Theses of Doctoral (PhD) Dissertation

Investigating the ecology and micrbiology of the urban and rural Hooded Crows (*Corvus cornix*) in Hungary

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1. INTRODUCTION

Urbanisation generates complex and diverse systems characterised by high levels of human disturbance, pollution, and landscape and environmental changes (SHOCHAT et al., 2006; LOWRY et al., 2012). Often modifying the ecological processes of a given ecosystem, urbanisation's effects range from disturbing the structure of the food chain by removing or introducing species, encouraging human tolerance and adaptation, increasing health risks for humans and wildlife, and modifying ecosystem services (MARZLUFF et al., 2001; VUORISALO et al., 2003). One of the most acknowledged urban dwellers are wild passerines like corvid species. Known for their world wide geographic distribution and adaptability to many habitats, corvid species are often described as suburban adaptors and even urban exploiters (BENMAZOUZ et al., 2021), making them ideal models for urbanisation-related investigations. Corvids' association with urbanised areas is not only due to their taxonomic and ecological diversity but also to their behavioural flexibility and high tolerance for human presence, resulting in their increasing interactions with humans (MATSYURA & JANKOWSKI, 2016). This flexibility and their ecological adaptability allow corvids to actively explore and colonise urban settlements.

In the present day, the different interaction routes and interconnections between humans, animals, and the environment are evident and have undeniably outlined the interdependence between the different health systems recognised currently under the one health concept. As one of the most important and urgent global health issues, resistance to antimicrobial (AMR) treatments has been extensively reported in the last few years. The origin of this phenomenon and the dissemination of antimicrobial-resistant bacteria (ARB) in the environment are subject to deep investigation. And numerous factors have been recognised as the probable reason for this emergence, mainly the level of interaction between wildlife and humans and human activities such as intensively managed livestock farms, landfills, and waste-water treatment facilities (LANTHIER et al., 2010; ALROY & ELLIS, 2011; ZURFLUH et al., 2016). Consequently, urban wild birds can be considered sentinels since they may easily pick up human and environmental bacteria (BONNEDAHL & JÄRHULT, 2014) and serve as reservoirs and vectors of ARB and resistance genes (DOLEJSKA & LITERAK, 2019; DOLEJSKA & PAPAGIANNITSIS, 2018).

In this study, we first aim to understand how the urban environment in our cities would affect the continuously increasing Hooded Crow (*Corvus cornix*) community by examining the

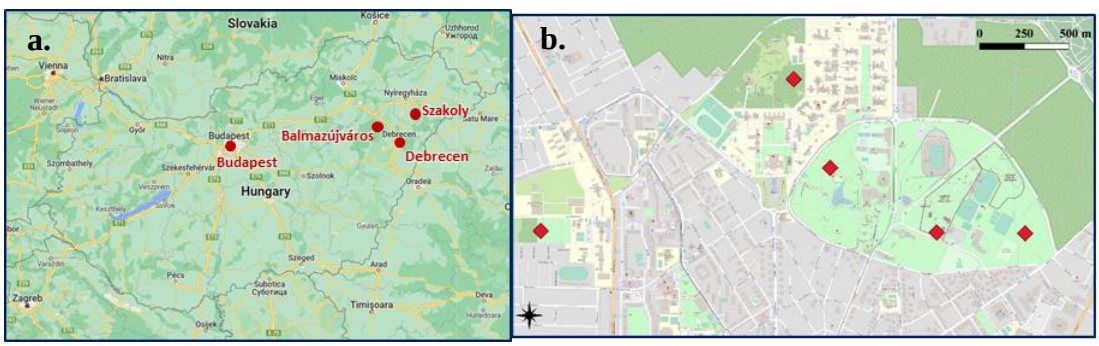
presence of any morphological and physical differences between Hooded Crows from rural and urban areas and thus exploring any phenotypic responses from the crows to urbanisation. Secondly, we aim to: (1) explore the occurrence of extended-spectrum β-lactamases (ESBLs) (providing resistance to third-generation cephalosporins, broad-spectrum beta-lactam antibiotics that are widely used in humans and veterinary medicine) producing Enterobacterales (EXNER et al., 2017) and vancomycin resistant enterococci (VRE); (2) identify the genes responsible for the resistance found and characterise AMR determinants and associated plasmids; and (3) investigate the virulence potential of the isolates using whole-genome sequencing (WGS). The goal is to estimate the degree of contamination with ARB of health and veterinary importance of an urban wildlife species in Hungary and to see if the success of Hooded Crow in urban environments is leading to the carriage of AMR by these wild birds in Hungary.

2. MATERIAL AND METHODS

2.1. Area of study

This project has been operating in the city of Debrecen (47.52997°N; 21.63916°E) (Figure 1a.), located at the centre of the Northern Great Plain region and situated nearby the Hortobágy National Park. We operate four separate traps in the city for our urban sample collection (Figure 1b.). Additional urban samples were collected from wild crows trapped at the Budapest Zoo (47°29′33″N; 19°03′05″E). Whereas, the rural samples are collected from the bordered hunting areas of Szakoly, a rural village 35 km north-east of Debrecen, and Balmazújváros, a rural town 25 km west of Debrecen, adjacent to the Hortobágy National Park (Figure 1a.).

Figure 1: **Maps showing the different study areas (a); and the locations of the traps in the city of Debrecen (b). Red coloured dots = Trap sites during the study period. Green colour = natural landscapes, Grey colour = urban infrastructure (e.g. buildings and roads).**



Hooded Crows were captured in all urban and rural areas (Figure 2) between March and August 2020. Trapping and bird handling happened twice a week in the city of Debrecen by IB and LK, where traps were visited twice a week, exclusively after sunset, during the entire study period. Whereas at the Budapest Zoo and rural areas, crow trapping was performed by a corresponding person from the local wildlife management units, and with their assistance, bird handling and sample collection were conducted during our daytime visits to trap locations. All traps had a fixed structure and were open continuously during the trapping occasions.

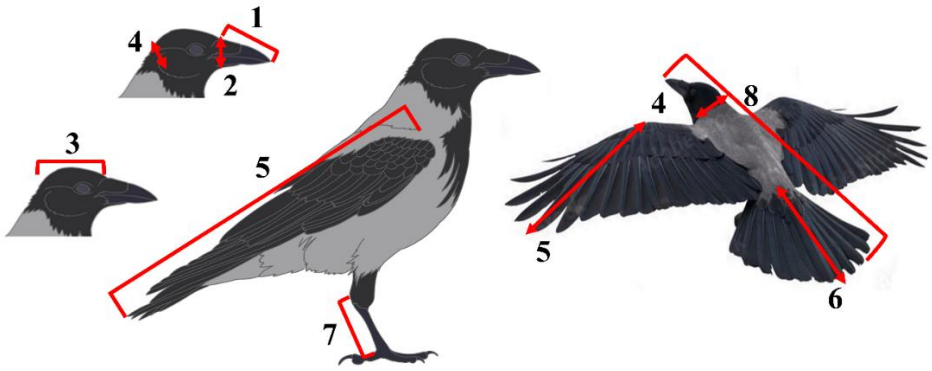
2.2. Sample collection

Wild Hooded Crows are caught using a 'Ladder entrance trap' (Figure 2) in both urban and rural environments continuously throughout the years (KÖVÉR et al., 2018). This kind of trap has been successfully used to catch Hooded Crow in rural areas and in urban zoos in Vienna (Austria), Sapporo (Japan), and Debrecen (Hungary) before (KÖVÉR et al., 2018).

Figure 2: **A ladder entrance trap used for Hooded Crow capture**.

Biometric data, including body mass, body condition, body length, head length, head width, wing length, bill length, bill width (thickness), tail length, and tarsus length, are measured for each captured and sampled individual from all areas (Figure 3).

Figure 3. **Diagram of quantified measurements of captured Hooded Crows. (1) bill length measured from tip to skull along the culmen; (2) bill width; (3) skull length measured from basis of bill to back of head; (4) head width; (5) wing length from carpal joint to wingtip; (6) tail length; (7) tarsus length and (8) body length (from tip of bill to tip of tail).**

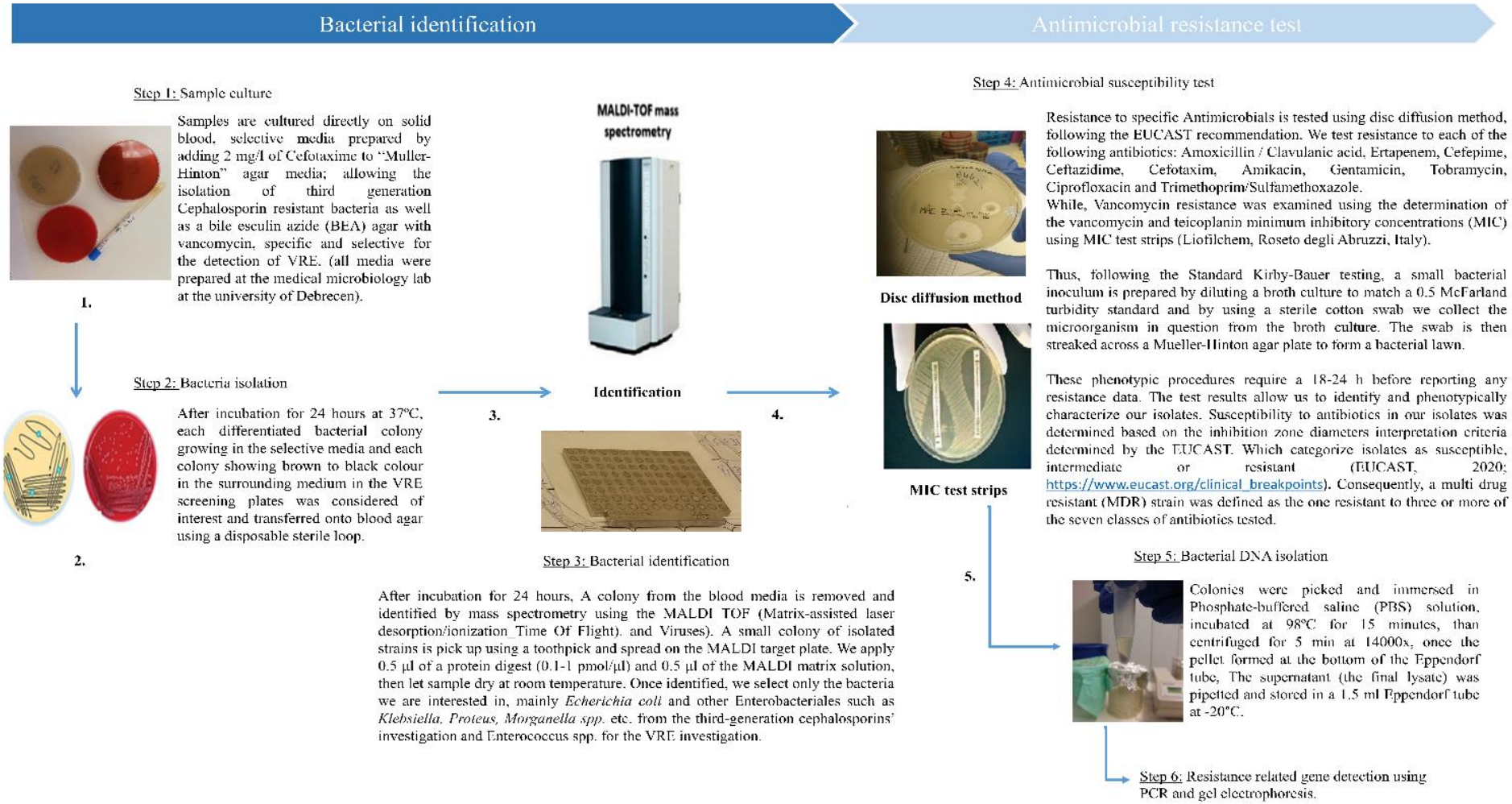


Faecal samples were collected for the trapped birds directly from the cloaca using a standard sterile cotton swab immersed in the Stuart transport medium, a semi-solid, non-nutritional transport medium for fastidious, pathogenic organisms. The swabs were transferred to the medical microbiology lab at the University of Debrecen, where they were immediately cultured.

2.3. Microbiological analysis

The different steps of the analysis of our samples are illustrated in the following figure (Figure4).

Figure 4: **Summary of the laboratory methods used for the analysis of AMR in Hooded Crow faecal samples. MALDI-TOF: matrix-associated laser desorption/ionization – time of flight. MIC: Minimum Inhibitory Concentration. DNA: deoxyribonucleic acid. PCR: Polymerase chain reaction.**



Resistance related gene detection using PCR and gel electrophoresis:

DNA was extracted, purified, and amplified using PCR, a fairly simple and rapid technique and a very practical and cost-effective tool. The main gene groups investigated were *bla-$_{CTX-M-1}$*, *bla-$_{CTX-M-2}$*, *bla-$_{CTX-M-9}$*, *bla-$_{CTX-M-8}$*, and *bla-$_{SHV}$*. The PCR master mix and the thermal cycling programmes used for each reaction mix were always prepared according to the method assigned to different genes. Once the PCR template was ready, following each protocol accordingly, we proceeded with the amplifications of our DNA samples. Once the PCR was completed, we proceeded with the gel electrophoresis to analyse our samples. Horizontal gel electrophoresis was used. A 1.5% agarose gel was prepared, and the electrophoresis was run for 60 minutes at 100 Volts. The results were viewed using Gel-Doc XR (Bio-Rad) and Image Lab 6.1 (Bio-Rad) software, and the results were recorded directly into a spreadsheet.

Whole genome sequencing of all ESBL-producing E. coli isolates:

1. Purified genomic DNA was extracted using the Quick-DNA™ Fungal/Bacterial Miniprep Kit (Catalogue no. D6005) using the Zymo-Spin technology ™ (ZYMO RESEARCH) following the manufacturer's instructions.
2. To prepare Illumina-specific libraries, the Illumina® Nextera XT DNA Library Preparation Kit (Illumina, San Diego, CA, USA) and the Nextera XT Index Kit v2 (Illumina, San Diego, CA, USA) were used.
3. Libraries were sequenced using an Illumina® NextSeq 500 sequencer (Illumina, San Diego, CA, USA).
4. FastQ files were quality trimmed using FastQC and filtered using FastQP, then errors were corrected using Bloocoo (a kmer-spectrum-based read error corrector).
5. Two assemblies were carried out using two different pipelines: unicycler (WICK et al., 2017) and megahit (LI et al., 2016). The assemblies were then merged using gam-ngs (VICEDOMINI et al., 2013).
6. The contiguity of genomes was checked using QUAST v5.2.0 (Quality Assessment Tool for Genome Assemblies by CAB (The Center for Algorithmic Biotechnology, SPbU)) and completeness was fulfilled using the Enterobacterales_odb10 core gene database.
7. The quality of the genome of our isolates was analysed using CeckM (PARKS et al., 2015) and Kraken2
8. The purity of isolates was analysed with checkm and kraken2 using the k2_pluspfp database (WOOD & LANGMEAD, 2019).

9. Gene resistance was screened using ABRicate (https://github.com/tseemann/abricate) using the CARD database. Serotypes were determined using ectyper (https://github.com/phac-nml/ecoli_serotyping), and sequence types (STs) were determined using mlst (https://github.com/tseemann/mlst).
10. PlasmidFinder 2.1 (CAMACHO et al., 2009; CARATTOLI et al., 2014), VirulenceFinder 2.0 (CAMACHO et al., 2009; JOENSEN et al., 2014; MALBERG et al., 2020) and cgMLSTFinder 1.2 (CLAUSEN et al., 2018; ZHOU et al., 2020), available from the CGE (Centre for Genomic Epidemiology) were used to identify plasmid replicon types, virulence factors, and genomic sequence types.
11. Phylogenetic groups were identified using the ClermonTyping web-interface (http://clermontyping.iame-research.center/ (BEGHAIN et al., 2018; CLERMONT et al., 2019)).

2.4. Anthropogenic food sources and temperature data

We surveyed the presence of anthropogenic food sources in urban and rural areas. We located different food establishments, pinpointed every single location on a map using QGIS 2.6.3.1 (2022), and created a multi-scale buffer from trap locations (10-200 m) in order to illustrate the probable range of the crows in the area and their possible overlap with these artificial food sources. Also, additional randomly selected 200-m circles were created to have a total of five circles in all study sites.

Temperature data was extracted from the Visual Crossing online weather database (Visual Crossing Corporation, 2022). Data on the annual average temperature over the last 10 years were obtained from the meteorological station in close proximity to all areas of study.

2.5. Statistical analysis

All statistical analyses were performed using R 4.2.1 software (R Core Team 2022) as well as PAST software (Paleontological Statistics Software Package) version 4.03 (HAMMER et al., 2001). Data from each area (urban and rural) will be pooled during all the study periods.

To explore the morphological divergence in Hooded Crows, we examined our data at three different scales: (1) between habitat types (urban vs. rural areas), (2) between urban areas (Budapest vs. Debrecen), and (3) between four urban sample collection sites within the city of

Debrecen (between the Zoo, Open-Air Theatre, Sport Complex, and Agriculture Faculty Campus (Agrár Campus)).

All morphological data were combined into PCA analysis using PAST version 4.03 (HAMMER et al., 2001). The first, second, third, and fourth PCA axes had values of 45.193, 26.221, 10.945, and 8.903, respectively. Therefore, we used the first four axes in our analysis (91.3%). The PC1 axis correlated positively with bill length and bill width. The PC2 axis correlated positively with linear body size measurements. The PC3 axis correlated positively with body length and skull width and negatively with tarsus length. Lastly, PC4 was positively correlated with skull width. Additionally, as an index of body condition, we calculated a regression residual between body mass and tarsus length following the literature (LABOCHA & HAVES, 2012; DULISZ et al., 2016). Thus, we tested the differences in the nine original morphological traits in the first four principal components obtained by a principal component analysis (PCA) conducted to reduce the original eight body size variables into four independent, non-correlating variables and the index of body condition.

Moreover, as this study was conducted between the months of April and November, we evaluated any possible monthly variations in our variables. We constructed linear models with the month and a factor of interest as the two main effects. We also tested the interaction between the month and the factor of interest to test for changes in the opposite direction (Figure 5).

Before fitting linear models, we checked the assumption of normality by the Shapiro-Wilk test and the assumption of homoscedasticity by the Bartlett-test. In case the assumptions were not met, we log-transformed the variables for the linear models. In addition to the linear models, we used non-parametric tests (Wilcoxon rank-sum test, Kruskal-Wallis test) to obtain distribution-free results and conclusions.

Finally, since several statistical tests were conducted on the same dataset, p-values were adjusted using the False Discovery Rate method (BENJAMINI & HOCHBERG 1995) in all statistical tests presented. Other than the PCA, all calculations and statistical testing were conducted in R version 4.2.2. (R Core Team 2022).

On the other hand, resistance rates were calculated for each area separately, and rates between urban and rural areas were compared. Using the chi-squared test conducted on PAST version 4.03 (HAMMER et al., 2001), the multi-resistant bacterial diversity was tested as a function of the independent variable habitat.

Figure 5. **Summary if the linear model construction and relative statistical tests applied.**

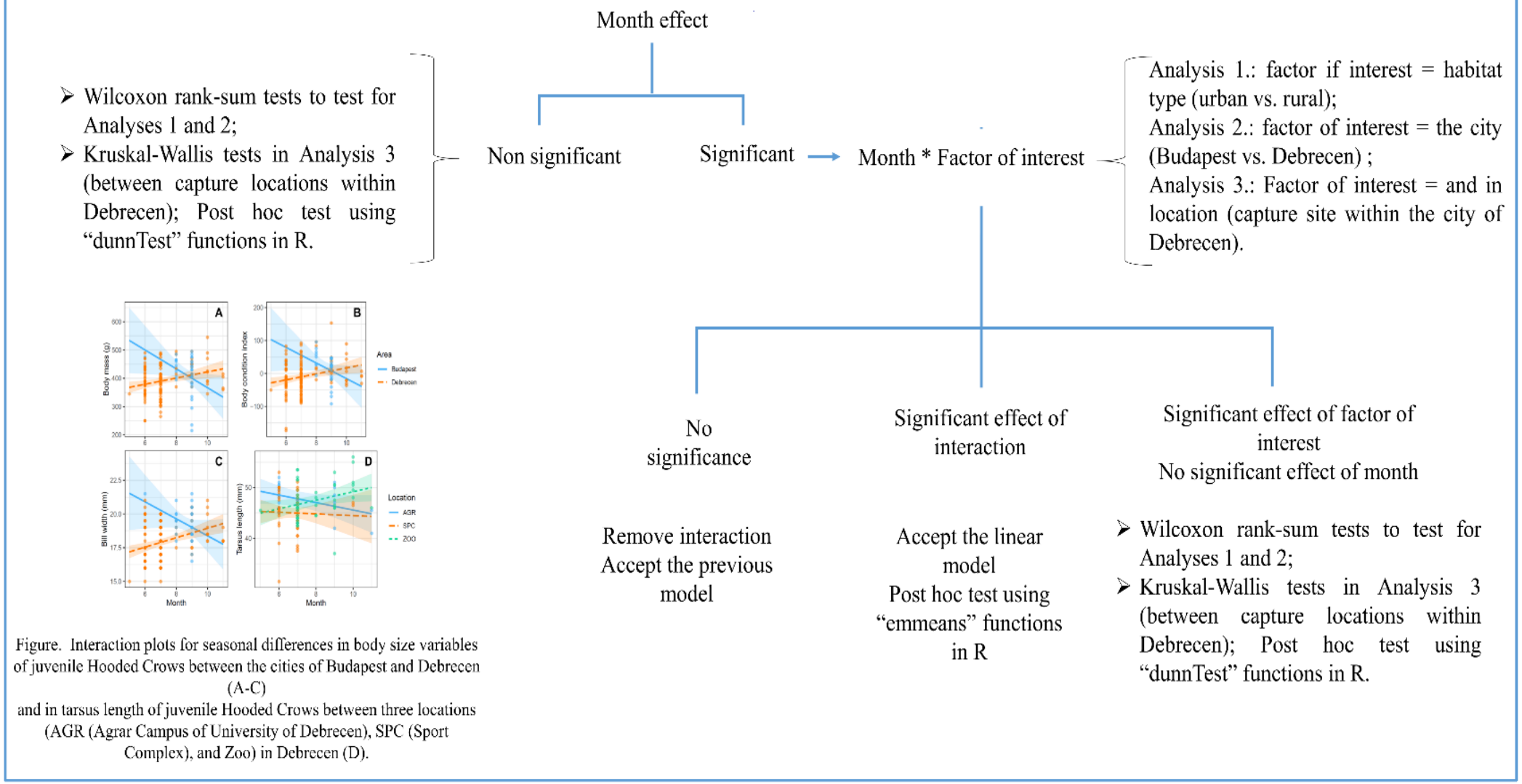


Figure. Interaction plots for seasonal differences in body size variables of juvenile Hooded Crows between the cities of Budapest and Debrecen (A-C)
and in tarsus length of juvenile Hooded Crows between three locations (AGR (Agrar Campus of University of Debrecen), SPC (Sport Complex), and Zoo) in Debrecen (D).

3. RESULTS

3.1. Interspecies morphological divergence in the Hooded Crow

3.1.1. Morphological differences across spatial scales and age categories

Since we captured both juvenile and adult Hooded Crows, we initially tested the effect of age on our data set, found an important age effect on several morphological traits measured, and decided to divide our data into two subsets based on age. Differences in morphological traits were explored between adults from different habitats, and despite the low numbers of juvenile measurements from rural areas, we proceeded with testing these variations between juveniles as well. We captured a total of 248 Hooded Crows. Our sample size included 43 birds from rural areas (Balmazújváros: two juveniles, 26 adults; Szakoly: one vs. 14) and 205 birds from urban areas (Budapest: 28 vs. 10; Debrecen: 128 vs. 39) (Table 1).

Urban and rural Hooded Crows differ in some of their morphological traits, with morphological divergence only being statistically significant for some traits. Particularly in juvenile Hooded Crows, 'bill size' and bill length were larger for urban birds than for rural ones, whereas 'lengthiness', 'skull width', tarsus length, and skull length were larger for rural birds than for urban ones (Table 2). This demonstrates that rural juvenile crows were bigger in size than urban ones, but the latter had strong bills. In adults, 'lengthiness' and tarsus length were greater for rural birds than for urban ones (Table 2), indicating smaller adult crows in urban areas. No additional differences were significant.

At a city level, Hooded Crows from Budapest and Debrecen exhibited inter-city differences where the direction of the effect of the area seemed age-dependent. Since no differences in any of the tested variables of adult crows from both cities were found. On the contrary, juvenile crows from the city of Debrecen displayed lower body conditions and smaller bill forms (smaller 'bill size', bill length, and bill width) than individuals from Budapest. Additionally, significant monthly variations were observed in several traits, including body mass (slope B = –33.4 ± 15.77, F = 5.796, p = 0.017), PC1 (0.6 ± 0.22, F = 6.693, p = 0.011), body condition (–23.7 ± 13.23, F = 6.211, p = 0.014), bill length (0.01 ± 0.004, F = 5.890, p = 0.016), and bill width (–0.63 ± 0.375, F = 12.259, p = 0.0006). While the interaction between month and city was significant for body mass (44.3 ± 16.20, F = 7.472, p = 0.007), body condition (32.8 ± 13.58, F = 5.838, p = 0.017), and bill width (0.98 ± 0.385, F = 6.453, p = 0.012) (Figure 12), suggesting that body mass, body condition index, and bill width increased along the season in Debrecen and decreased in Budapest (Figure 5).

Table 1

Results of the statistical computation of means and standards of deviation of 12 different body size variables, for both adult and juvenile Hooded Crows, at three different spatial scales: habitat level (urban and rural habitats), city level (Debrecen and Budapest), and capture location level in the city of Debrecen (three locations (juveniles) and four locations (adults)).

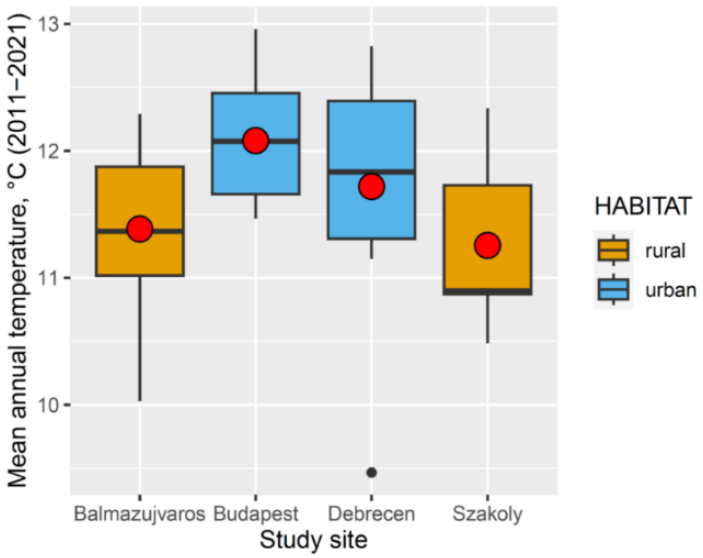
Age group	Variables	Between Habitats (mean ± SD)		Between cities (mean ± SD)		Between three locations (juveniles) or four locations (adults) in the city of Debrecen (mean ± SD)			
		Urban	Rural	Budapest	Debrecen	Zoo	SPC	Agrar	OST
Juvenile	Body mass	**398.6 ± 56.11**	**466.7 ± 35.12**	**425.7 ± 52.27**	**393 ± 55.63**	**418.7 ±55.13**	**366.3 ± 51.68**	**398.5 ± 37.73**	-
	Body length	**43.0 ± 3.70**	**43.2 ± 1.89**	**43.7 ± 2.55**	**42.8 ± 3.9**	**43.9 ± 2.30**	**41.9 ± 5.40**	**42.7 ± 1.38**	
	Body condition	**9.4 ± 2.59**	**10.8 ± 0.39**	**17.3 ± 44.4**	**9.35 ± 2.8**	**11.9 ± 53.44**	**-26.1 ± 46.11**	**-10.4 ± 34.82**	-
	Bill length	**51.4 ± 3.44**	**39.8 ± 15.05**	**53.09 ± 2.36**	**50.8 ± 3.4**	**50.9 ± 2.86**	**50.8 ± 3.68**	**50.4 ± 3.90**	-
	Bill width	**18.2 ± 1.50**	**16.7 ± 0.29**	**19.3 ± 1.26**	**18 ± 1.46**	**18.5 ± 1.26**	**17.6 ± 1.44**	**17.8 ± 1.64**	-
	Wing length	**30.6 ± 1.37**	**29.7 ± 0.58**	**30 ± 1.76**	**30.7 ± 1.26**	**30.8 ± 1.55**	**30.7 ± 1.04**	**30.4 ± 0.87**	-
	Tail length	**18.5 ± 0.96**	**18.3 ± 1.44**	**18.2 ± 0.9**	**18.5 ± 0.97**	**18.3 ± 1.05**	**18.6 ± 0.93**	**18.7 ± 0.86**	-
	Tarsus length	**46.6 ± 4.17**	**52.5 ± 1.73**	**47.6 ± 5.27**	**46.4 ± 3.9**	**47.3 ± 3.72**	**45.0 ± 3.88**	**47.7 ± 2.93**	-
	Head length	**90.7 ± 3.67**	**82.7 ± 15.7**	**92.7 ± 3.34**	**90.3 ± 3.61**	**90.8 ± 3.31**	**90.1 ± 3.87**	**89.7 ± 3.35**	-
	Skull length	**39.3 ± 2.21**	**42.8 ± 3.2**	**38.8 ± 2.09**	**39.4 ± 2.24**	**39.8 ± 2.55**	**39.3 ± 1.95**	**39.3 ± 2.14**	-
	Skull width	**34.4 ± 1.59**	**35.8 ± 0.76**	**35 ±1.27**	**34.2 ± 1.63**	**34.9 ± 2.08**	**34.0 ± 1.26**	**34.0 ± 1.30**	-
Adults	Body mass	**426.7 ± 47.47**	**417.2 ± 50.13**	**416.3 ± 24.96**	**427.7 ± 49.33**	**441.3 ± 49.26**	**418.6 ± 55.51**	**409.5 ± 26.82**	**434.5 ± 60.16**
	Body length	**43.9 ± 2.26**	**44.9 ± 2.23**	**44.8 ± 0.5**	**43.8 ± 2.36**	**45.3 ± 2.75**	**42.5 ± 2.31**	**42.7 ± 2.11**	**44.4 ± 2.35**
	Body condition	**17.47 ± 39.74**	**-3.5 ± 48.84**	**4.4 ± 17.62**	**18.8 ± 41.29**	**5.0 ± 52.1**	**20.1 ± 32.17**	**17.0 ± 40.01**	**27.8 ± 40.25**
	Bill length	**51.8 ± 2.69**	**51.8 ± 5.53**	**52.5 ± 1.42**	**51.8 ± 2.79**	**51.8 ± 3.45**	**49.9 ± 2.11**	**50.8 ± 2.73**	**52.4 ± 2.54**
	Bill width	**19.1 ± 1.19**	**19.1 ± 1.09**	**19.0 ± 0.82**	**19.1 ± 1.23**	**19.9 ± 0.99**	**19.2 ± 1.07**	**18.7 ± 1.11**	**19.8 ± 1.38**
	Wing length	**30.3 ± 1.64**	**30.5 ± 1.66**	**28.9 ± 0.63**	**30.4 ± 1.65**	**31.4 ± 1.18**	**31.1 ± 1.59**	**30.4 ± 1.07**	**29.1 ± 1.66**
	Tail length	**18.6 ± 1.59**	**19.1 ± 0.99**	**19.3 ± 0.65**	**18.5 ± 1.65**	**16.1 ± 0.68**	**19.0 ± 1.0**	**19.6 ± 1.16**	**18.3 ± 0.9**
	Tarsus length	**47.7 ± 4.51**	**49.8 ± 2.62**	**48.1 ± 2.95**	**47.7 ± 4.67**	**52 ± 3.9**	**46 ± 5.35**	**45.1 ± 2.91**	**47.3 ± 4.47**
	Head length	**93.1 ± 3.29**	**93.4 ± 5.59**	**95.4 ± 2.10**	**92.9 ± 3.31**	**93.9 ± 3.40**	**91 ± 2.69**	**91.1 ± 2.62**	**94.1 ± 3.72**
	Skull length	**41.25 ± 2.27**	**41.6 ± 1.25**	**42.9 ± 1.65**	**41.1 ± 2.28**	**42.2 ± 1.98**	**41.1 ± 1.51**	**40.3 ± 1.78**	**41.7 ± 2.18**
	Skull width	**35.52 ± 1.70**	**35.6 ± 6.44**	**36.1 ± 0.25**	**35.5 ± 1.78**	**35.3 ± 1.13**	**34.8 ± 1.60**	**34.9 ± 0.99**	**36.0 ± 1.76**

At a local level, within the city of Debrecen, interesting differences in body size were found in juvenile crows. For instance, individuals captured at the Zoo were generally heavier, lengthier, and had better body conditions, more fat reserves and wider skulls and bills than individuals caught elsewhere, especially those captured at the Sport Complex (SPC) (Table 2). What is more interesting is that such a difference between juveniles was observed between two trapping sites that are very close to each other (the Zoo and SPC traps were less than 500m apart).

3.1.2. Environmental factors: temperature and anthropogenic food availability

The annual average temperature was higher in urban (average value of Debrecen and Budapest) than in rural (average value of Szakoly and Balmazújváros) study areas (t-test: t = -2.8152, df = 39.74, P = 0.008) (Figure 6). The annual temperature between urban Debrecen and Budapest did not significantly differ over the period of ten years (t = 1.1509, df = 15.811, P = 0.2669) (Figure 6).

Figure 6. **Mean annual temperature between 2011 and 2021 in the four study areas. The plot depicts the median (thick horizontal line), 25% and 75% quartiles (box), min and max values (whiskers) and mean values (red dots).**



The availability of anthropogenic food sources, such as restaurants and café shops, were greater in urban than in rural study areas (Table 3).

Table 2

Results of statistical tests for the difference in body size variables of Hooded Crows between urban and rural habitats, between cities (Debrecen and Budapest), and between three locations (juveniles) or four locations (adults) in the city of Debrecen. F statistics are from linear models, and H statistics are from Kruskal-Wallis tests. P values were adjusted by the FDR method. Significant differences are in bold.

Age group	Variable	Between habitats (Urban vs. Rural habitats)			Between urban areas (Budapest vs. Debrecen)			between three locations (juveniles) or four locations (adults) in the city of Debrecen			
		Test statistic	Adjusted p	Interpretation	Test statistic	Adjusted p	Interpretation	Test statistic	Adjusted p	Post-hoc	
										Interpretation	p-value
Juveniles	Body mass	F = 4.341	0.084		F = 2.867	0.197		**F = 13.883**	**0.0015**	**Zoo > SPC**	**0.001**
	Fat reserves	W = 252	0.883		W = 1596.5	0.223		**F = 4.089**	**0.036**	**Zoo > SPC**	**0.01**
	PC1 ‘bill size’	**F = 22.508**	**0.0008**	**Urban > Rural**	**F = 22.680**	**0.0005**	**Budapest > Debrecen**	F = 0.480	0.715		
	PC2 ‘lengthiness’	**F = 7.768**	**0.03**	**Rural > Urban**	W = 1957	0.696		**F = 6.800**	**0.0075**	**Zoo > SPC**	**0.006**
	PC3 ‘body length’	W = 89	0.099		W = 1904	0.83		**H = 8.168**	**0.036**	**SPC > AGR** **Zoo > AGR**	**0.017** **0.023**
	PC4 ‘skull width’	**W = 439**	**0.033**	**Rural > Urban**	W = 2032	0.493		H = 0.337	0.845		
	Body condition	F = 1.204	0.343		**F = 6.623**	**0.041**	**Budapest > Debrecen**	**F = 8.335**	**0.003**	**Zoo > SPC**	**0.005**
	Body length	W = 225	0.9		W = 2203	0.218		F = 3.523	0.055		
	Wing length	W = 111	0.16		F = 4.098	0.123		H = 3.045	0.297		
	Tarsus length	**F = 6.069**	**0.038**	**Rural > Urban**	W = 2119.5	0.293		**F = 6.830**	**0.008**	**AGR > SPC**	**0.015**
	Tail length	W = 191	0.662		W = 1589.5	0.293		H = 3.590	0.249		
	Skull length	**F = 7.313**	**0.03**	**Rural > Urban**	W = 1563	0.276		H = 1.526	0.583		
	Skull width	W = 384.5	0.099		F = 4.588	0.102		**H = 8.121**	**0.036**	**Zoo > SPC**	**0.021**
	Bill length	**F = 42.997**	**0.0008**	**Urban > Rural**	**F = 21.794**	**0.0005**	**Budapest > Debrecen**	F = 0.193	0.845		

Table 3.

Number of anthropogenic food sources within a 200 m radius circle in trapping and random sites in different study areas.

		Number of anthropogenic food sources within a 200 m radius study circle				
		Site 1	Site 2	Sit 3	Site 4	Site 5
Urban habitat	Budapest	5	30	6	2	20
	Debrecen	0	2	0	5	9
Rural habitat	Balmazújváros	0	0	0	0	0
	Szakoly	0	0	0	0	0

The mean annual temperature between 2011 and 2021 differed significantly between the study areas (Figure 6, one-way ANOVA, $F_{3,40} = 3.049$, $p = 0.040$), mainly because Budapest, the largest city in Hungary, had a significantly higher mean annual temperature than Szakoly, the smallest of the studied settlements (contrast: 0.83 ± 0.30, Tukey-adjusted $p = 0.042$). When the two urban and two rural areas were pooled, the difference was also significant, with urban areas being significantly warmer than rural areas (Welch two-sample t-test, $t_{40.489} = 2.751$, $p = 0.009$).

Potential anthropogenic food sources were most abundant in 200-m-radius circular buffers located around trapping locations in Budapest, followed by Debrecen, whereas they were completely missing from the rural areas in Balmazújváros and Szakoly. The mean number of such food sources was 12.6 per buffer in Budapest, 3.6 in Debrecen, and 0 in both rural areas.

3.2. Occurrence of AMR in Hooded Crows in Hungary

3.2.1. Occurrence ESBL Producing Enterobacterales in Hooded Crows, Susceptibility test results and PCR

Overall, 51% of the sampled Hooded Crows carried ESBL-producing Enterobacterales (135/264). Four samples harbouring ESBLs were recovered from rural birds (7.7% of all 52 rural captures; exclusively from Balmazújváros as no ESBL isolates were found in samples collected from Szakoly) and 130 samples from urban ones (61% of all 212 urban samples; with 108/171 and 20/41 isolates from Debrecen and Budapest, respectively). Overall, 221 ESBL-producing strains were isolated, 197 and 24 of which were *E. coli* and other Enterobacterales,

respectively (89% vs. 10%). The *bla*$_{CTX\text{-}M\text{-}1}$ group genes were predominant in the Hooded Crow samples (218/222), followed by *bla*$_{SHV}$ and *bla*$_{CTX\text{-}M\text{-}9}$, found in two samples each. A number of isolated ESBL strains showed resistance to ceftazidime (72%, 160/221, with 156/218 urban vs. rural 4/4 and 142/197 *E. coli* vs. 18/24 Enterobacteriales), polymyxin B (29%, 65/221), and fluoroquinolones (9%, 20/221). Additionally, fewer isolates showed resistance to aminoglycosides (1-7%). While only 1.4% were resistant to ertapenem (3 exclusively urban isolates), was found.

3.2.2. Characteristics of ESBL producing *E. coli* in Hooded Crow

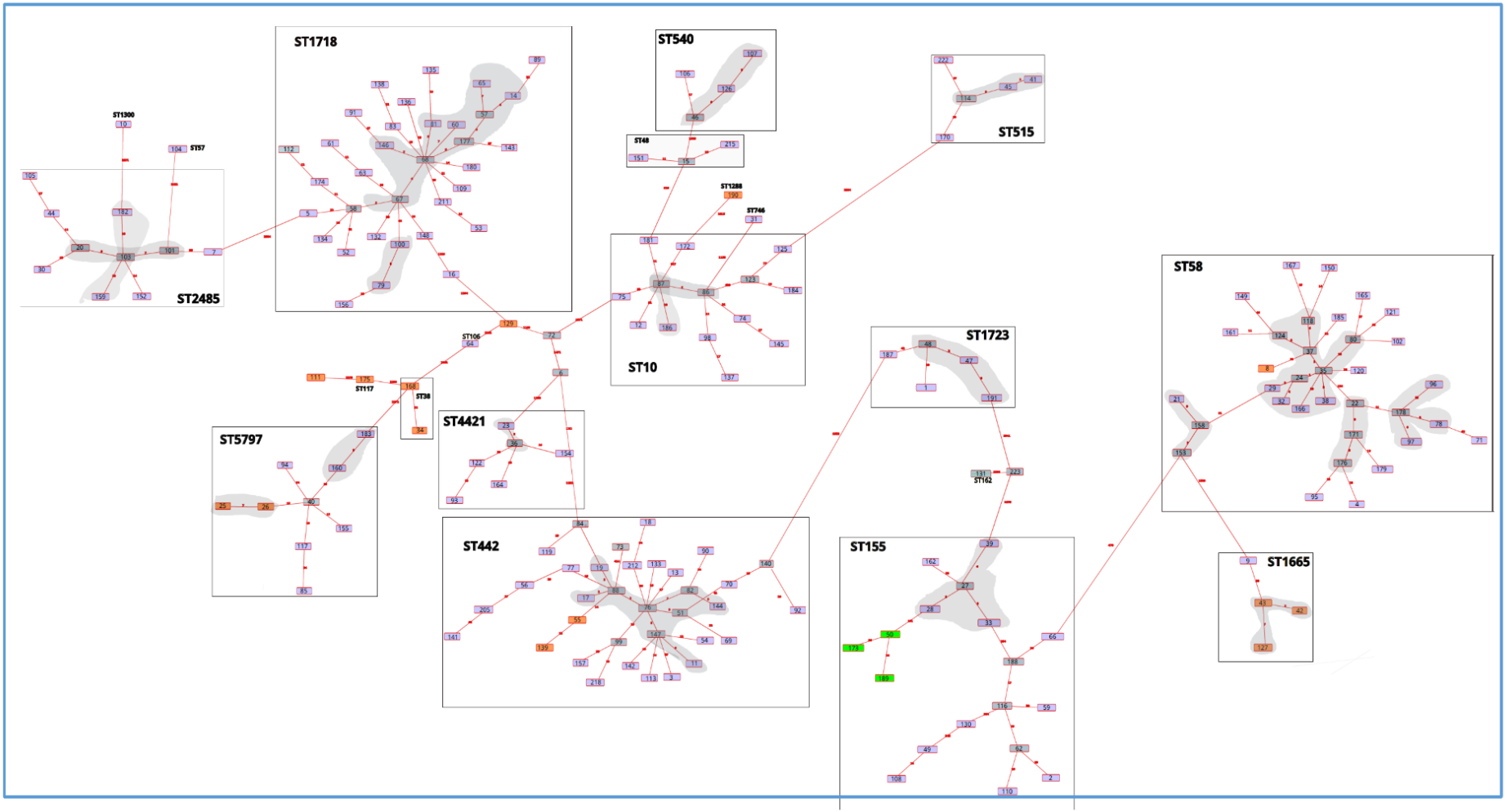
In this study, the 197 sequenced ESBL-producing *E. coli* belong to 22 sequence types (ST) (Table 3). The most prevalent ST was ST58, found in 34 isolates. Most of the STs found have been previously described in human and animal isolates, including ST10, ST155, and ST442, previously associated with human and animal diseases (MELLMANN et al., 2009; ZHUGE et al., 2019; EWERS et al., 2021), ST58, and ST162, also found in wild species, as well as ST38, ST48, ST746, and ST1723 (reported by WANG et al., 2017). However, some STs described in our crow samples, such as ST1718, ST4412, and ST5798, have yet to be reported in humans and/or animals. The pandemic ST131 *E. coli* clonal lineage was not detected in Hooded Crow isolates. For comparison, over 170 various sequence types (ST) have been described in wildlife around the world (WANG et al., 2017). While core-genome (cg) MLST revealed that these ESBL isolates belong to 33 distinct cgSTs, some STs were represented by multiple cgMLST types, showing a marked diversity. Isolates collected from different habitats (urban vs. rural) were always found to be genetically different. In contrast, within a city, some strains were found in high abundance, without any pattern between capture sites. Surprisingly, close genetic connections were observed between isolates belonging to the same STs from different urban areas (Budapest vs. Debrecen) (Table 3, Figure 7). Multiple cgMLST types were found in both cities (ST58 cgMLST46030; ST442 cgMLST28154; ST1665 cgMLST 210846; ST1718 csMLST143413; ST2485 cgMLST161907). This may point to the occasional transmission of strains between the two populations, in which case this may be due to vagrant juveniles, as adults are highly territorial and are highly unlikely to leave their nesting area; or it may indicate that these *E. coli* strains are ubiquitous in Hungarian crows. All of the more common STs appeared in juveniles, but there were differences in their distribution in adults; ST10, for example, did not appear at all in adults, or ST58 was found in 1 adult and 33 juvenile birds; in contrast, ST155 was found in 7 and 10 adults and juveniles, respectively.

Table 3

Distribution of the most frequent STs and cgSTs amongst ESBL E. coli isolated from free-ranging Hooded Crows based on crow age groups (Juveniles and adults) and study areas (urban areas (Budapest and Debrecen)

ST	cgST	Adult (%)	Juvenile (%)	Budapest (%)	Debrecen (%)
10		0 (0,0%)	12 (7,1%)	0 (0,0%)	12 (6,6%)
	196882	0 (0,0%)	9 (5,4%)	0 (0,0%)	9 (5,0%)
58		1 (3,4%)	33 (19,6%)	1 (8,3%)	33 (18,2%)
	45951	0 (0,0%)	11 (6,5%)	0 (0,0%)	11 (6,1%)
	46030	1 (3,4%)	22 (13,1%)	1 (8,3%)	22 (12,2%)
155		7 (24,1%)	10 (6,0%)	0 (0,0%)	17 (9,4%)
	136648	1 (3,4%)	6 (3,6%)	0 (0,0%)	7 (3,9%)
442		5 (17,2%)	28 (16,7%)	2 (16,7%)	31 (17,1%)
	28154	3 (10,3%)	27 (16,1%)	2 (16,7%)	33 (18,2%)
1718		5 (17,2%)	27 (16,1%)	1 (8,3%)	31 (17,1%)
	143413	1 (3,4%)	33 (19,6%)	1 (8,3%)	33 (18,2%)
2485		0 (0,0%)	10 (6,0%)	1 (8,3%)	9 (5,0%)
	161907	0 (0,0%)	10 (6,0%)	1 (8,3%)	9 (5,0%)
4421		0 (0,0%)	7 (4,2%)	0 (0,0%)	7 (3,9%)
	44522	0 (0,0%)	7 (4,2%)	0 (0,0%)	7 (3,9%)
5797		0 (0,0%)	9 (5,4%)	0 (0,0%)	9 (5,0%)
	4894	0 (0,0%)	9 (5,4%)	0 (0,0%)	9 (5,0%)
Others		11 (37,9%)	58 (34,5%)	8 (66,7%)	57 (31,5%)
all STs		29 (100,0%)	168 (100,0%)	12 (100,0%)	181 (100,0%)

Figure 7. **Minimum spanning tree based on core-genome multilocus sequence typing allelic profiles of 197 sequenced ESBL-producing *E. coli* isolates. Each square represent an allelic profile based on sequence analyses of 2513 cgMLST target genes. The number on the connecting lines illustrate the number of target genes with different alleles in a pairwise comparison. The squares are named with isolate ID numbers and coloured according to their sampling area (grey = Debrecen, orange: Budapest, and green= Balmazújváros). Closely related genotypes are shaded (<10 alleles differences).**



Most ESBL isolates belonged to commensal phylogenetic groups (Table 4). With B1 group being dominant (112/197, 56.7%; Budapest: 6/14; Debrecen: 102/179; Balmazújváros: 4/4), seconded by phylogenetic group A (55/197, 27.9%_exclusively urban isolates (Budapest: 2/14; Debrecen: 53/179)). Fewer strains belonged to phylogenetic group E (12/197, 6.0%, exclusively urban isolates from Debrecen (12/179)), phylogenetic group F (9/197, 4.6% exclusive urban isolates (Budapest: 2/14; Debrecen: 7/179)), and phylogenetic group D (3/197, 1.5% exclusive urban isolates (Budapest: 2/14; Debrecen: 1/179)). Only one isolate was found to belong to the phylogenetic group B2 from Debrecen (1/179), and another belonged to the phylogenetic group G from Budapest (1/14). Four strains were not affiliated with any phylogenetic group (4/197, 2.0% exclusively urban isolates (Debrecen: 3/179; Budapest: 1/14)). Our finding showed that sampled Hooded Crows carry commensal ESBL-producing *E. coli* that most likely act as reservoirs for ESBL genes, which is alarming and challenging for healthcare. Previously found in several hosts (SANTOS et al., 2014; RHADOUANI et al., 2014), the prevalence of these commensal ESBL producers could contribute to the spread of ESBLs and possible related gene transmission to other pathogens, making them a good indicator for the possible selective pressure behind such occurrence, thus providing insights on AMR emergence and dissemination. Additionally, since most of these strains belonged mostly to the B1 and A phylogenetic groups, we can say that Hooded Crows host ESBL strains of primary importance.

Table 4

Different MLST (STs) and core-genome MLST (cgMLST) profiles, phylogenetic groups and associated serotypes among isolated ESBL-producing E. coli isolated from Hooded Crows, determined using WGS reads.

STs	**cgMLST**	**Phylogenetic groups**	**Associated serotype**	**Number of isolates**
10	196882	A-B1	ONT:H32	8
	177042	A		3
	85754	B1		1
38	52633	D	ONT:H12	2
57	4209	E	ONT:H26	1
58	46030	B1-A-E	O8:H25	23
	45951	B1-A	O8:H10	11
106	26761	A	O15:H1	1

117	85789	G	O114:H4	1
155	136648	A-B1-E	O109:H51	7
			ONT:H51	
	16956	B1	O116:H9	4
	81555	B1	O9:H10	3
	27078	B1-A	ONT:H21	2
	9117	B1	O9:H51	1
162	171743	B1	O8:H19	1
442	28145	B1-A-F	ONT:H21	27
		B1-A	O91:H21	5
		B1	O9:H21	1
515	198763	B1	O98:H2	5
			ONT:H2	
540	96445	A-B1	O9:H30	4
746	130005	A	ONT:H19	1
1288	184707	A	O9:H9	1
1300	203111	E	-	1
1665	210846	B1	O162/O101:H7	4
1718	143413	A-B1-D-E	O9:H31	33
1723	56789	B1	O141:H16	5
			ONT:H16	
2485	161907	A	ONT:H45	1
		E-B1	O4:H45	8
		E	ONT:H47	1
4421	44522	B1-F	ONT:H7	5
5797	4894	F-B1-A	ONT:H42	9
UnKnown	28154	A – B1	ONT:H21	2
	44522	B1	O91:H7	1
	76074	B1	ONT:H21	1
	143413	B1	O9:H21	1
	169654	B1	-:H9	1
	196882	A	O69:H32	1
	100607	A	O8:H6	1
	-	-	-	2

3.2.3. Co-resistance in ESBL-*E. coli* in Hooded Crows

A total of 197 ESBL-producing *E. coli* isolates were subjected to WGS, in which *bla*$_{CTX\text{-}M\text{-}1}$ (151/197) was largely the most dominant ESBL gene, followed by *bla*$_{CTX\text{-}M\text{-}55}$ (20/197) and *bla*$_{CTX\text{-}M\text{-}15}$ (10/197) and, to a lesser extent, *bla*$_{CTX\text{-}M\text{-}32}$ (3/197), while *bla*$_{CTX\text{-}M\text{-}14}$ was found in a single isolate. CTX-M Groups 2, 8, and 9 were not detected. Some isolates showed co-resistance, mostly to aminoglycosides, fluoroquinolones, and trimethoprim–sulfamethoxazole, as some of the genetic elements and plasmids, which tend to carry CTX-M group genes, also harbour other resistance genes, including other beta lactamases encoding genes (e.g., *bla*$_{TEM}$), particularly CTX-M-1 and CTX-M-55 groups (Table 5).

To our knowledge, this is the first study to report the carriage of ESBL-producing *E. coli* in wild Hooded Crows and to report the WGS analysis of such strains. Investigating the prevalence of AMR in wild birds like the Hooded Crow is crucial, as such carriers may be reservoirs of MDR bacteria that can be of both human and animal importance. Our findings are in accordance with previous reports of such ESBL types in wildlife, particularly wild birds (ZURFLUH et al., 2019). Hooded Crow isolates showed interesting co-resistance rates to non-beta-lactam antibiotics. For instance, in contrast to rook isolates (Nagy et al., 2021), Hooded Crow-derived isolates carrying *bla*$_{CTX\text{-}M\text{-}15}$ showed co-resistance to aminoglycosides, fluoroquinolones, and trimethoprim–sulfamethoxazole; this is not surprising as such strains tend to carry the CTX-M-1 group, particularly *bla*$_{CTX\text{-}M\text{-}15}$. Similarly, isolates carrying *bla*$_{CTX\text{-}M\text{-}55}$ displayed higher levels of resistance to non-beta-lactam antibiotics. All this genetic material might help the *bla*$_{CTX\text{-}M}$ genes face co-selection pressures. In contrast, carbapenem resistance was not detected in the recovered isolates.

Interestingly, the prevalence of *bla*$_{CTX\text{-}M\text{-}55}$ in our Hooded Crows is noteworthy, considering its infrequent presence in Europe. Nonetheless, recently, *bla*$_{CTX\text{-}M\text{-}55}$ was reported in wintering rooks in Hungary (Nagy et al., 2021), which might explain *bla*$_{CTX\text{-}M\text{-}55}$ carriage in our crows. Hooded Crows in Hungary are sedentary in nature, found all year long in their corresponding habitats, with successful urban populations in constant contact with different anthropogenic sources. We speculate that these birds acquire strains carrying ESBL genes prevalent in humans and animals from their surroundings, including visiting rooks.

Table 5

Whole genome sequencing results showing different antibiotic resistance genes harboured by ESBL *E. coli* isolated from Hooded Crows.

ESBL genes	Other resistance genes	Number of isolates
bla$_{CTX-M-1}$	-	122
	APH(3'')-Ib;APH(6)-Id; QnrS1; sul2	1
	ANT(3'')-IIa;APH(3'')-Ib;APH(6)-Id; dfrA1; sul1;sul2;	1
	aadA2; dfrA12; sul1; tet(A)	8
	aadA2; bla-CTX-M-1; tet(A); dfrA12; sul1	1
	APH(3'')-Ib;APH(6)-Id; QnrS1; sul2	1
bla$_{CTX-M-1}$/*bla*$_{TEM-1}$	-	24
bla$_{CTX-M-55}$	*AAC(3)-Iid; ANT(3'')-Iia; APH(3'')-Ib; APH(6)-Id; tet(A); QnrS1; sul2; floR*	1
	APH(3'')-Ib; APH(6)-Id; QnrS1	8
	QnrB5	1
	AAC(3)-IId; QnrS1	2
bla$_{CTX-M-55}$/*bla*$_{TEM-150}$	*AAC(3)-Iid; ANT(3'')-Iia; APH(3'')-Ib; APH(6)-Id; tet(A); QnrS1; sul2; floR*	6
	AAC(3)-Iid; APH(3'')-Ib; ANT(3'')-Iia; APH(6)-Id; tet(A); sul2	5
bla$_{CTX-M-15}$	*tet (A), QnrS1,*	3
	APH tet (B)	1
bla$_{CTX-M-15}$/*bla*$_{TEM-1}$	*APH, tet (A), QnrS1, dfrA14, sul2*	2
	-	3
bla$_{CTX-M-32}$	*APH(3'')-Ib; APH(6)-Id; tet(A); QnrS1; Sul2*	1
	APH(3'')-Ib;APH(6)-Id; sul2	1
	APH(3'')-Ib;APH(6)-Id; QnrS1	1
bla$_{CTX-M-14}$	*tet (B)*	1

Although our work focused mainly on ESBLs, the additional resistance patterns to different antibiotic classes of critical importance found in our strains are comprehensive. Such results provide the genotypic characterization of ESBL producers carried by Hooded Crows in Hungary. Our results revealed that Hooded Crows harbour MDR *E. coli*, making them potential reservoirs of critically important ARB.

3.2.4. Plasmid prediction

In the isolated ESBL *E. coli* strains, fourteen plasmid groups were represented in the prediction: IncI1-I (Alpha) (141 isolates, 71.6%), IncFII (97 isolates, 49%), IncFIB (94 isolates, 47.7%), IncFIA (88 isolates, 44.7%), IncFIC (II) (43 isolates, 21.8%), IncQ (24 isolates, 12.2%), IncX1 (19 isolates, 9.6%), IncI2 (16 isolates, 8.1%), IncN (14 isolates. 7.1%), IncY (11 isolates, 5.6%), ColpVC (25 isolate, 12.7%), p0111 (16 isolates, 8.1%), col156 (14 isolates, 7.1%) and Col (pHAD28) (one isolate, 0.5%). However, we did not identify any possible associated plasmids in seven of our isolates (3.6%), which indicates that the resistance genes are carried on the chromosome.

Table 6

List of Plasmid predicted by Plasmidfinder 2.1 determined by using WGS reads and its association to resistance gene identified in isolated ESBL producing *E. coli*.

ESBL genes	Other resistance genes	Associated plasmids	Number of isolates
CTX-M-1		col156 / IncY	1
		IncFIB / INCI1-I	5
		IncFIC / INC1I-I	26
		IncFIA / FIC / INC1I-I	8
		IncFIA /FIB / FIC / INC1I-I	24
		IncFIB / INCI1-I / IncX1	6
		IncFIB / IncFIC/ IncI1-I	9
	APH(3")-Ib;APH(6)-Id; QnrS1; sul2	IncFIB / IncFIC/ IncI1-I	1
		IncFIB / IncFIC/ IncI2	1
		colVC / IncFIA / FIB / FIC / IncI1-I	1
		IncFIB / IncFIC / IncI1-I / p0111	3

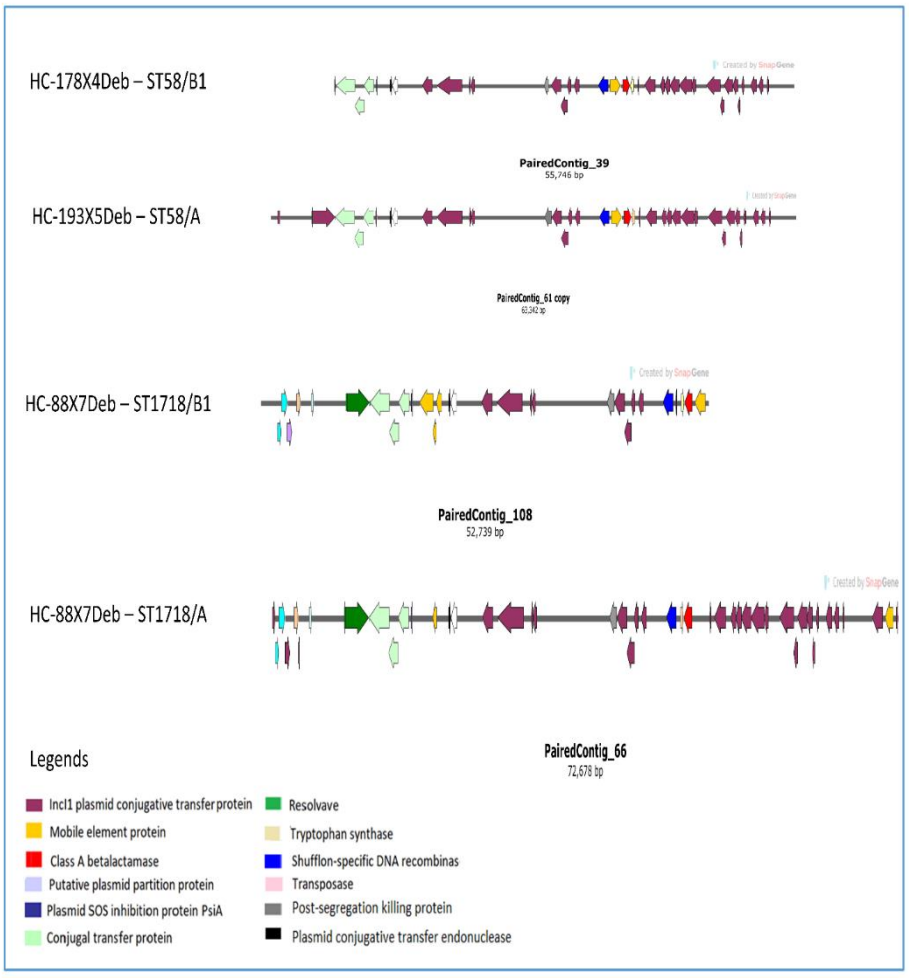
	ANT(3")-IIa;APH(3")-Ib;APH(6)-Id; dfrA1; sul1;sul2;	IncFIB / IncFIC / IncI1-I / p0111	1
	aadA2; dfrA12; sul1; tet(A)	IncFIB / IncFIC / IncI1-I / p0111	8
	aadA2; tet(A); dfrA12; sul1	IncFIB / IncFIC / IncI1-I / p0111	1
		col15 / Inc FIB / IncFIC / IncI1-I	2
		IncB / IncN	1
		col156 / IncFIB / IncFIC / incI1-I / IncN	1
		col156 / Inc FIC / IncI-1I	1
		IncFIB / IncFIC/ IncI1-I / IncX1	2
		col156 / IncI1-I / IncY	4
		IncFIA / IncFIB / IncI1-I	1
		IncFIA / IncI2	2
		IncFIA/ FIB/ FIC	7
		IncFIC	1
		IncFIA/ FIB/ FIC / IncI1-I / IncI2	2
		IncFIA/ FIB/ IncI1-I / IncI2	7
		IncFIB	1
		IncFIB/ FIC / IncI1-I / IncI2	1
	APH(3")-Ib;APH(6)-Id; QnrS1; sul2	IncFIB/ FIC / IncN / IncQ1	1
		col156 / IncFIB / IncFIC / incI1-I	1
		IncFIB / IncFIC/ P0111	1
		-	2
		col / IncFIB/ IncFIC / IncI1-I / p0111	1
CTX-M-1 / TEM-1		IncFIB / IncFIC/ INCI1-I / IncQ1	17
		col156 / IncN / IncQ1 / IncX1	1
		IncFIB / IncI1-I / IncQ1	4
		Inc FIB / IncFIC / IncQ1	2

CTX-M-55	AAC(3)-Iid; ANT(3")-Iia; APH(3")-Ib; APH(6)-Id; tet(A); QnrS1; sul2; floR	IncFIA FIB	1
	APH(3")-Ib; APH(6)-Id; QnrS1	IncN	4
	APH(3")-Ib; APH(6)-Id; QnrS1	-	1
	APH(3")-Ib; APH(6)-Id; QnrS1	IncFIC (IncFII)/ IncI2 / IncN	2
	APH(3")-Ib; APH(6)-Id; QnrS1	IncI2 / IncN	1
	QnrB5	IncFIB/C	1
	AAC(3)-IId; QnrS1	-	1
	AAC(3)-IId; QnrS1	IncN	1
CTX-M-55 / TEM-150	AAC(3)-Iid; ANT(3")-Iia; APH(3")-Ib; APH(6)-Id; tet(A); QnrS1; sul2; floR	IncFIA / FIB/ FIC / IncX1	4
	AAC(3)-Iid; ANT(3")-Iia; APH(3")-Ib; APH(6)-Id; tet(A); QnrS1; sul2; floR	IncFIA / FIB/ IncX1	2
	AAC(3)-Iid; APH(3")-Ib; ANT(3")-Iia; APH(6)-Id; tet(A); sul2	IncFIA / IncX1	1
	AAC(3)-Iid; APH(3")-Ib; ANT(3")-Iia; APH(6)-Id; tet(A); sul2	IncFIA/ FIB/ FIC / IncX1	2
	AAC(3)-Iid; APH(3")-Ib; ANT(3")-Iia; APH(6)-Id; tet(A); sul2	IncFIA/ FIC / IncX1	2
CTX-M-15	tet (A), QnrS1,	-	3
	APH tet (B)	IncFIB / IncY	1
CTX-M-15 / TEM-1	APH, tet (A), QnrS1, dfrA14, sul2	IncY	2
		col156 / IncY	3
CTX-M-32	APH(3")-Ib; APH(6)-Id; tet(A); QnrS1; Sul2	IncF / IncN	1
	APH(3")-Ib;APH(6)-Id; sul2	IncN	1
	APH(3")-Ib;APH(6)-Id; QnrS1	IncN	1
CTX-M-14	tet (B)	IncF FIA/ FIB / FIC	1

The presence of *IncI1* in association with other resistance genes warns of the possible dissemination of AMR genes, which leads to higher risks to human and animal health. Additionally, our findings revealed that bla_{CTX-M}-containing contigs share a high degree of similarity among the strains with the same STs (Figure 8). Plasmid prediction revealed that plasmids such as Incl1, IncF, and col156 were located near or in the same conting as the bla_{CTX-}

M genes in most of the strains, suggesting the possible transfer of these ESBL genes via plasmids.

Figure 8. **Linear view of the genetic environment of *bla*$_{CTX\text{-}M\text{-}}$ in 4 *E. coli* isolates (each pair share the same ST). GenBank accession number of the contig containing CTX-M- were given below each scheme. ORFs were illustrated with arrows. The colour of arrows indicates the function of the gene. The annotation of the whole gene was done using RAST 2.0 online platform and visualized using SnapGene 6.2 software (https://www.snapgene.com/).**



From a One Health perspective, the presence of these genes must be monitored, especially in bacteria with zoonotic potential. Here, we found that ESBL *E. coli*, harbouring AMR genes, isolated from free-ranging Hooded Crows living mostly in close contact with anthropogenic sources, could have a significant role in the dissemination of AMR genes between bacteria, especially considering the prevalence of STs in large animal and human occurrences. Additionally, the potential presence and coexistence of mobile plasmids

(PATERSON et al., 2006; BUSH et al., 2020) might explain the co-resistance observed in these ESBL isolates, highlighting the possible dissemination of these ARB and AMR genes into the wild.

3.2.5. Virulence characteristics

Based on the WGS results, *E. coli* isolated from Hooded Crows had a high prevalence of several virulence genes (VFs), including *hlyE, hlyF, iss, iroN, gad, cib, cvaC, ompT, mch B,C,F, IpfA, iucC, iutA,* and *afaD*. Amongst them, VFs prevalence ranged from 0% (*PAI, vat, stx1, stx2, ipaH*) to 97% (191/197) (*terC*, an iron transport protein), followed by *hlyE*, a pore-forming haemolysin (also known as Cytolysin A (*CytA*) or silent haemolysin), found in 93.4% (184/197).

Previous studies have shown that avian pathogenic *E. coli* (APEC) and extra-intestinal pathogenic *E. coli* (ExPEC) strains are phylogenetically close and share some VFs, and that commensal *E. coli* may acquire additional virulence, suggesting the potential role of commensal *E. coli* as a reservoir of virulence genes of APEC and ExPEC strains, which is a serious health risk to humans and animals (RODRIGUEZ-SIEK et al., 2005; BELANGER et al., 2011; MANGES, 2016). RODRIGUEZ-SIEK et al. (2005) suggested a possible plasmid exchange and thus virulence gene exchange between human and avian *E. coli* strains.

Out of the APEC-associated genes we found, iss, hylF, ompT, iroN, and iutA, and to a lesser extent, irp2, papC, Cva, and tsh genes. In addition, VFs such as adhesins, toxins (temperature-sensitive haemagglutinin (tsh)), iron acquisition mechanisms (iroN, feoB, chuA, fyuA, ireA, irp2, iucD, and sitD), invasins, increased serum survival genes (iss), protectins, and plasmids are included. Furthermore, the presence of plasmids like colicin V (ColV, also known as IncF), previously associated with these virulence genes, suggests the potential exchange of VFs between isolates (NEWMAN et al., 2021).

To the best of our knowledge, this is the first study to provide insight on ESBL-producing Enterobacterales prevalence in wild Hooded Crows and WGS analysis of such strains. The occurrence of CTX-M genes, along with others encoding resistance to five other antimicrobial classes (fluoroquinolones, trimethoprims, sulphonamides, aminoglycosides, and macrolides), highlights the potential role of these free-ranging birds in the spread of MDR bacteria and emphasises the need for more surveillance studies. Over the last decades, ESBL-producing *E. coli* strains have been spreading rapidly in humans, animals, and the environment, making them an important risk factor threatening public health (RAMOS et al., 2020). Our

findings provide a first look into the carriage of a highly urbanised wild bird and subsequently some insights into an understudied part of the eco-system that deserves more recognition in AMR investigations.

3.2.6. Absence of Vancomycin resistant Enterococci

None of the collected samples demonstrated bacterial growth on the VRE screening media, indicating that none of the sampled Hooded Crows in any group carried VRE strains. Enterococci were detected on nonselective media that were not vancomycin-resistant, demonstrating that these crows carry Enterococci in general but not VRE strains.

Previous findings of VRE among birds of the genus *Corvus* (ORAVCOVA et al., 2016) indicate that carriage of VRE is indeed possible and that crows can be used for monitoring the emergence of VRE, particularly in urban settlements. Wild birds in contact with anthropogenic factors, for example, crows and gulls, are potential carriers of clinically important VRE (ORAVCOVA et al., 2016). Moreover, although a low prevalence of VRE was previously reported among hospital settings in Debrecen (DOMBRÁDI et al., 2012), more recent reports showed an increase in VRE prevalence in Hungary (MELEGH et al., 2018). Thus, with (1) the widespread distribution of the Hooded Crow in North and Eastern European countries, including Hungary; (2) its constant association with human-made environments; (3) the multi-resistance observed in hospital isolated strains; and (4) the prevalence of VRE in wintering rooks in different European cities (ORAVCOVA et al., 2016), a potential environmental spread of VRE should be foreseeable, warranting monitoring of these urban bird populations.

4. NEW SCIENTIFIC RESULTS

1. Urban Hooded Crows have generally smaller body size than rural ones (Tarsus length (cm): 47.7 ± 4.51 vs. 49.8 ± 2.62, p= 0.015; 'Lengthiness': p= 0.015).

2. Urbanisation influences the morphology of Hooded Crow especially post-fledging individuals (Tarsus length (cm): 46.6 ± 4.17 vs. 52.5 ± 1.73, p=0.038, 'Lengthiness': p= 0.03; Skull width (cm): 34.4 ± 1.59 vs. 35.8 ± 0.76, p=0.033, 'Bill size': p=0.0008).

3. Adult Hooded Crows in urban habitats are very successful in maintaining their body condition with highly anthropogenic sources (Body condition (cm): 17.47 ± 39.74, -3.5 ± 48.84 of urban and rural crows respectively, p=0.934). Urban adult crows show morphological adaptations to live in urban environments, especially during their adulthood (Body mass (gr): 426.7 ± 47.47, 417.2 ± 50.13 of urban and rural crows respectively, p=0.351).

4.-Post-fledging Hooded Crows in urban areas of different scale expressed the likelihood of rapid local responses to spatially varying factors (environmental and non-environmental). Juvenile crows from Debrecen displayed weaker body condition (9.35 ± 2.8 vs. 17.3 ± 44.4, p=0.041), smaller bill form (Bill length (cm): 50.8 ± 3.4 vs. 53.09 ± 2.36, p=0.0005; Bill width (cm): 18 ± 1.46 vs. 19.3 ± 1.26, p=0.0005, 'Bill size': p= 0.0005) than crows from Budapest, with significant monthly variations observed in several traits, and a significant interaction between 'month' and 'city' for body mass (gr) (44.3 ± 16.20, F = 7.472, p = 0.007), body condition (cm) (32.8 ± 13.58, F = 5.838, p = 0.017), and bill width (cm) (0.98 ± 0.385, F = 6.453, p = 0.012).

5. Post-fledging Hooded Crows captured at the Zoo in Debrecen were generally heavier, lengthier, and had better body condition and wider skull and bill forms than those captured elsewhere in the city, especially than young crows captured at the SPC (Body mass (gr): 418.7 ±55.13 vs. 366.3 ± 51.68, p= 0.001; Body condition: 11.9 ± 53.44 vs. -26.1 ± 46.11, p= 0.005; 'Lengthiness': p= 0.006; Skull width (cm): 34.9 ± 2.08 vs. 34.0 ± 1.26, p= 0.021; Bill width b(cm): 18.5 ± 1.26 vs. 17.6 ± 1.44, p=0.085).

6. A higher frequency of ARB carriers was found in the urban population of the Hooded Crow (61% of urban crows harboured ARB vs. 7.7% of rural ones, p<0.0001).

7. Most *E. coli* isolates belonged to commensal phylogenetic groups, primarily phylogenetic group B1 (112/197, 56.7%; Budapest: 6/14; Debrecen: 102/179; Balmazújváros: 4/4), and A

(55/197, 27.9%_exclusively urban isolates (Budapest: 2/14; Debrecen: 53/179)), making Hooded Crows possible reservoirs of ESBL-producers and ESBL-encoding genetic elements.

8. A high predominance of the $bla_{\text{CTX-M-1}}$ ESBL-encoding gene was found in sedentary Hooded Crows, followed by $bla_{\text{CTX-M-55}}$ and $bla_{\text{CTX-M-15}}$ respectively.

9. Prevalence of MDR-ESBL-producing *E. coli* showing resistance to aminoglycosides (65/197 isolates, 33%), fluoroquinolones (33/197, 16.7%), trimethoprim–sulfamethoxazole (35/197, 17.8%), Sulphonamides (52/197, 26.4%), and tetracycline (31/197, 15.7%), harbouring both APEC and ExPEC virulence genes (including *hlyE, hlyF, iss, iroN, gad, cib, cvaC, ompT, mch B,C,F, IpfA, iucC, iutA,* and *afaD*), as well as a range of plasmid replicons (including, IncI1-Iα (141 isolates, 71.6%), IncFII (97 isolates, 49%), IncFIB (94 isolates, 47.7%), IncFIA (88 isolates, 44.7%), IncFIC (II) (43 isolates, 21.8%), IncQ (24 isolates, 12.2%), IncX1 (19 isolates, 9.6%), IncI2 (16 isolates, 8.1%), IncN (14 isolates. 7.1%), IncY (11 isolates, 5.6%), ColpVC (25 isolate, 12.7%), p0111 (16 isolates, 8.1%), col156 (14 isolates, 7.1%), and Col (pHAD28) (one isolate, 0.5%), highlights the potential role of Hooded Crows in the dissemination of ESBL producers into the environment.

10. Molecular characteristics of ESBL-producing *E. coli* carried by Hooded Crows almost exclusively from urban areas reinforce future investigations into AMR and epidemiological traits in GNB. Hooded Crows harboured ESBL-producing *E. coli* belonging to 22 different sequence types (STs), among which globally disseminated STs were found including ST58, ST10, ST155, and ST38. While some STs described in *E. coli* isolates have yet to be found in human and/or animal isolates (ST1718, ST4412, and ST5798).

5. PRACTICAL RESULTS

This study is the first to subject the Hooded Crow to such ecological and microbiological scrutiny. Providing thus preliminary data, for future studies.

1. Using the ladder trap for trapping is an effective method to capture free-ranging wild birds such as Hooded Crows, especially in urban areas.

2. Anthropogenic food sources in urban areas can be a target factor for efficient wildlife management in urban settlements.

3. The consequences of living in urban settings observed provide a background for future experimental studies to investigate the mechanisms of different factors, which will help understand Hooded Crows' responses to urbanisation.

4. The necessity of multiple spatial scales and different age-category-based studies in order to understand wildlife adaptations to urban environments has been established. The presence of local environmental factors that may attenuate or exacerbate the large-scale impact of the urban-rural gradient on the morphology of Hooded Crows.

5. The occurrence of ESBLs supports the perception that urbanisation increases the exposure of Hooded Crow to AMR microorganisms, which makes it an ideal interface to study and monitor the occurrence of AMR.

6. Hooded Crows from urban areas are currently important carriers of ESBL-producing Enterobacterales of human importance. These findings add to the knowledge on the dissemination of these high-priority microorganisms and highlight the potential role of the Hooded Crow in the spread of AMR, consequently contributing to the current AMR surveillance results as well as to responsive approaches to the increasing spread of these organisms.

7. The high prevalence of ESBL producers in urban Hooded Crows reflects the emergence of such bacteria in urban areas in Hungary, supporting future investigations on AMR trends and links between its emergence and anthropogenic sources.

8. The determination of the full spectrum of antibiotic resistance genes and virulence determinants acquired by *E. coli*, collected from a synanthropic bird, might reinforce future investigations of MDR patterns and epidemiological traits of ESBL producers.

9. Conducting a complex, multidisciplinary study helps to get a more holistic understanding of the effect of urbanisation on wildlife in line with the One Health principle. Similarly, understanding the urban adaptation of wild birds such as the Hooded Crow can be useful for managing AMR prevalence and may lend insights into the dynamics of other pathogens.

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List of publications related to the dissertation

Hungarian scientific articles in Hungarian journals (1)

1. **Benmazouz, I.**, Kövér, L., Kardos, G.: Az antimikrobiális rezisztencia terjedése a vadon élő madarak körében: A vadon élő madarak mint AMR-rezervoárok és -terjesztők Irodalmi áttekintés = The rise of Antimicrobial resistance in Wild birds: Potential AMR sources and Wild birds as AMR reservoirs and disseminators : Literature review.
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In: 2. Urbanizációs Ökológia Konferencia : Absztrakfüzet, [s.n.], Győr, 28, 2021.

Total IF of journals (all publications): 24,158
Total IF of journals (publications related to the dissertation): 9,431

The Candidate's publication data submitted to the iDEa Tudóstér have been validated by DEENK on the basis of the Journal Citation Report (Impact Factor) database.

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DEBRECENI EGYETEM * Egyetemi és Nemzeti Könyvtár *