality (Chandrappa and Kulshrestha, 2015). The largest contemporary military attack on a European state, Russia's invasion of Ukraine, is having a major impact on local air quality due to massive troop movements and the destruction of over 1800 buildings (according to Reuters – as of March 2022) in many large Ukrainian cities such as Odessa, Kiev, and Mariupol. A major threat is the possible attacks on existing nuclear power plants in Ukraine, along with the threat posed by nuclear bombs from Russia's arsenal (The Guardian, 2022).

Monitoring of air pollution

Methods for measuring air pollutants

Over the past half-century, developed countries monitored concentrations of the primary pollutants known to damage health and destroy the environment. They focused on the most populated areas to assess daily, monthly or annual concentrations. Although greater spatial and temporal assessment of the pollution was desired, the costs of acquiring and operating sufficiently robust and accurate instruments are not feasible from an economic point of view. The motivation to develop cheap, responsive air quality monitoring devices that can be deployed in large numbers in specific areas can provide enough data to obtain the expected air quality resolution. Indeed, over the last decade, many researchers, entrepreneurs and manufacturers have been pursuing the development, implementation and evaluation of lower-cost devices measuring air pollution (Cross *et al.,* 2017). Two main methods are used in assessing the degree of air pollution: an analytical method based on using sensors for measuring the chemical and physical properties of the air (Michulec *et al.,* 2005), and a relatively new technology that uses living systems such as microorganisms to measure the concentration of contaminants in the atmosphere (Gavrilescu *et al.,* 2015). The analytical methods resort to techniques that can measure more precise concentrations of pollutants. The applied analytic approaches can be chosen regarding the volatility of the air contaminant (Michulec *et al.,* 2005). These techniques are sensor-based systems that monitor the pollutants such as NOx, O_3, SO_2, CO (Lewis *et al.,* 2016). These consist of a sensor element that can

detect, interact and measure the pollutants, a transducer that transforms the sensor's response into an electrical signal, and a device that stores and displays the data (Snyder *et al.,* 2013). The primary type of sensors that are used for measuring the air quality is those that detect and measure the interaction between the sensor material and compound of interest, or the photometrical method that can display the absorption or emission of light (Michulec *et al.,* 2005; Snyder *et al.,* 2013). The analytic method implies using an electrochemical cell and metal oxide semiconductors, and the photometrical method resorts to instruments that measure the non-dispersive infrared absorption and ultraviolet absorption (Snyder *et al.,* 2013). The sensors are desired to become more accessible and easier to use for most people because, in this case, every person can monitor the quality of air around the location they live in and contribute to a more extensive database regarding the situation of pollution (Sun *et al.*, 2016).

Biosensors represent one specific sensor that gained attention over the past years. Biosensors are instruments capable of detecting analytes by using a biological pathway or system (Nigam and Shukla, 2015). These consist of the following components: a recognition element of biological nature (genetically modified microorganisms, antibodies, proteins, DNA fragments, enzymes), a transducer or detector (optical, electrochemical, piezoelectrical, thermal or calorimetric) and a signal processing system (Fig. 3) (Salgado *et al.,* 2011).

This method is accessible and low cost, given that microorganisms have a rapid growth rate and can quickly adapt to any living conditions. The bacterial systems can also be engineered to detect a specific kind of air pollutant, making this method highly efficient in monitoring the air quality. Enzymes isolated from microorganisms can also be incorporated into the sensors for more precise assessment. Furthermore, these devices are quite small and can be easily installed in different areas to monitor air pollution. They can detect air contaminants instantly, without preparing a sample or separating the analyte from its matrix. One major disadvantage would be that biosensors are usually designed only to recognize one pollutant per biosensor (Nigam and Shukla, 2015).

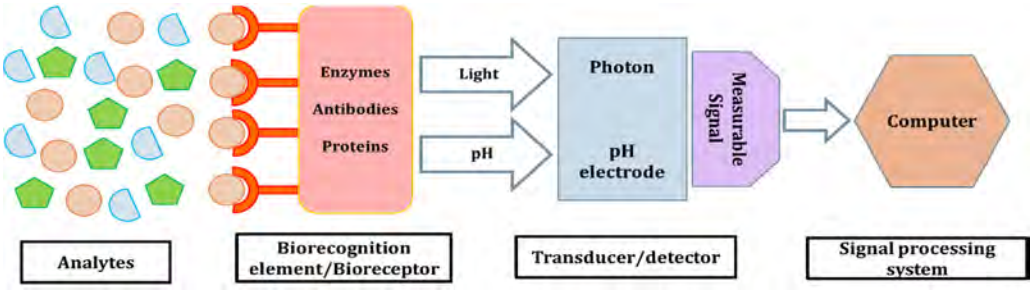

Figure 3. Elements of a biosensor

Health and environmental effects

Human health problems (direct and indirect)

The increasing concern about the consequences of air pollution led to more research on the subject. Epidemiological studies have shown a direct correlation between decreased lung function, cardiovascular disease, increased hospital admissions, mortality and concentrations of pollutants in the air (Kelly, 2003). One of the most disquieting outcomes of air pollution is the negative impact on pregnant women, newborns and children, considered more vulnerable (Vizcaíno *et al.*, 2016; Mannucci and Franchini, 2017). Numerous studies show that exposure to polluted air may cause miscarriages, reduce women's fertility, or trigger health problems in newborns due to mother transmission of pollutants (Vizcaíno *et al.,* 2016). The respiratory system is also heavily affected. Irritations of the respiratory tract and damaged alveoli are common health issues triggered by air pollution (Kampa and Castanas, 2008; Mannucci and Franchini, 2017). Cardiovascular disease is caused mainly by carbon monoxide that binds to haemoglobin, reducing the capacity to transfer oxygen and diminishing the transfer of oxygen in the organism (Kampa and Castanas, 2008). Dioxins and heavy metals are dangerous pollutants that can induce neuropathies and damage the kidney or liver cell affection (Kampa and Castanas, 2008). PAH (polycyclic aromatic hydrocarbons) are strongly genotoxic and carcinogenic and are found in high concentrations in the air of urban areas (Singh *et al.*, 2007). Air pollution also causes atherosclerosis in long exposure (Künzli *et al.,* 2011). More research is still in process to increase the knowledge regarding the interaction between air pollution and the human body. Effective methods to prevent such health problems are also being investigated (West *et al.,* 2016).

Global warming

Air quality has become a severe concern in many industrialized countries and a growing one for the rest of the globe due to the enormous increase in emissions pollutants due to economic and industrial expansion (D'Amato *et al.*, 2016). Owing to the growing relevance of air quality, many scientists have begun to link air pollution to global warming (Manisalidis *et al.,* 2020), as the climate is an important factor that may affect air quality (Orru *et al.*, 2017). High quantities of greenhouse gases, such as CO2, methane, tropospheric ozone, and aerosols, build up in the Earth's atmosphere and influence the amount of solar radiation received, which can contribute to climate change and global warming (D'Amato *et al.*, 2016; Orru *et al.*, 2017; Manisalidis *et al.,* 2020). As a result, climate change is predicted to worsen air quality, and because the quantity of

solar energy we receive is reduced, the temperature of the Earth is rising, which may result in melting ice and an increase in the prevalence of diseases in many modern populations (Gibson, 2015; Orru *et al.*, 2017; Manisalidis *et al.,* 2020). Therefore, it seems to be likely that climate change may impact air pollution exposure through modifying weather patterns, human-caused air pollution levels, and biogenic emissions, as well as the type and distribution of allergens released into the air (Bernard *et al.,* 2001; Orru *et al.,* 2017).

Acid rains

Acid rain refers to the atmospheric deposition of acidic constituents that impact the Earth in the rain, snow, particulates, gases, and vapour (Burns *et al.*, 2016). That was one of the most critical environmental issues during the last decades of the twentieth century and the largest environmental threat (Grennfelt *et al.,* 2020).

Coal is a source that contains the substances necessary for the formation of acid rain. It is currently the largest energy source on Earth, used in electricity generation. The main components of coal are carbon, sulfur, oxygen, hydrogen, small compounds of nitrogen. After combustion, these react with oxygen and produce carbon dioxide and monoxide, sulfur dioxide and trioxide, nitrogen dioxide and nitric oxide, forming acid rains. The emission of the gases has been directly and indirectly correlated with skin, cardiovascular, brain, blood and lung diseases and different types of cancers (Munawer, 2018). Furthermore, acid rains negatively affect soil fertility and the normal functioning of plants at all stages of growth and development by acidifying the soil and surface water, depositing on leaves and changing the symbiotic microbial community (Xalxo and Sahu, 2017). Marine life is also affected by harming the food chain and interferential acid and oxygen circulation, damaging the gills and causing heart problems in fish (Shammas *et al.,* 2020).

Ozone depletion

Ozone depletion has a significant impact on environmental climate change. These changes harm the environment and the globe's population by affecting human health, fauna, and crops (Barnes *et al.,* 2019). It can lead to higher exposure to UV radiation since stratospheric ozone is known to absorb considerable amounts of UV radiation. This type of radiation triggers various problems, such as photo-ageing, skin cancer, cataracts and photosensitivity disorder (Lucas *et al.,* 2015; Barnes *et al.,* 2019). Despite past worries about the harmful effects of UV-B radiation on global plant production due to stratospheric ozone loss, this radiation is an energetic driver of a wide variety of plant responses. Rapid advancements across a variety of organizational scales indicate that

essential plant responses to UV-B radiation, such as alterations in secondary metabolism, improved photoprotection, up-regulation of the antioxidative response, and changed pest and disease attack resistance, could be exploitable in the sense of a sustainable contribution to the strengthening of global food supply (Wargent and Jordan, 2013). In low concentrations, it favours vitamin D synthesis, which is beneficial for human health (Lucas *et al.,* 2015; Barnes *et al.,* 2019).

The effects of the COVID-19 pandemic on air quality

The COVID-19 pandemic that has led to nationwide lockdowns has had a massive impact on everyday life by challenging the healthcare system, forcing businesses to close down, limiting transportation and halting international travel (Berman and Ebisu, 2020). As a result, lower air pollutants such as CO2, CO, SO2, NO2, particulate matter (PM), ozone and other volatile organic compounds (VOCs) have been registered. Daily human activities such as burning fossil fuels, car emissions, biomass burning, and other industrial processes are the main sources of these air pollutants in the atmosphere, thus the most logical assumption would be that halting activities would decrease these compounds in the atmosphere. This hypothesis has been confirmed by several studies which tracked the decline of most air pollutants in the atmosphere since the outbreak of the COVID-19 pandemic (Ghahremanloo *et al.,* 2021). In addition, countries like China, USA, and several European cities have registered a significant drop in nitrogen dioxide and other air pollutants compared to data collected in early 2020 and previous years (Berman and Ebisu, 2020). Moreover, research data provided by Le Quéré *et al.* (2020) suggests that the daily global emissions of CO2 have decreased by -17% since the beginning of the lockdowns in march 2020 compared to CO2 emissions registered in 2019. Interesting but not surprising is that the emissions of air pollutants have drastically decreased in the regions that were epicentres of the COVID-19 pandemic, such as China, Italy, Spain, and USA, where down to 30% were registered (Barua and Nath, 2021).

Solutions for remediation of air quality

Energy saving

Energy saving is viewed as a favourable solution to reduce air pollution by developing new technologies that can operate with a smaller quantity of energy and resources. One aspect of lifestyle that can reduce the amount of energy is to create buildings design that can optimize the use of sunlight. For example, using atrium is shown to save energy and decrease carbon footprint

(Sher *et al.,* 2019). Furthermore, natural ventilation can save energy as well. A better natural ventilation strategy in buildings can reduce energy consumption (Tong *et al.*, 2016). The development of automated vehicles could also contribute to energy saving by using advanced sensors that analyse and interact with other automated vehicles offering the most economical driving (Vahidi and Sciarretta, 2018). However, the best method to reduce air pollution by energy-saving is implementing new technology in a big factory that optimizes energy use and introduces a law regulating emission (Zhang *et al.,* 2018).

Material recycling

Recycling is the process of converting waste into new products. This process reduces fresh raw materials consumption, the use of energy, air and water pollution and the emission of greenhouse gases (Banerjee, 2015). Particles, sulfur dioxide, nitrogen oxides, ozone, carbon monoxide, volatile organic compounds (VOCs) and polycyclic aromatic hydrocarbons (PAHs) are among the most important pollutants of outdoor air results from zootechnical farms, burning fossil fuels, wood, factories, and others. (Saxena and Naik, 2018; Domingo and Rovira, 2020). Reducing the amount of waste sent to sites and burners would significantly improve the air quality. Some researchers found that methods such as collection types, curbing, dropping, a single strike, or pay-as-you-throw (PAYT) impact the success of a recycling program. Increased service departure from the PAYT system in municipalities could improve air quality (Giovanis, 2014).

Use of public transport

In populated urban areas, the main contributor to air pollution is heavy traffic, leading to vehicular emissions (Sun *et al.,* 2019). Several studies outline that pollution stemming from motor vehicles in urban areas increases rapidly and represents a considerable percentage of the total pollution in cities (Pan *et al.,* 2016). Different solutions are being implemented to decrease the high level of pollution caused by the car traffic. One effective method is the investment and promotion of public transport infrastructures. A plethora of studies and evidence prove that establishing and expanding a public transport system significantly reduces CO_2 and NOx (nitrogen oxides) emissions generated by cars (Sun *et al.,* 2019). In cities such as Taipei, Mexico City and urban areas in China, the implementation of a public transport system has shown to actively contribute to the decrease of the pollution generated by car emissions (Chen and Whalley, 2012; Bel and Holst, 2018, Sun *et al.*, 2019). Further research conducted in London suggests that the active use of public transport is responsible for a noticeable decrease in gases such as CO_2 and NOx concentration in the air of

urban areas (Ma *et al.,* 2021). Moreover, according to Rojas-Rueda *et al.* (2012) replacing cars with public transport in the city of Barcelona, Spain could help reduce the level of mortality linked to the inhalation of fine particulate matter, which is released into the atmosphere. CO2 emissions are also estimated to drop significantly by using means of public transportation, which could lead to an even lower death rate caused by air pollution (Rojas-Rueda *et al.,* 2012).

Air pollution control technologies

Biofiltration is a technology that allows the control of air pollution. It implies transforming gases and vapours that can oxidize into mostly innocuous end products with the help of microorganisms. It was used successfully in many industries, such as the chemical and food sectors, but further research is needed to implement this method in other fields to stop air pollution (Janni *et al.,* 2001). Another effective method for controlling air pollution would be cloth filtering, which uses baghouses to filter dust particles. These baghouses can filter large amounts of different dust particles and have a modular design that requires large floors. Another disadvantage is that it cannot operate in moist environments or under fire hazards (Cooper and Alely, 2010).

Sustainable approaches

Air pollution is a significant concern in both developed and developing countries. It is critical to minimize air pollution and raise public consciousness to create sustainable cities for the future; however, this remains an open challenge (Kumar *et al.,* 2014). Citizens' science initiatives have been introduced over the years to track the environment and raise public awareness, but most of these works are of a contribution type, in which trained researchers design, prepare, and analyse tasks while citizens participate (Silva and Medes, 2012). By engaging local communities and stakeholders, citizen science can be used as a "tool" to increase public awareness of air pollution (Mahajan *et al.,* 2020). Every citizen can mitigate air pollution through behavioural changes in their lifestyles, such as reducing energy consumption in transport, households and supplies (Rickenbacker *et al.*, 2019).

Furthermore, it is well recognized that motor vehicle transportation is responsible for approximately 70% of all environmental emissions since exhaust gases are known for being the source of various pollutants (Sofia *et al.,* 2020). As a result, initiatives aimed at improving travel behaviour are critical. Every resident should take advantage of public transportation such as buses, trams, metros, and trains as much as possible and travel actively using alternatives like walking and cycling (Xia *et al.,* 2015). On a global scale, the joint effort to minimise carbon dioxide emissions will improve air quality. When

fossil fuels are burned, oxygen combines with hydrogen to produce water or with carbon to produce carbon dioxide. These reactions release heat, which is used as energy. Therefore, a significant reduction in carbon dioxide emissions, together with increased energy efficiency, will contribute to achieving the energy security goals of countries and regions by encouraging a more efficient, versatile and diversified energy mix (Ibáñez-Forés *et al.,* 2013). Society and its decision-makers, who prioritize various valuable energy goals such as climate change mitigation, environmental pollution, and energy security, may shape the future energy system in various directions (Harlan and Ruddell, 2011; Sofia *et al.,* 2020).

Conclusions

Scientists have addressed the poor air quality topic more frequently in recent years, especially since the environmental capacity of self-purification does not cope with the number of pollutants that humans produce. Sources such as burning fossil fuels, agriculture, mining, household activities, natural disasters are just a few of the main causes that affect human health and cause global warming and acid rains. More people are aware that air pollution is a big problem, and they try to remediate this by energy saving, recycling, using public transport and finding technologies that control air pollution. Air pollution monitoring is necessary for environment and population as well. Scientific research is vital to help the public understand the effects of air pollution and spread awareness about this topic and its consequences.

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=== BOOK REVIEW ===

NYÁRÁDY Erazmus Iulius (Gyula): Geografia, flora și vegetația Băilor Sărate Sovata din perioada 1940-1945 (Geography, Flora and Vegetation of Sovata Baths between 1940-1945), Edited by Roman Anamaria & Bartók Katalin, Kriterion Publishing, Cluj-Napoca, 2021, 267 pp. (5 chapters, 37 photographs, 21 plant drawings, 113 bibliographical sources, 3 appendices)

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Keywords: Lake Ursu, Sovata Baths history, Nyárády's plant drawings and field diary

Botanical literature was significantly enriched by an outstanding volume printed in 2021. We welcomed the publication of the nearly 8-decade-old manuscript of the famous botanist Erazmus Iulius Nyárády (original title in Hungarian: *Szovátafürdő és környékének monográfiája*), which deals with the geography, flora and vegetation of Sovata Baths, based on field surveys in the period 1943-44. Nyárády was asked to do this scientific work by Sovata Baths Council.

Erazmus I. Nyárády (1881-1966) is perhaps the most prominent figure of flora research in the 20th century Romania, also recognized at European level. He is the author of several monographs, especially known as a coeditor-in-chief of the Romanian Flora in 13 volumes (original title in Romanian: *Flora Republicii Populare Romîne - Flora Republicii Socialiste România*, 1952-1976, Editura Academiei R.P.R.-R.S.R.) and as a specialist in several critical plant genera (e.g. *Hieracium, Allysum, Rubus*). In recognition of his work, he was elected in the Romanian Academy of Sciences in 1948.

The current monograph was published posthumously. During his lifetime, Nyárády unsuccessfully tried several times to publish his work in Sovata's flora.

His plans were changed by World War II, when the manuscript submitted to the Capuchin Press in Budapest was destroyed in 1944, during the siege of the Hungarian capital. Based on his detailed field notes, he reconstructed the manuscript in both Hungarian and Romanian. The flora of Sovata and the development of wooden vegetation on the salt belt was also the subject of his acceptance speech for academic chair, but despite his constant efforts, he failed to publish his study eventually.

This unique volume offers an accurate image of the geographical and vegetation conditions in Sovata in the early 1940s. The editors redrew some lost maps of the original manuscript, added recent photographs and put this manuscript in today's context.

One novelty of this volume is Chapter 2, dealing with the history of Lake Ursu and Sovata Baths. This chapter was included in the first version of Nyárády's manuscript, but he gave up publishing it later on. The editors agreed to publish it in its original form, thus increasing the value of the book. From this chapter, the reader finds out that the lake was formed in 1875 during a heavy rain. A local landowner, *sófalvi Illyés Lajos* (1839-1926), visited Sovata in 1893 and "discovered" Lake Ursu. Later, he bought the land nearby, built bath cabins and opened the lake for public bathing. The author describes in an easy-to-read way how the spa town grew until the First World War. There are also statistics on the number of guests: while in 1902 the number of tourists was 311, in 1911 it increased to 4,000 and continued to increase until the First World War. It reveals how the forests around the lake were declared protected areas by a ministerial decree in 1902 and logging was banned. These historical data come from the former archives of Sovata Baths, whose documents were partially destroyed during the nationalization of the building. That is why this historical chapter is so valuable.

The 3rd chapter discusses the area around the spa and salt lakes from geographical and geological perspectives. Nyárády had also a degree in geography, therefore such detailed descriptions were an important part of his monographs. He did the field mapping work himself and after that he drew the maps. It is interesting that many of them, such as the map made in Sovata, were used by the Romanian army for further development of the military maps between 1959-1961. Unfortunately, the original 1:2,000 high-resolution map of Sovata was lost, but the editors redrew it. Nyárády also collected the toponyms of the area and, where they did not exist, as he himself noted, he was forced to adopt new toponyms in Hungarian, which he later translated into Romanian. This chapter contains several original black and white images of the landscape and coloured map sketches from 1943-1944 (Fig. 1). It also contains detailed geographical descriptions of the lakes and climatic data from 1905–1913 and 1923–1926, respectively.

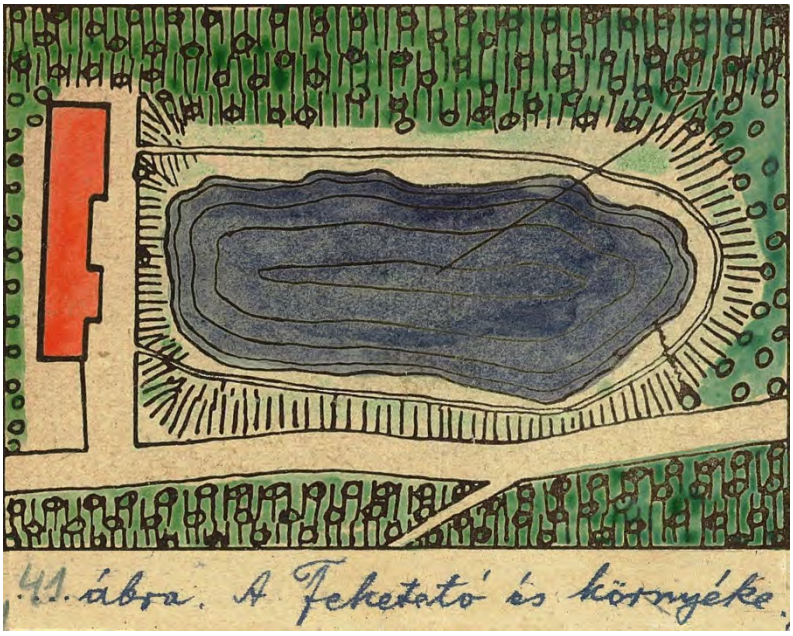

Figure 1. Map sketch with Nyárády's handwriting (with the permission of the Editors).

The main part of the volume is the 4th chapter, which contains a detailed description of the flora and vegetation of Sovata, based on Nyárády's original manuscript. The author describes in detail the vegetation in the vicinity of the lakes, saline ravines and sinkholes (dolinas) and analyzes the relationship between the salt bedrock and the vegetation formed on it. He identified 818 plant taxa belonging to 73 families from an area of 255 ha. In the next chapter, the editors assign the Latin names of taxa used by Nyárády to those in use today and indicate their distribution on the map they have redrawn, with appropriate coding. Topographical nomenclature has also been assigned to the codes in a table, so that the occurrence of a certain plant species can be easily identified both on the map and in the field. This can be very useful for those researchers who would like to study the changes of vegetation cover during the last century around salt lakes in Sovata.

The value of the publication is increased by the 21 art plates that contain drawings of plants (Fig. 2), made by Nyárády himself, as well as by the author's field diary, attached as a CD. This 100-page notebook is a real jewel, contains its own map sketches, accurate drawings and the work and results of each day spent in the field. Also, we are given personal information regarding the help received from his son, Tóni: "Thursday, July 15, 1943. Tóni and I continued the

survey from *Nagytó* until the *Keskenyút*."- page 29; or "I hired the salt guard Pál Márton, with whom we visited the surroundings and from whom we learnt the names of the places and noted them down." - page 39.

Figure 2. Plant drawings by E. I. Nyárády (with the permission of the Editors).

Undoubtedly, Erazmus I. Nyárády's scientific work and heritage in the field of flora research is remarkable. This valuable volume may serve as a very good example of what meticulous and hard work really means. To have this impressive study finally published, it took 77 years and the effort of a few dedicated editors who embraced the cause of this manuscript and passed it to the posterity. We hope to arouse the interest of the reader for this special volume.

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