A SURVEY OF THE PRESENCE AND SEX RATIO OF CULICOIDES (DIPTERA CERATOPOGONIDAE) IN ROMANIA

PREZENŢA ŞI RAPORTUL MASCULI / FEMELE LA UNELE SPECII ALE GENULUI CULICOIDES (DIPTERA CERATOPOGONIDAE) ÎN ROMÂNIA

Gh. DĂRĂBUŞ[1)], Elena TILIBAŞA[1)],
I. OPRESCU[1)], Narcisa MEDERLE[1)],
S. MORARIU[1)], M.S. ILIE[1)], Mirela IMRE[1)]

ABSTRACT | REZUMAT

Culicoides (Diptera: Ceratopogonidae) are known in the literature as vectors for bluetongue virus.

The presence of the population of *Culicoides* was evaluated in Western Romania using CDC Onderstepoort UV black-light traps set in seven locations. The traps were settled starting from May and up to October, for three nights in a row, four times a month.

During the three years study (2012-2014), in western Romania, 14,006 insects belonging to *Culicoides* genus were captured. The species were: *C. pulicaris* complex (38.31%), *C. obsoletus* complex (33.19%), *C. nubeculosus* (27.13%) and *C. imicola* (1.37%).

Comparing the total number of culicoides captured significant differences between the species ($p<0.001$) were registered. Overall, the number of females was significantly higher ($p<0.001$) compared with males in species: *C. obsoletus* complex*, C. pulicaris* complex and *C. nubeculosus* and not significant for the specie *C. imicola*. The sex ratio females/males vary from one specie to another: *C. obsoletus* (1/3.01), *C. imicola* (1/2.27), *C. pulicaris* (1/2.10) and *C. nubeculosus* (1/1.58). In October 01-04, 2014, the specie *C. imicola* was captured for the first time in Romania. Most insects were captured in southern locations. No statistical differences were found between the number of culicoides captured in cattle farms compared to sheep.

Keywords: *C. obsoletus*, *C. pulicaris*, *C. imicola, C. nubeculosus*

Genul *Culicoides* (Diptera: Ceratopogonidae) este cunoscut în literatura de specialitate ca vector pentru virusul bluetongue. Prezența populațiilor de *Culicoides* a fost evaluată în șapte locații din vestul României utilizând capcane luminoase, de tip CDC Onderstepoort UV. Capcanele au fost montate din mai până în octombrie, trei nopți la rând, de patru ori pe lună.

În timpul celor trei ani de studiu (2012-2014), în vestul României au fost capturate 14.006 insecte din genul *Culicoides.* Speciile identificate au fost: *C. pulicaris* complex (38.31%),*C. obsoletus* complex (33.19%), *C. nubeculosus* (27.13%) și *C. imicola* (1.37%).

Comparând numărul total de culicoizi capturați, între diferitele specii, s-au înregistrat diferențe semnificative ($p<0.001$). Pe ansamblu, numărul de femele a fost semnificativ mai mare ($p<0.001$) in comparație cu masculii la speciile *C. obsoletus* complex, *C. pulicaris* complex și *C. nubeculosus*, și nesemnificativ pentru specia *C. imicola*. Raportul masculi/femele a variat de la o specie la alta: *C. obsoletus* (1/3.01), *C. imicola* (1/2.27),*C.pulicaris* (1/2.10),*C. nubeculosus* (1/1.58). În anul 2014, în perioada 01-04 octombrie, a fost capturată pentru prima dată în Romania specia *C. imicola*. Cele mai multe insecte au fost capturate în locațiile sudice. Nu au fost identificate diferențe statistice între numărul de culicoizi capturați în fermele de bovine comparativ cu cele de ovine.

Cuvinte cheie: *C. obsoletus*, *C. pulicaris*, *C. imicola, C. nubeculosus*

Culicoides (Diptera: Ceratopogonidae) are the smallest insect vectors for important infectious agents (protozoa, viruses, bacteria) in humans and animals. Approximately 50 viruses have been identified in different culicoid species (8). In addition, these insects cause discomfort and allergic dermatitis (5, 11, 19).

Currently over 1340 species of *Culicoides* (4), which have a great number of subgenera, were described (3).

In western and central Europe, until 2010, five species are known to be involved in the transmission of the bluetongue virus, namely: *C. obsoletus* (Meigen 1919), *C. scoticus* (Downes and Kettle 1952), *C. chiopterus* (Meigen 1830), *C. pulicaris* (Linnaeus 1758), *C. dewulfi* (Goetghebuer 1935) and *C. imicola* (Kieffer 1913).

Additionally, in the Mediterranean area, one important specie from Africa is involved - *C. imicola* (14).

1) Banat's University of Agricultural Sciences and Veterinary Medicine "King Michael I of Romania", Faculty of Veterinary Medicine, Timişoara, Romania, E-mail: gheorghe.darabus@fmvt.ro

Very important is the involvement of *Culicoides* genus in the transmission of the Bluetongue virus, the African horse sickness virus and the Schmallenberg virus (6). *C. obsoletus* complex, *C. pulicaris* complex and *C. imicola* seem to be the most involved vectors, depending also on the climatic zone (6, 10). According to this study *C. imicola* is not found in Romania's climate. In Western Romania, the dynamics of *C. obsoletus* and *C. pulicaris* are positively correlated with average and maximum temperatures and negative correlated with wind speed (17). Another study in south-west of Romania reveals the existence of two species, *C. obsoletus* and *C. pulicaris* (12). The study of culicoids has become important in Romania, with the first cases of bluetongue in 2014, in cattle and sheep (21). Preliminary data published in 2014 reveal that, in Romania, the species potential involved in the transmission of bluetongue are *C. obsoletus, C. pulicaris* and *C. nubeculosus*. The population dynamics of the culicoids is influenced by several abiotic factors, such as: maximum temperature, mean temperature and minimum temperature, wind speed and relative humidity (20).

METHODS

Geographic area studied

The study of the presence of the culicoids was carried out in three counties in western Romania during the period 2012-2014. There were 7 locations, within the three counties, suitable for capturing and monitoring the culicoids: Timiș County - 3 locations; Arad County - two locations; Caraş-Severin County - two locations. The coordinates of the seven locations were determined by GPS (Global Positioning System). These locations have one or more common features that favor the emergence and development of *Culicoides* dipters, namely the presence of hosts and animal waste, the existence of forests and abundant vegetation, the existence of water courses and standing water, low pollution of the atmosphere, etc.

Timiș County is in the west of the country, on the border of Romania with Yugoslavia and Hungary. To the east is neighboring Hunedoara County, to the southeast with Caraş-Severin County and to the north by Arad County. The extreme points of the County are: to the west, east longitude 20° 21`(Beba Veche), east, longitude east 22°15`(Poieni), to the south, north latitude 45°15`(Lăţunaş) and to the north, north latitude 46°10 (Cenad) (24). The three locations were coordinated: location A (cattle farm) - lat. 45.782298; long. 21.212271; Location B (waste discharging basin) - lat. 45.389889; long. 21.139279; Location C (bovine farms) - lat. 46.089258; long. 20.604901.

Arad County is situated in the western extremity of Romania, in the plain of Tisza, at 46°11' lat. N and 21°19' long E, in the alluvial plain of Arad, part of the West Plain, at an average altitude of 107 m (22). The two locations had the following coordinates: location D (cattle farm) - lat. 46.160144; long. 21.275539; Location E (sheep farm) - lat. 46.507467; long. 21.534233.

Caraş-Severin County is situated in the south-western part of Romania at 45°18'0 "lat. N and 21°53' 25"long. E. Bordering northwest with Timiș County, northeast with Hunedoara County, east with Gorj County, southeast with Mehedinți County, in the west with the Republic of Serbia and Montenegro,on a length of 70 km, and in the southwest with the Danube River, on a length of 64 km (23). The two locations were coordinated: Location F (sheep farm, lake) - lat. 45.279525; Long. 21.898499; Location G (sheep farm, forest edge) - lat. 45.034327; Long. 21.695595.

Capturing equipment

The *Culicoides* were captured using shiny traps, CDC Onderstepoort UV type, equipped with 4W tubes with black-blue light and a 320-420 nm UV emission, a photo-optic sensor, a suction fan motor, a 3 mm metal mesh frame, an insect collection container, a nylon mesh and a 12 volt power supply. The traps were set starting from May and up to October, for three nights in a row, four times a month. For each location, 24 samples/year were collected. In total, in the seven locations, over the three years study, 504 samples were obtained. The traps were set in the evening, between the hours 19-20 and taken down the next day between the hours 7-8. The sample containers were discarded every morning and transported to the laboratory. There were washed with water twice or three times and filtered into other glasses. After the first wash the insects were transferred in 70% ethylic alcohol and disposed in Petri dishes, marked in quarters, for counting. After processing, the samples were stored in the refrigerator at 4°C until the species identification process.

Identification of Culicoides species

The identification, separation according to species and the sexing of the *Culicoides* have been achieved using a MOTIC SMZ-140 stereomicroscope and a Leica DM500 microscope equipped with an ICC50 HD camera. The microscopic and macroscopic exams had the aim of enhancing various morphological characteristics such as: the size of the body, the aspect of the pro-

boscis, the wings and the location of the black-greyish spots on them, the hairs on the main nerve, the innervation of the wings (the drawing), the position of the eyes, the thorax, the antennae, the spermatheca position and the structure of the legs. The captured *Cullicoides* species were identified based on identification keys proposed by Rawlings (18), Goffredo and Meiswinkel (9) and based on the identification keys available online (13). The statistics were made using Microsoft Excel 2016, Data Analysis Tools, statistic and t-Test: Paired Two Sample for Means.

RESULTS

In 2012, 4453 *Culicoids* (males and females) were captured, the number of insects being very close between locations. Three species have been identified: *C. obsoletus, C. pulicaris* and *C. nubeculosus*. Comparing the number of individuals captured there were significant differences between *C. obsoletus* and *C. nubeculosus* ($p < 0.001$) on the one hand and between *C. pulicaris* and *C. nubeculosus* ($p < 0.05$), on the other hand. The number of females was higher than of males, for all three species ($p < 0.001$). The female male ratio was in favor of females ranging from 1/2.17 (*C. nubeculosus*) and 1/3.08 (*C. obsoletus*) (Table1). Comparing the M/F ratio between the different culicoid species, it was found to be very significant between *C. pulicaris* and *C. nubeculosus* ($p < 0.001$) and between *C. obsoletus* and *C. nubeculosus* ($p < 0.001$).

In 2013, the highest number of collected culicoides (5790) was recorded, but with large variations between locations. Abundance was very high in locations B, C, F, G, all southern locations. All species were captured: *C. obsoletus, C. pulicaris* and *C. nubeculosus*. Although females were dominant in number, the M/F ratio increased in favor of males, especially in *C. pulicaris* and *C. nubeculosus* (Table 2). The numerical differences between males and females were significant only for *C. obsoletus* ($p<0.001$). Comparing the M/F ratio, between the different species identified, the differences were significant between *C. obsoletus* v *C. pulicaris* ($p <0.05$) and between *C. obsoletus* and *C. nubeculosus* ($p<0.01$). The differences between *Culicoid* species, with respect to the total number of captured insects, were significant among the *C. obsoletus* v. *C. nubeculosus* species ($p<0.001$) and C*. pulicaris* v *C. nubeculosus* ($p <0.001$). In 2014, 3763 culicoids were captured from the same sites, with the lowest abundance reported in the years under study. In addition to the species captured in previous years, a new species has been identified, with a lower abundance, *C. imicola*.

This year, the male/female ratio increased in favor of females (Table 3). If for the three species identified in previous years, the numerical differences between males and females were significant ($p<0.001$), for *C. imicola* the numerical differences between males and females were insignificant. By statistically analysis the differences in M/F ratios between species, the following

Table 1

The presence of *Culicoides* in 2012 and male/female ratio

Location	*C. obsoletus*			*C. pulicaris*			*C. nubeculosus*			*C. imicola*			Total
	M	F	R(M/F)	M	F	R(M/F)	M	F	R(M/F)	M	F	R(M/F)	
A	56	193	1/3.45	57	172	1/3.02	66	155	1/2.35	0	0	-	699
B	53	160	1/3.02	50	150	1/3	58	117	1/2.02	0	0	-	588
C	54	171	1/3.16	63	205	1/3.25	61	129	1/2.11	0	0	-	683
D	58	188	1/3.24	56	163	1/2.91	61	156	1/2.56	0	0	-	682
E	56	156	1/2.79	51	153	1/3	64	134	1/2.09	0	0	-	614
F	51	140	1/2.75	49	143	1/2.92	55	104	1/1.89	0	0	-	542
G	54	167	1/3.09	56	182	1/3.25	59	127	1/2.15	0	0	-	645
Total	**382**	**1175**	**1/3.08**	**382**	**1168**	**1/3.05**	**424**	**922**	**1/2.17**	0	0	-	**4453**

were found: *C. obsoletus* v *C. pulicaris* (p <0.001); *C. pulicaris* v *C. nubeculosus* (p<0.001); *C. obsoletus* v *C. nubeculosus* (p<0.001); *C. obsoletus* v *C. imicola* (p< 0.01); *C. pulicaris* v *C. imicola* (p<0.01); *C. nubeculosus* v *C. imicola* (NS). By comparing the total number of culicoids captured for the different species, statistically significant differences were found (p<0.001).

Throughout the study (2012-2014), 14006 males and females (nulliparous, parous and gravid) of the genus *Culicoides* were captured. Most insects of the genus *Culicoides* captured belonged to the species *C. pulicaris* - 5365 with 38.31% abundance, followed by *C. obsoletus* - 4648 (33.19%), *C. nubeculosus* - 3800 (27.13%) and *C. imicola* - 193 (1.37%) (Table 4).

Overall, the number of females was significantly higher (p<0.001) compared to males in *C. obsoletus, C. pulicaris* and *C. nubeculosus* and insignificant for *C. imicola*. By comparing the total number of culicoids captured in the 3 years of study, there were significant differences between the species identified (p<0.001).

In 2014, during the period 01-04 October, the species *C. imicola* was captured in Timiș County for the first time in Romania. In the same year, *C. imicola* was identified in all locations.

Most of the insects were captured in the southern locations (A, B, C, F and G) belonging to the Counties Caraş-Severin and Timiş. Analyzing the total number of culicoids captured, the differences were statistically insignificant between the southern counties (Caraș-Severin and Timiș), but they were statistically significant (p<0.05) when these counties were compared with the northern county (Arad). Also, comparing the total number of culicoids captured, regardless of species, between the farms of cattle and sheep, although there were small differences, they are statistically insignificant. The dynamics of the culicoid population in the location - waste basin - followed similar developments with the locations represented by sheep and cattle farms. In this location, in 2013, the only exception was recorded, the number of males was higher than that of females (Table 2).

DISCUSSION

Tables 1, 2 and 3 highlight the difference in the dynamics of the populations of different species of culicoides from one year to another and from one location to another. In 2013, the variations among culicoid population were very high both between locations and species found. Analyzing the variation of the temperature and wind velocity large variations were observed (20). For the three years studied, the abundance was higher for *C. pulicaris*, followed by *C. obsoletus*, *C. nubeculosus* and *C. imicola*. The higher abundance in the southern parts (locations A, B, C, F and G) seems to be explained by a favorable biotope for the development of the life cycle stages of the culicoids, given the influence of the Mediterranean climate. Another study (12), more limited, conducted in Gorj County (Romania) in

Table 2

The presence of *Culicoides* in 2013 and male/female ratio

Location	*C. obsoletus*			*C. pulicaris*			*C. nubeculosus*			*C. imicola*			Total
	M	F	R(M/F)	M	F	R(M/F)	M	F	R(M/F)	M	F	R(M/F)	
A	80	217	1/2.71	79	166	1/2.10	62	141	1/2.27	0	0	-	745
B	103	250	1/2.43	201	166	1/0.83	154	151	1/0.98	0	0	-	1025
C	107	301	1/2.81	210	220	1/1.05	169	193	1/1.14	0	0	-	1200
D	32	101	1/3.16	38	97	1/2.55	27	61	1/2.26	0	0	-	356
E	26	73	1/2.81	33	105	1/3.18	26	60	1/2.31	0	0	-	323
F	102	266	1/2.61	188	198	1/1.05	159	155	1/0.97	0	0	-	1068
G	104	273	1/2.63	192	204	1/1.06	165	135	1/0.82	0	0	-	1073
Total	**554**	**1481**	**1/2.67**	**941**	**1156**	**1/1.23**	**762**	**896**	**1/1.18**	0	0	-	**5790**

2012, shows the identification of only two species: *C. obsoletus* and *C. pulicaris*. These data prove that all the conditions for emerging bluetongue are created by identifying of at least three species recognized in the literature as vectors for this disease *C. obsoletus*, *C. pulicaris* and *C. imicola* (16).

A study published in Portugal by Capela et al. (7) reveals that higher abundances were obtained for *C. pulicaris* and lower for *C. imicola*, similar with the results published in our study. Another study (1), analyzes the potential of vectors for bluetongue in Sweden finding a low abundance for *C. pulicaris*, and high abundance for *C. obsoletus* /*C. scoticus*.

The presence of vectors from *C.obsoletus*, *C. pulicaris* and *C. nubeculosus* complexes is important for bluetongue transmission which, although monitored since 2000, was diagnosed in Romania only in 2014 (20).

In a map made by Brugger and Rubel (6), based on the Köppen-Geiger climate classification, Romania is represented as an area where the conditions for the development of the *C. obsoletus* and *C. pulicaris* complexes are present. Until now, there was no mention of *C. imicola* species in the peri-Mediterranean areas, this study demonstrates that, probably due to global warming, the species began to migrate further north.

Comparing the culicoid populations, based on the numerical difference between the sexes, the females were always more numerous. Except 2013, an atypical year, in the other two years the M/F ratio was over 3 for *C. obsoletus* and *C. pulicaris*, close to 2 or more for 2 for *C. nubeculosus* and over 2 for *C. imicola*. In 2013, for the B, C, F and G locations there was observed a significant increase in abundance, and a reduction in the M/F ratio by increasing the number of males. Further research could prove that, under favorable conditions, when culicoid populations grow, the M/F ratio is balanced in favor of males. Analyzing the situation of the M/F ratio for three years, differences are observed from one species to another: *C. obsoletus* (1/3.01), *C. imicola* (1/2.27), *C. pulicaris* (1/2.10) and *C. nubeculosus* (1/1.58). The sex ratio found in this study is different from that reported by Gonzales et al. (10) in Spain for *C. obsoletus*. A higher number of females than males is found by Meiswinkel et al. (15) in a study in the southeast of the Netherlands. Statistically comparing the abundance of culicoid populations in the cattle farms (A, C, D) and sheep farms (E, F, G) it was found that the animal species did not influence the size of the population. Other authors (2) find, on the other hand, that some culicoids prefer some animal species.

CONCLUSIONS

Four species of culicoids have been identified in Romania: *C. obsoletus* complex, *C. pulicaris* complex, *C. imicola* and *C. nubeculosus*. The first three species are recognized as bluetongue vectors. The abundance was significantly different between these species. The presence was higher in southern areas, perhaps due to a more favorable climate. No abnormal differences were found between bovine and sheep farms. Determining

Table 3

The presence of *Culicoides* in 2014 and male/female ratio

Location	*C. obsoletus*			*C. pulicaris*			*C. nubeculosus*			*C. imicola*			Total
	M	F	R(M/F)	M	F	R(M/F)	M	F	R(M/F)	M	F	R(M/F)	
A	31	124	1/4	59	197	1/3.34	44	73	1/1.66	3	4	1/1.33	535
B	35	113	1/3.23	61	196	1/3.21	42	75	1/1.79	3	4	1/1.33	529
C	31	110	1/3.55	60	179	1/2.98	40	66	1/1.65	16	49	1/3.06	551
D	31	129	1/4.16	58	190	1/3.28	42	75	1/1.79	3	4	1/1.33	532
E	29	110	1/3.79	49	155	1/3.16	33	71	1/2.15	3	4	1/1.33	454
F	34	123	1/3.62	60	197	1/3.28	44	72	1/1.64	14	16	1/1.07	560
G	32	124	1/3.88	62	195	1/3.15	43	76	1/1.77	17	53	1/3.12	602
Total	**223**	**833**	**1/3.74**	**409**	**1309**	**1/3.20**	**288**	**508**	**1/1.76**	**59**	**134**	**1/2.27**	**3763**

Table 4

The presence of *Culicoides* between 2012 and 2014 and male/female ratio

Location	*C. obsoletus*			*C. pulicaris*			*C. nubeculosus*			*C. imicola*			Total
	M	F	R(M/F)	M	F	R(M/F)	M	F	R(M/F)	M	F	R(M/F)	
A	167	534	1/3.20	195	535	1/2.74	172	369	1/2.15	3	4	1/1.33	1979
B	191	523	1/2.74	312	512	1/1.64	254	343	1/1.35	3	4	1/1.33	2142
C	192	582	1/3.03	333	604	1/1.81	270	388	1/1.44	16	49	1/3.06	2434
D	121	418	1/3.45	152	450	1/2.96	130	292	1/2.25	3	4	1/1.33	1570
E	111	339	1/3.05	133	413	1/3.11	123	265	1/2.15	3	4	1/1.33	1391
F	187	529	1/2.83	297	538	1/1.81	258	331	1/1.28	14	16	1/1.07	2170
G	190	564	1/2.97	310	581	1/1.87	267	338	1/1.27	17	53	1/3.12	2320
	1159	3489	1/3.01	1732	3633	1/2.10	1474	2326	1/1.58	59	134	1/2.27	14006
Total	4648 (33.19%)			5365 (38.31%)			3800(27.13%)			193 (1.37%)			

the sex ratio has shown the existence of a greater number of females in all identified culicoids. Differences were significant among the sex ratio found in the four species.

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