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Investigating the ecology and microbiology of the urban and rural Hooded Crow (*Corvus cornix*) in Hungary

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INVESTIGATING THE ECOLOGY AND MICROBIOLOGY OF THE URBAN AND RURAL HOODED CROW (Corvus cornix) IN HUNGARY

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List of Abbreviations

AGR = Agrár Campus of the University of Debrecen

AmpC = Cyclic adenosine monophosphate

AMR = Antimicriobal resistance

AMB = Antimicrobial resistant bacteria

BEA = Bile esculin azide

bla = Beta lactamase

Bp = Base pair

C = Celcius

cgMLST = Core genome multilocus sequence type

DNA = Deoxyribonucleic acid

E. coli = *Escherichia coli*

ECDC = European Centre for Disease Prevention and Control

EEA = European Economic Area

E.M.A = European Medicines Agency

ESBL = Extanded spectrum beta lactamase

EU = European Union

EUCAST = European Committee on Antimicrobial Susceptibility Testing

ExPEC = Extraintestinal pathogenic Escherichia coli

GNB = Gram negative bacteria

GPB = Gram positive bacteria

km = Kilometre

m = Metre

MALDI TOF= Matrix-assisted laser desorption/ionization_Time Of Flight

MDR = Multi drug resistant

mg/l = Milligramm per litre

MGE = Mobile genetic elements

MIC = Minimum inhibitory concentration

Min = minute

MLST = Multilocus sequence type

MRSA = Methicillin-resistant *Staphylococcus aureus*

ng/µL = Nanogram per microlitre

OXA = Oxacillinases

PBP = Penicillin-binding protein

PBS = Phosphate-buffered saline

PCA = Principal component analysis

Qnr = quinolone resistance

SPC = Sport complex

ST = Sequence type

TBE = Tris/Borate/EDTA

tet = Tetracyclin

UN = United Nations

VRE = Vancomycin resistant enterococci

WHO = World Health Organisation

WNV = West Nile Virus

µL = Microlitre

1. INTRODUCTION

First human civilizations dating from thousands of years before Christ observed a significant growth in human populations leading to the rise of cities; however, by the 18th century, the rise of the industrial revolution led to the massive urbanisation we are observing today. The industrial revolution saw an upsurge in infrastructure and a growth in human migration to metropolitan areas. This growth continued throughout the 20th century and increased dramatically all over the world. Nowadays, more people live in urban than rural areas, and by 2050, 66% of the world population is estimated to be urban (UN, 2019). This rapid urbanisation poses challenges for sustainable development, as expanding cities corresponds to higher demands for land, not only for housing but also for agricultural allocation to sustain the continuously growing populations, resulting in the loss of natural landscapes. In addition to landscape modifications, humans also made use of natural water resources in urban settlements, such as the construction of dams and artificial canals and even reversing the flow of natural rivers like the Chicago River (BESEK, 2017). These different modifications have been influencing the environment in various ways, causing changes in a range of nature's ecologies, from climate change to decreasing species diversity, thus creating new environmental conditions and exposing wildlife to new man-made stress. This rapid urban expansion is a new selective force that modifies tremendously the composition of animal communities. Urbanisation generates complex and various systems defined by great levels of pollution, human disturbance, landscape alterations, and environmental changes (SHOCHAT et al., 2006; LOWRY et al., 2012). These changes can alter the biology, behaviour, morphology, reproductive, and survival traits of wildlife, causing the disappearance of some native animal species that cannot adapt to the fast changes, and in parallel, other species, like crows and pigeons, can move to urban settings or increase in numbers (BENMAZOUZ et al., 2021). Urban development can also cause a loss of biodiversity (SERESS & LIKER, 2015), 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 wildlife, and modifying ecosystem services (MARZLUFF et al., 2001; VUORISALO et al., 2003). Generally, the first response to human-made environmental changes in many wildlife species is behavioural adjustments made in efforts to adapt to the new ecosystem. For example, many animals were reported to have altered their breeding, nesting, and foraging

patterns, diet composition, as well as vigilant behaviour and vocalisation in response to human-made environments (LOWRY et al., 2012). This may either potentially improve or deteriorate the survival and/or reproductive rates of an organism in a changing world. Additionally, urbanisation is considered by many to be an overwhelming evolutionary force influencing the life-history traits and population genetics of species (LIKER, 2020). Presently, urbanisation is still growing at an acceleration rate (UN, 2019), unfortunately resulting in a continuous loss of habitat, calling for a better understanding of the current situation and to work on the most appropriate measures to implement for the best outcome. And though urbanisation's known for its negative impacts on wildlife, several authors have highlighted numerous advantages of the urban environment, including warmer winters, the availability of anthropogenic foodstuffs, and a lack of predation (HEISS et al., 2009; KRAUSMAN et al., 2014), of which several wildlife species have been reported to have benefited (SZALA et al., 2020). Additionally, the presence of wildlife in our cities creates more opportunities for direct or indirect interactions with humans, which may also affect humans; frequent contacts also result in an increased risk of zoonotic disease transmission.

Animals in urban environments represent the connection to nature for people; therefore, they may seek the company of such animals. Considering the latest records on wildlife-borne zoonotic diseases (KRUSE et al., 2004; HASSELL et al., 2016), it is believed that these animals may serve as reservoirs or vectors and may become the sources for such infections (BORGES et al., 2017). However, data on the microbiology of these interactions is scant. 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. Although not novel, the One Health approach has gained attention in recent years considering the increasing interactions observed between people, animals, plants, and the environment, thus calling for collaboration between public health, animal health, and environmental professionals. Subsequently, potential disease transmission between these interdependent settings is disconcerting, and it is crucial to include wildlife in the One Health complex network. According to the World Health Organisation (WHO, 2014), One Health issues include zoonotic disease, foodborne diseases, antimicrobial resistance (or AMR), and other threats shared between animals, humans, and the environment.

As one of the most important and urgent global health issues, resistance to antimicrobial treatments have been extensively reported in the last few decades. Reports of its emergence and spread among different wildlife species are sounding alarms for health officials. The origin of this phenomenon and the dissemination of Antimicrobial-resistant bacteria (ARB) in the environment necessitate deep investigations. Numerous factors are recognised as the probable reason for this emergence, mainly the level of interaction between wildlife and humans and human activities such as intensive livestock farming, wastewater treatment facilities, and landfills (LANTHIER et al., 2010; ALROY & ELLIS, 2011; ZURFLUH et al., 2016). Food and water seem to be major sources of transmission of AMR of human or veterinary importance to wildlife (BAQUERO et al., 2008; ATTERBY et al., 2017; AHLSTROM et al., 2018).

Considering the general use of the same classes of antimicrobials in both human and veterinary medicines, it is believed that the release of bacteria from anthropogenic sources into the environment and their potential contact with environmental bacteria exhibit ideal ecological and selective conditions for the survival of resistant bacterial strains of human and veterinary importance in the wild (DOLEJSKA et al., 2008; BAQUERO et al., 2008; SKURNIK et al., 2016; ATTERBY et al., 2017). Accordingly, in cooperation with the Food and Agriculture Organisation of the United Nations (FAO), the World Organisation for Animal Health (OIE), and the WHO, the latter developed a list of critically important antimicrobials (the "WHO CIA" list) to be used as a reference to help manage AMR. So based on AMR mechanisms, the availability of alternative treatments, and the possibility of transmission from non-human sources, antimicrobials used in human medicine are categorised as "critically important", "highly important", and "important". Additionally, the current WHO CIA list includes the highest priority critically important antibiotics like 3rd, 4th, and 5th generation cephalosporins, macrolides, polymexins, quinolones, and the high priority critically important ones like aminoglycosides and carbapenems (WHO, 2019). Hence, one of the problems with antimicrobial medications lies in the use of third-generation cephalosporins, broad-spectrum beta-lactam antibiotics that are widely used in humans and animals. Resistance to these drugs is mediated by extended-spectrum β-lactamases (ESBLs) and AmpC beta-lactamases (EXNER et al., 2017), and ESBL genes (as well as certain AmpC genes) are highly mobile and are transmitted on plasmids, transposons, and other genetic elements, increasing the chances of their spread in different ecosystems. For instance, resistance to

third-generation cephalosporins has been frequently reported amongst *Escherichia coli* (*E. coli*) (MAEYAMA et al., 2018; VOUNBA et al., 2019), a common inhabitant of the gastrointestinal tract of humans and animals, including birds, also broadly found in plants, soil, and water worldwide (TOUCHON et al., 2020). *E. coli* is an important faecal contamination indicator commonly used to survey the evolution of the AMR in humans and animals (DOLEJSKA & LITERAK, 2019). In addition, the clonal transmission of ESBL-producing *E. coli* and horizontal dissemination of genes responsible of such resistance in *E. coli* were reported in numerous human, animal, and environmental strains, causing a serious public health concern.

Consequently, we believe that the resistance to third-generation cephalosporins is a good example of a One Health approach when addressing AMR, considering their critical importance for both animal and human health. Additionally, the increased proliferation of ARB observed in the environment has opened the door for researchers to consider the role of the environment and wildlife as bio-indicators and sentinels (LARSSON et al., 2018; SWIFT et al., 2019).

Urban wildlife makes an ideal example to proceed with such investigations, as animals living in urban settlements would, at some point in their lives, interact with humans and be subjected to different anthropogenic pressures that could potentially expose them to different shared nuisances, including AMR microorganisms. One of the most acknowledged urban dwellers are wild passerines like corvid species. Known for their worldwide geographic distribution and adaptation to various habitats, corvid species are regularly 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. As such, these wild birds can be considered sentinels since they may easily acquire human and environmental bacteria (BONNEDAHL & JÄRHULT, 2014) and serve as reservoirs and vectors of ARB and resistance genes (DOLEJSKA & LITERAK, 2019).

2. LITERATURE REVIEW

2.1. Urbanisation from the ecological perspective

Urbanisation is a process involving the conversion of natural habitats into anthropogenic, unnatural ones. Strongly associated with the degradation, fragmentation, and loss of natural ecosystems, urbanisation leads to various, complex systems defined by high levels of human disturbances, landscape alterations, and environmental changes (SHOCHAT et al., 2006; LOWRY et al., 2012). Urbanisation affects nature in every way. Altering land cover, drainage, and nutrient cycles, as well as climate parameters such as temperature. These changes can affect the morphology, biology, behaviour, and reproductive traits, as well as the survival of wildlife, and can result in the disappearance of native species and the appearance of non-native ones. Accordingly, urban ecologists commonly assess urbanisation effects on ecological systems and their consequences by examining human activities in urban landscapes (SHOCHAT et al., 2006), which calls for the quantification of anthropogenic changes relevant to ecological processes within an area in order to adequately define habitat type within a study (THEOBALD, 2004). Therefore, researchers often subjectively categorise urbanised areas using terms like urban, suburban, exurban, and rural (THEOBALD, 2004), while the degree of urbanisation is usually quantified based on human population density and concrete-like surface coverage, which facilitate comparison studies among areas.

In ecology, an urban development is an ecological alteration that often modifies the services of a given ecosystem by affecting predators through their removal or increase, altering the structure of the food chain, encouraging human tolerance and adaptation, increasing health risks for humans and wildlife, and other ecological processes (MARZLUFF et al., 2001; VUORISALO et al., 2003). Therefore, understanding these effects is essential for successful wildlife conservation and management. The negative impact of human-made landscapes and infrastructure on wildlife was emphasised on many occasions. However, it was also described how certain species benefit from these novel environments (KÖVÉR et al., 2015, 2019; BENMAZOUZ et al., 2021). And although considered an important factor resulting in biodiversity loss (SERESS & LIKER, 2015), urbanisation is also considered a strong evolutionary force on population genetics and life-history traits of species (LIKER, 2020). Wildlife has been subject to different human-related stress and disturbances, and wild animals and plants are bound to

adapt to these everlasting changes or face local extinction. Some wildlife move to urban areas such as deer, raccoons, and birds (VUORISALO et al., 2003; MARZLUFF et al., 2001; KRAUSMAN et al., 2014). These animals, capable of surviving in manmade settlements, are called synanthropes. For wild animals, the benefits of urban environments lie in the presence of a warmer climate, the availability of anthropogenic food, less predation, and the constitution of animal protection regulations (HEISS et al., 2009; KRAUSMAN et al., 2014). Still, even though urbanisation provides some protection to wildlife, the number of stressors is thought to have outweighed the benefits.

One of the main characteristics of urbanisation is buildings, usually associated with high human activity, elevated noise, light disturbances and reduced vegetation, which might be avoided by susceptible species. However, ones that are more tolerant may gain benefits from it (MILLER et al., 2001). For example, overwintering arthropods might find a thermal shelter in adjacent buildings (SERESS & LIKER, 2015), while some bird species prefer roosting or nesting on houses. Indeed, due to the presence of impervious surfaces such as asphalt and concrete and the consequent decrease in tree cover in urban areas become warmer day and night throughout the year, a phenomenon known as the urban heat island effect (ZITER et al., 2019). This heat effect can eventually attract different species to the city. Ecological light and noise disturbances are other characteristics of urban settlements that have complex and deep effects on animal behaviour by affecting animals' orientation, migration, communication, foraging, and reproduction (DOMINONI et al., 2013). While the availability of household trash may be another factor that can influence the composition of wildlife communities by providing food for species that thrive on trash (such as raccoons, skunks and corvids) (BALTENSPERGER et al., 2013). Such food sources were previously reported to affect wildlife behaviour (e.g., reproductive strategies and movement patterns) (KRAUSMAN et al., 2014; SALLEH HUDIN et al., 2016).

Thus, numerous bird species exist in increasing numbers in urban environments (LOWRY et al., 2012; MEILLÈRE et al., 2015). Birds such as magpies, ravens, jays, and crows of the *Corvidae* family are synanthropic birds that succeed in manmade settlements and in large populations dominating the habitat (LOWRY et al., 2012; BALTENSPERGER et al., 2013; MEILLÈRE et al., 2015; KÖVÉR et al., 2015; BENMAZOUZ et al., 2021). However, how these species could adapt is still largely debated (LOWRY et al., 2012; MEILLÈRE et al., 2015). Various theories are implied in

an effort to explain this process including genetic adaptation, phenotypic plasticity, or certain temperaments and personalities being preferred in such an environment (LOWRY et al., 2012; LIKER, 2020). Moreover, extensive work has been exploring how urban environments affect the functional composition of avian assemblages. Factors driving morphological and physical differences within a given species and thus phenotypic responses to urbanisation are investigated (EVANS et al., 2009; MEILLÈRE et al., 2015; AMIOT et al., 2022). For instance, considering food availability in cities all year round, species abundant in urban areas are expected to benefit from their environment and to have an overall good body condition, yet, according to MEILLÈRE et al. (2015), high food predictability could have a negative impact on body condition. Though storing body reserves counter sudden food shortage in birds (BRODIN, 2007; SALLEH HUDIN et al., 2016), it also increases important metabolic and mobility costs, thus increasing predation risks (SWANSON, 2010). Thus, individuals are expected to exhibit lower body mass when living in areas with high food predictability, such as urban environments (i.e., adaptive mass regulation; reviewed in BRODIN, 2007).

Furthermore, although urban environments could provide easily accessible food, the growing density of urban avian populations could cause a strong competitive atmosphere between individuals, resulting in low food availability overall (SHOCHAT, 2004; SOL et al., 2013). In addition, anthropogenic food may be insufficient to sustain all nutritional requirements for the adequate survival of birds (HEISS et al., 2009). Therefore, urban diets may be inadequate and may lead to poor body condition in urban wildlife. Finally, the change in food composition driven by urbanisation pressures, such as the high availability of anthropogenic food, has been reported to be a major force shaping bill traits in different bird species, as feeding ecology has been closely linked with bill morphology in birds (BADYAEV et al., 2008; CORBIN, 2008). For instance, BADYAEV et al. (2008) reported that urban finches (*Carpodacus mexicanus*) developed deeper and wider bills in adaptation to feeding on harder seeds than their rural counterparts, suggesting that stubbier bills may be developed as an adjustment to hard ground surfaces usually found in urban areas (EVANS et al., 2009).

In addition, urban areas are particularly characterised by an increased human presence as well as non-native potential predators, which may induce morphological adjustments, adaptations, or exert selection in birds based on the morphological traits that are directly related to the aerodynamics or locomotor performances during escape

(MØLLER et al., 2015). For example, according to SWADDLE & LOCKWOOD (2003), wing shape may influence the angle for take-off and thus affect antipredator response, while tarsus length may be relatively linked to the risk of predation as a longer tarsus can facilitate take-off leap, whereas high body mass negatively impacts flight abilities, take-off velocity, and angle, as well as reduces flight speed (MØLLER, 2015). In contrast, some authors reported no intra-species differences between different habitat types (GRUNST et al., 2014) nor any difference in condition indices or locomotor morphology (AMIOT et al., 2022).

2.2. Urban ecology from corvid perspective

The *Corvidae* family includes very urbanised large passerines with highly documented presence in different cities and towns (BENMAZOUZ et al., 2021). Corvid species are often considered ideal subjects for investigating urbanisation and are considerably scrutinized. Often described as urban adaptors and even exploiters, these birds have displayed over the years their adaptability to continuous habitat changes all over the world. Due to their flexibility and ecological adaptability, corvids actively explore and colonise urban settlements and are largely common in cities where they usually cause conflicts with human populations (reported by MATSYURA & JANKOWSKI, 2016). Thus, numerous studies explored the effect of urbanisation on wildlife and the adaptation of synanthropic birds like corvids' species to habitat, environmental changes, and human expansion (BENMAZOUZ et al., 2021).

Corvids are very intelligent animals, and despite all the possible sources of stressors and disturbance in an urban environment, these birds are relocating to urban areas. Among them, crows have been showing huge ecological plasticity, being able to survive in different habitats (MARZLUFF et al., 2001). Such plasticity was noted by SLABBEKOORN (2013), where urban birds were recorded to be louder than their rural counterparts, suggesting this phenomenon to be a response to the loudness of city environments. Substantial increases in urban populations of the American Crow were also recorded in different regions of the United States compared to rural ones. Even though rural habitats have the capacity to accommodate population growth, rural crow populations do not seem to be increasing (MARZLUFF et al., 2001). This increase in the urban crow population could be explained by the dispersal of breeding populations from rural and wild land areas into urban areas. Regardless, the reason behind this increase in

adaptation and use of urban landscapes is still puzzling. Yet, several factors can be implied; for example, cities provide warmer winter climates, great nesting opportunities, and plenty of food sources (VUORISALO et al., 2003; BENMAZOUZ et al., 2021). Cities can offer habitat opportunities for crows when there is competition for food resources and nesting sites in rural areas. Cities can also ensure fewer risks of predation for adults (VUORISALO et al., 2003), since main predators like Pine Martens (*Martes martes*) tend to avoid human settlements, and regulation of human activities decreases persecution, which can also be an influential factor (MARZLUFF et al., 2001).

2.3. Corvids in urban environment: management efforts

Corvids in urban areas are the object of management and conservation efforts around the world. Mainly considered pests and sources of nuisance. From garbage scattering, roosting on roofs and parks, and the use of infrastructure for nesting to predation on prey species, which makes them targets of persecution (CHONG et al., 2012). One way to control corvids is to trap and translocate them, and given their intelligent nature, trapping is often important in many research programmes (KÖVÉR et al., 2018). In a study, KÖVÉR et al. (2018) tested and compared four trap types: bow trap (or bow net), Larsen trap, Swedish trap, and ladder trap, used to capture corvids in urban areas, where the Larsen and ladder entrance traps were found to be the most effective in capturing crows and Rooks in urban areas. Defining in the process a number of practical points for a successful trapping:

1. The location: it is necessary to select sites with frequent crow appearance; oftentimes, such places have attractive food sources such as accessible garbage cans, pens in zoos, and fruit trees (KÖVÉR et al., 2018).
2. Human avoidance: crows are very vigilant creatures; traps should be set in large open areas that are rarely, or not frequented by people and that present easy access to birds with little or no human interference.
3. Bait: the use of bird decoys can increase the attraction of corvids towards the traps; some even argue that the use of live decoys is necessary.
4. Trapping period: winter is the best time for corvid trapping due to the lack of food resources and the increased number of resident and winter crows in the city. Yet, during nesting season, it is easier to capture adult crows actively defending their nest and the hatchlings after leaving the nests.

5. Trap-checking time: corvids are very intelligent and attentive birds; they can recognise a threat from a distance. Therefore, traps should be checked when it is dark to decrease the risk of being spotted by birds.

Notoriously difficult to capture, a number of trapping methods were used to capture crows, ranging from traps to cannon nets, net launchers, and net guns (LEO & MANLEY, 2018). Net guns and launchers are reported to be the most successful capture techniques but have limitations; both techniques require the constant presence of an observer, and these methods depend on factors such as budget, time, personnel, and location (LEO & MANLEY, 2018). However, the current population status of these species indicates the limited success of the management effort so far. Perhaps controlling the features influencing corvid populations may be more successful at reducing them than direct population control. For example, CHONG et al. (2012) believe that Singapore's efficient waste management contributed largely to House Crow (*Corvus splendes*) population control, as also suggested by Kurosawa et al. (2003) in Jungle Crow (*Corvus macrorhynchos*) management. Thus, controlling access to food sources may be a management tool for corvid population growth by focusing on increasing control of garbage, animal husbandry practices, and bird feeding activities around residential areas.

2.4. Consequences of Urbanisation, One Health concept

Human impact on the different ecosystems has occurred for as long as humans have existed, however, current levels of human-ecosystem interaction have reached an unprecedented level of natural resource exploitation and agricultural practices, causing an increase in environmental encroachment and land use. Today, urbanisation is considered a critical factor in land-use change and is frequently associated with the emergence of wildlife-borne zoonotic diseases. The spatial overlap observed between humans and wildlife in urban habitat plays a key role in the emergence of such pathogens (HASSELL et al., 2016). Though some studies expressed potential links between the emergence of wildlife-borne zoonosis and human activities, fewer discussed the role of the urban environment in wildlife-pathogen interactions.

For years, wildlife has been described as an undeniable source of zoonotic agents. This constitutes a major public health problem affecting both humans and animals, and today such a phenomenon has gained increasing attention. It was reported that 62% of the 1,415 known human pathogens were of zoonotic origin (KRUSE et al., 2004). Nowadays,

wild animals seem to be associated with the epidemiology of most zoonosis and can be important reservoirs for such pathogens (HASSELL et al., 2016). Accordingly, the continuously increasing presence of synanthropic species in various cities around the world, like the Hooded Crow, not only increases levels of wildlife-human conflicts but can also lead to diverse wildlife-livestock-human interfaces where each part overlaps constantly, thus creating critical points of cross-species transmission and the emergence of pathogens between different species (HASSELL et al., 2016). For example, avian roosts and communal feeding locations in cities serve as encampments for urban birds like corvids and thus increase disease transmission possibilities, which is of great concern for human and animal health (MARZLUFF et al., 2001; VUORISALO et al., 2003).

Several authors addressed the potential role of different wild birds (e.g., mallards and pigeons) as vectors and spreaders of important zoonosis and human-related pathogens in urban areas (WILLE et al., 2017; BORGES et al., 2017). The most researched zoonosis in crow species is the West Nile Virus (WNV), which causes high mortality in corvids, especially the American Crow (*Corvus brachyrhynchos*) (LADEAU et al., 2011); thus, crows and ravens are biosensors used as an early indicator of the presence of this virus in a given area. The role of cities in the dissemination of human infectious disease has been established; however, little is known about how urban landscapes influence wildlife-pathogen interactions. Yet, the obvious interconnection between humans, animals, and the environment is undeniable and has undoubtedly drawn the attention of the scientific society, calling for a multidisciplinary collaboration between public health, animal health, and environmental professionals. According to the WHO (2019), One Health issues include “zoonotic disease, foodborne diseases, AMR, and other threats shared between animals, humans, and the environment”. Hence, one of the concurrent health problems is the continuous rise of antimicrobial-resistant pathogens, especially their emergence in various wildlife species (WANG et al., 2017), causing concern particularly for health professionals.

2.5. Antimicrobial resistance

Antimicrobial resistance (from here on “AMR”) is an urgent global health problem. The occurrence of AMR bacteria in humans and human settlements and their presence in the environment is of great concern, but their emergence in extremely remote areas is even more concerning (SJÖLUND et al., 2008).

An ancient phenomenon, AMR is the result of interactions between dynamic microorganism communities and the antimicrobial compounds found in their environment. Thus, bacteria developed mechanisms in order to counter antimicrobial actions for their own survival. Under the selective pressure of antimicrobials, susceptible bacteria are killed or inhibited, while naturally resistant bacteria (often referred to as "intrinsic AMR") or ones that have acquired AMR traits (often referred to as "acquired AMR") have greater chances of survival.

The origin of this phenomenon is subject to extended investigations in order to understand the emergence, selection, and dissemination of ARB in the environment. Although antimicrobials have revolutionised modern medicine and are considered to be one of the most important discoveries of our time, the routine administration of these drugs is believed to be one of the most important causes of the emergence, rapid evolution, and distribution of AMR. The extensive use of antimicrobial drugs in clinical and agricultural settings facilitates the spread of bacteria carrying resistance genes in the environment. Moreover, the ecology of AMR is further complicated by the possible development of resistance gained by previously susceptible bacteria through mutations in chromosomal genes or horizontal transmissions of AMR genes between bacteria of different phyla via genetic determinants such as plasmid conjugative transposons, most likely obtained from environmental microorganisms. These elements usually contain multiple genes responsible for the response to antimicrobial actions and other environmental stressors, thus threatening the efficiency of many antimicrobial drugs, including last-resort medications (e.g., carbapenems) (DAVIES & DAVIES, 2010; WELLINGTON et al., 2013).

On the other hand, the importance of the environment in the spread of AMR has been widely recognised. Antibiotic-producing bacteria exist naturally in terrestrial and aquatic environments (DAVIES & DAVIES, 2010), living within soils, plants, and animals. The contact of these bacteria with newly introduced ones originated from anthropogenic sources such as farmlands and waste plants demonstrates the ideal ecological and selective conditions for the emergence of novel resistant strains. Different environmental habitats can be considered hotspots for horizontal transfer of resistance genes (ALLEN et al., 2010). Retrospective studies have shown that genetic determinants of antibiotic resistance existed even in bacteria that did not produce these molecules long

before the extensive use of antimicrobial drugs (DAVIES & DAVIES, 2010; ARZANLOU et al., 2017; GOGRY et al., 2021).

2.5.1. Genetic basis of AMR

Bacteria are capable of responding to a great number of threats, including the presence of antimicrobial molecules, due mainly to their own extraordinary genetic flexibility (MUNITA & ARIAS, 2016). Bacterial species can maintain intrinsic resistance capacity, a natural trait that is shared within a species and that is not related to horizontal gene transfer, most frequently involving different mechanisms such as the natural activity of efflux pumps. On the other hand, bacteria can develop resistance (acquired) either through a genetic change (mutations) or by acquiring resistance-related genetic materials from an already resistant bacterium. As such, bacteria use two major strategies to evolve their response to antimicrobials (MUNITA & ARIAS, 2016):

1) Gene Mutation: Bacteria susceptible to antimicrobial activity can develop mutations in genes that influence the actions of the drug. When the mutant bacteria emerge, the antimicrobial drugs eliminate the sensible organisms while the resistant ones survive.
2) Horizontal gene transfer: Acquisition of external genetic determinants of resistance.

Acquiring extraneous genetic materials through horizontal transfer is one of the most important factors in AMR evolution. Most antimicrobial compounds are found in different environments, and bacteria living in the same environment as these molecules harbour genetic materials encoding resistance to these products. There is evidence suggesting that the AMR genes can be transferred and mobilised between different bacteria from different phyla through mobile genetic elements (MGE) (ALLEN et al., 2010). The implication of this dissimilation in the widespread resistance against a number of frequently used antimicrobial drugs has been implied. This exchange happens through three main actions:

- Transformation (by naturally incorporating free DNA).
- Transduction (via bacteriophages).
- Conjugation (via direct cell-to-cell contact) by using mobile genetic elements to share genetic information, such as plasmids.

Some MGEs are limited to a restricted host range, but others have a broader range and can therefore promote exchange between clinical and environmental bacteria

(RAMOS et al., 2012; TOUCHON et al., 2020), which creates an environmental reservoir of resistance with potential effects on human and animal health (WANG et al., 2017; DELPECH et al., 2019).

2.5.2. Antimicrobial resistance mechanisms

Bacteria have developed complex mechanisms for AMR as a response to antimicrobial action. A single bacterium may use a line of mechanisms to resist antibiotic attacks by using multiple different routes against a given antibiotic (MUNITA, & ARIAS, 2016), which may coexist within the same bacteria at the same time. The different AMR mechanisms can be categorised according to the biochemical routes involved:

a. Modification of the antimicrobial molecule

Altering the chemical composition of the drug by producing specific enzymes causing chemical addition to the antibiotic is a common mechanism in both Gram-negative (from now on GNB) and Gram-positive bacteria (GNP). This method mostly affects antimicrobials that act by inhibiting protein synthesis at the ribosome level (WILSON et al., 2015). One of the best examples is the aminoglycoside-modifying enzymes that equally change the hydroxyl or amino groups of the aminoglycoside molecule. Usually, these enzymes are found in mobile genetic elements, but genes coding these molecules have also been found to be part of the chromosome in some bacteria.

Another type of modification is destroying the drug molecule; this is the main mechanism used against beta-lactams by the action of beta-lactamases. These enzymes target the lactam bond of the beta-lactam ring, enabling the lysis and inactivation of the antimicrobial. Beta-lactamase was first described in the early 1940s, and ever since, novel generations of these enzymes have appeared with each new antimicrobial compound (DAVIES & DAVIES, 2010). Genes responsible for beta-lactamases are referred to as *bla*, followed by the name of a specified enzyme. They have been found in mobile genetic elements as well as part of the chromosome of bacteria. These genes were also found as part of integrons, which facilitate their circulation between microorganisms (MUNITA & ARIAS, 2016).

b. Prevention to attain the target site

The mechanisms of resistance may prevent the antibiotic from reaching its target site by decreasing the permeability of the outer membrane. Hydrophilic molecules such as tetracycline and beta-lactams are affected since they usually cross the membrane through water-filled diffusion channels or porins (PAGÉS et al., 2008).

Bacteria can also resist antimicrobial activity by producing a complex bacterial system capable of expelling a foreign molecule out of the cell. One of the first to be identified was an efflux system able to extrude tetracycline out of the cytoplasm of *E. coli* in the early 1980s (MUNITA, & ARIAS, 2016). Since then, a number of efflux pumps have been described in both GNB and GPB. This mechanism can affect a wide range of antimicrobial classes. The genes encoding this type of resistance were found in mobile genetic elements as well as on chromosomes (MUNITA & ARIAS, 2016).

c. Change or bypass target sites

Changing the target site in order to avoid the action of an antimicrobial is a common method used by bacteria to express AMR. To do so, microorganisms have developed different ways to protect the target. (1) By producing proteins that mediate target protection. Drugs like tetracycline (DÖNHÖFER et al., 2016) and fluoroquinolones (ALDRED et al., 2014) are affected by this method. (2) By simply changing the target site, which is one of the most common ways to resist antimicrobial activity in pathogenic bacteria. Almost all families of antimicrobials are affected by it. These modifications include:

- ⇨ Mutations of the target site, for example: the development of resistance to oxazolidinones (a synthetic drug that inhibits protein synthesis by acting on bacterial ribosomes) (MENDES et al., 2014).
- ⇨ Enzymatic modification for binding sites, for example: the acquisition of *erm* genes responsible for the erythromycin ribosomal methylation that allows macrolide resistance (ROBERTS, 2008).
- ⇨ Complete replacement or bypass of the original target site. Bacteria can develop new targets with similar function to the original ones and that are not susceptible to antimicrobial attacks. For example, the acquisition of an exogenous penicillin-binding protein (PBP2a) by *Staphylococcus aureus* against methicillin

(HIRAMATSU et al., 2013) and the modifications of the peptidoglycan structure in enterococci by the van gene clusters against vancomycin (MILLER et al., 2014). Another way is to overproduce the antibiotic target to overburden the active molecules and evade the different reactions targeted by the antimicrobial actions (MUNITA, & ARIAS 2016).

d. Global cell adaptation:

Bacteria were under constant attack from various environmental stressors in the most hostile surroundings, including the human body. Within a particular habitat, a microorganism needs to adapt to different stressful situations. Throughout the years, bacteria have been developing very sophisticated mechanisms to escape the disruptions of important cellular structures and maintain cell integrity against continuous attacks such as cell wall synthesis and membrane homeostasis. For example, clinical *Staphylococcus aureus* strains were found to have developed a global cell adaptive response against daptomycin (BAYER et al., 2013) and vancomycin (GARDETE & TOMAZS, 2014) actions, although at a low level.

2.5.3. Antimicrobial resistance in Gram negative Bacteria

GNBs were first described by Hans Christian Gram in 1884, hence their name. Distinguished by their pink coloration while counterstaining with safranin, GNB differs from GPB thanks to their outer membrane (MOYES et al., 2009). This outer membrane is the main cause of resistance against a range of antimicrobials, including beta-lactams, quinolones, colistin (or polymyxin E), and more, most likely due to the outer-membrane permeability barrier limiting access of antimicrobial agents to their cellular targets (BREIJYEH et al., 2020). GPB do not possess this outer layer, which makes them more vulnerable to antimicrobial actions than GNB (EXNER et al., 2017).

GNB can be the cause of a large number of AMR infections in humans and animals. Often causing high-risk infections such as pneumonia and bloodstream infections, GNB are a common cause of rapidly progressive septic shock, particularly in immunocompromised patients. Currently, treatment of GNB infection is frequently ‘empirical’ in the first 48 hours, requiring use of broad-spectrum antibiotics until culture and susceptibility results become available (STEWART et al., 2019). Among these bacteria are also some of the most successful environmental organisms. Mainly due to their ability to adapt to various niches, their inherent resistance to antimicrobials, and

their capacity to acquire resistance mechanisms and to develop or acquire AMR-encoding genes, especially in the presence of antimicrobial selection pressure (DAVIES & DAVIES, 2010; ARZANLOU et al., 2017; GOGRY et al., 2021). These organisms hold a number of resistance mechanisms, often using multiple mechanisms in response to the same antibiotic or using a single mechanism against multiple ones (BREIJYEH et al., 2020). These mechanisms of resistance are not exclusive, and the interplay of several mechanisms causes high levels of resistance (ARZANLOU et al., 2017). Additionally, these different resistance mechanisms are sometimes easily transmitted across different bacterial species, generating multiple-drug-resistant (MDR) microorganisms.

Common resistance mechanisms among GNB include beta-lactamase production leading to lysis of beta-lactam antibiotics; target-enzyme mutations; porin mutations; and drug efflux pumps (BREIJYEH et al., 2020). The clinically most important beta-lactamase-producing bacteria are those that hydrolyze third-generation cephalosporins by producing extended-spectrum beta-lactamases (ESBLs) or ampC lactamases, and those that hydrolyse carbapenems such as meropenem, known as carbapenemase producers (BREIJYEH et al., 2020). Thus, resistance to third-generation cephalosporin may be caused by mutations of genes encoding beta-lactamases of class A, like TEM-1, TEM-2, and SHV-1, responsible for ampicillin, amoxicillin, and early-generation cephalosporin resistance, thus giving birth to new β-lactamases that can hydrolyze them. CTX-Munich, another type of ESBL enzyme that hydrolyzes cefotaxime more efficiently than ceftazidime, can also be expressed by Enterobacterales, while carbapenem hydrolyzing oxacillinases (OXA), mainly found in *Pseudomonas aeruginosa*, are rare in Enterobacterales (ABDALLAH et al., 2015). Lately, in the United States and Europe, 15–25% of Enterobacterales responsible for hospital-acquired infections produce ESBLs, with rates much higher reported in Asia and South America (WHO, 2014). According to the ECDC report (2022), the resistance to third-generation cephalosporins in Enterobacterales is over 20%, and up to 1% for carbapenems in Hungary. Currently, third-generation cephalosporins-resistant Enterobacterales are subject to extensive research.

2.5.3.1. Extended spectrum Beta lactamase producing Enterobacterales

Extended-spectrum β-lactamases, or ESBLs, are a rapidly evolving group of β-lactamases that demonstrate the ability to hydrolyze third-generation cephalosporins

(RAWAT & NAIR, 2010). They have been established since the 1980s as a cause of hospital-acquired infections (EXNER et al., 2017).

Enterobacterales comprises over 40 genera and more than 180 identified species, most of which are ubiquitous in nature as well as commensal inhabitants of the gastrointestinal tract of humans and animals, including birds (TOUCHON et al., 2020). One of the most commonly surveyed members of the Enterobacterales is *E. coli*. Regularly used as an indicator for faecal contamination, species of this genus are mostly used to monitor AMR in wildlife (DOLEJSKA & LITERAK, 2019). For instance, the first recorded AMR in wildlife was a resistant *E. coli* in wild pigeons (SATO et al., 1978), and the first CTX-M type enzyme encoding for third-generation cephalosporin resistance in ESBL producers was also isolated from *E. coli*. To date, *E. coli* is still the principal host of these enzymes (CANTÓN et al., 2008). Yet, the prevalence of these enzymes in other Enterobacterales has increasingly been reported over the years as well (CANTÓN et al., 2012).

So far, over 200 ESBLs have been characterised (http://www.lahey.org/studies/webt.htm), and several studies reflect the global distribution of these microorganisms. Encoded by genetic materials in large plasmids, ESBL producers often carry genes responsible for resistance to other antimicrobials such as aminoglycosides, trimethoprim, sulphonamides, tetracycline, and chloramphenicol, as well as fluoroquinolones (CANTÓN & RUIZ-GARBAJOSA, 2011). Frequently characterised by a broad spectrum of AMR extending to different antibiotic classes, Enterobacterales are of particular concern, and due to the emergence of third-generation cephalosporin-resistant strains, these microorganisms are listed under the primary category of 'critical' priority by the WHO, and pose a major health problem.

2.5.4. Antimicrobial resistance in Gram positive bacteria: vancomycin resistant enterococci (VRE)

Enterococci is a large genus of GPB consisting of commensal microorganisms normally found in the intestinal tract of various beings, including humans, mammals, birds, reptiles, and insects (WADA et al., 2019). These commensal bacteria can become pathogenic and cause serious nosocomial infections, including urinary tract infections and sepsis (WADA et al., 2019; AYOBAMI et al., 2020). Enterococcal infections are usually treated with beta-lactams, or in cases of beta-lactam resistance, by glycopeptide

antibiotics (AYOBAMI et al., 2020; WERNER et al., 2020), like vancomycin, which is listed in the WHO's list of essential medicines, classified as critically important for human medicine (WHO, 2019), and is reserved to treat serious life-threatening infections caused by GPB that are nonresponsive to other treatments. However, in recent times, resistance to vancomycin has emerged among enterococci (DELPECH et al., 2019). Believed to have first appeared in hospital environments (WADA et al., 2019; WERNER et al., 2020), VRE were reported in companion and farm animals as well (NILSSON et al., 2009; RAMOS et al., 2012).

As with any emerging AMR, the occurrence of VRE outside hospital-like facilities is also believed to be facilitated by the extensive use of similar antimicrobial drugs in clinical and agricultural settings (KRUSE et al., 1999). In Europe, the use of avoparcin, a glycopeptide antibiotic sharing the same mechanism of action as vancomycin, as a feed additive was strongly associated with the rise of VRE strains in livestock, especially poultry (KRUSE et al., 1999; WERNER et al., 2020), which called for the avoparcin ban by the European Union in 1997. However, decades later, VRE is still occurring, and lately records of these bacteria in wildlife have been documented, as such strains were found in free-ranging gulls (SJÖLUND et al., 2008) and corvids (ORAVCOVA et al., 2013, 2016).

2.5.5. Emergence of AMR in the wild

Several studies have established that bacteria collected before and after the introduction of antibiotics were resistant to a number of drugs such as ampicillin and tetracycline and could transfer different AMR determinants, suggesting that genes encoding resistance occurred naturally and were horizontally transferred between natural bacteria long before the antibiotic era (ALLEN et al., 2010). This predisposition to the genetic transfer of resistance has most likely eased the emergence of AMR in pathogenic bacteria.

Currently, many factors are linked to this phenomenon, mainly the misuse of antimicrobials in human and veterinary medicine and 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). The fact that wild mammals and birds are not subject to antimicrobial therapy highlights the importance and complexity of bacterial resistance in wild animals and the possibility of interspecies transmission between

humans, domestic animals, the environment, and wildlife. As such, food and water seem to be major sources of transmission of resistant bacteria of human or veterinary origin to wildlife (BAQUERO et al., 2008; ATTERBY et al., 2017; AHLSTROM et al., 2018). In addition, antibiotic use in agriculture and intensive farming can be up to four times higher than in clinical treatments (SIMOES et al., 2010; SMITH et al., 2014), and manure and biological materials like fertilisers used in farming probably contain antimicrobial residuals and AMR bacteria (BAQUERO et al., 2008). Accordingly, many antimicrobial molecules were reported to be disseminated in the environment in their active form (DAVIES & DAVIES, 2010), and continuous exposure to these drugs might promote the proliferation of AMR determinants and resistant bacteria. Wildlife in contact with these sources could be at a higher risk of acquiring resistance genes, although empirical data to support this idea is scarce (VITTECOQ et al., 2016). This also opens the door to the assumption that commensal bacteria carrying AMR-related genes may act like reservoirs and transmit resistance traits to pathogenic microbes (BONNEDAHL & JÄRHULT, 2014). The impact on human and animal health rises when the genes naturally amplify and are horizontally transferred between different types of bacteria (BONNEDAHL & JÄRHULT, 2014). Thus, areas with increasing human and livestock populations encompass a multitude of AMR sources and amplifiers (ARNOLD et al., 2016). Such places have a high bacterial diversity, provide s large host abundance, and provide ideal conditions for horizontal transmission of AMR mobile genetic elements from commensal bacteria to pathogens.

On that note, due to their well-studied ecology and their relative closeness to the urban environment, wild birds are considered sentinels since they easily pick up human and environmental bacteria (BONNEDAHL & JÄRHULT, 2014). In fact, the first antibiotic-resistant bacteria detected in wildlife were from wild birds, in 1975, strains of *E. coli* resistant to multiple antibiotics were isolated in pigeons (SATO et al., 1978). Since then, numerous studies have recorded the presence of AMR in wildlife, including wild birds (RADHOUANI et al., 2014; WANG et al., 2017). For example, ARB were found on many occasions in wild species such as ducks, geese (COLE et al., 2005), gulls (ALROY & ELLIS, 2011; ATTERBY et al., 2017), doves (BORGES et al., 2017), and passerines (DOLEJSKA et al., 2008). ARB were detected among commensal gut bacteria of wild mammals, birds, reptiles, and fish with different patterns across species and habitats (SJÖLUND et al., 2008; CABELLO et al., 2013). In addition, beta-lactam and

tetracycline-resistant bacteria are believed to be common in the gut microbiome of birds, especially in scavenging and aquatic species such as waterfowl, gulls, and waders (ATTERBY et al., 2017; AHLSTROM et al., 2018; MARCELINO et al., 2019). For instance, *E. coli* producing ESBL was first found in wild birds in 2006 (COSTA et al., 2006). In recent years, many reports followed, mostly from European countries, as ESBL-producing Enterobacterales were found in almost 80 wildlife species (mostly birds) (reported by WANG et al., 2017). WANG et al. (2017) revealed that the ESBL-encoding genes identified in wildlife are frequently identical to those in human and veterinary medicine, suggesting possible interspecies gene transfer between humans, pets, production, and wild animals.

Alarmingly, this does not exclude the emergence of the ARB in wildlife in remote areas such as the Azores Archipelago (SANTOS et al., 2013) and the Arctic (SJÖLUND et al., 2008). Therefore, it is important to analyse the epidemiology and mechanisms of the emergence and spread of AMR in wildlife while taking into account the role of ecological factors like migratory behaviour and high population densities in increasing the likelihood of the presence of AMR genes in birds, even in the most isolated habitats (WELLINGTON et al., 2013).

On the other hand, more and more facts support the idea that AMR is a natural characteristic of bacteria and that environmental organisms are natural reservoirs of AMR genes (DAVIES & DAVIES, 2010; ARZANLOU et al., 2017; GOGRY et al., 2021). Suggesting that environmental antimicrobials may exercise selective pressure on co-resident organisms (ALLEN et al., 2010), plus their contact with newly introduced microorganisms, demonstrate the ideal ecological and selective conditions for the emergence of novel resistant strains of bacteria. Additionally, ALLEN et al. (2010) highlighted the influence of environmental conditions on the emergence and spread of ARB; they believe that environmental factors such as soil, wind, and water play an important role in the dissemination of AMR genes. Therefore, the different environmental habitats can be considered hotspots for horizontal transfer of resistance genes (ALLEN et al., 2010).

2.5.5.1 Wild birds as the carriers and spreaders of AMR

Once wildlife acquire ARBs, they become a source of contamination themselves, serving as reservoirs, vectors, and bio-indicators for bacterial resistance and the genetic

materials responsible (DOLEJSKA & LITERAK, 2019; DELPECH et al., 2019). The frequency of ARB in wildlife is generally linked to human activities (ALROY & ELLIS, 2011). A number of authors argue that individuals living near manmade settlements have a higher frequency of AMR (COLE et al., 2005; DOLEJSKA et al., 2008; SKURNIK et al., 2016; ATTERBY et al., 2017). Many factors are involved in the emergence of AMR in wildlife, as mentioned above.

Numerous bird species exist in an increasing manner in urban environments (LOWRY et al., 2012; MEILLÈRE et al., 2015). Synanthropic birds such as magpies, jays, and crows of the *Corvidae* family thrive in manmade settlements and are present in large populations dominating the habitat (LOWRY et al., 2012; MEILLÈRE et al., 2015). However, the way these species are able to adapt is still largely debated (LOWRY et al., 2012; MEILLÈRE et al., 2015). For example, the Hooded Crow (*Corvus cornix*), a totally urbanised bird species in several European settlements, is an ideal species to study several of such key issues on the effects of humans and human settlements on birds. There have been some studies regarding differences between rural and urban populations of wild birds, e.g., density, clutch size, and behaviour, but very few focused on the role of this species as a reservoir of pathogens. Such anthropophilic birds might harbour a diverse and potentially unexplored community of pathogenic and symbiotic microorganisms. Thriving in different cities around Europe, these species may spread pathogens to domestic animals and to humans, and therefore could be a source of AMR for humans and other animal species. Animals in urban environments have more opportunities for direct or indirect interactions with humans that may also affect them, since the greater wildlife's contact with humans and human-related activities, the higher interspecies transmission and exchange opportunities of different diseases and infections become (BORGES et al., 2017). Although some studies focus on the epidemiology of zoonosis involving wildlife (KRUSE et al., 2004), the role of the latter in the ecology and evolution of AMR is poorly addressed, especially in highly active (mobile) animals like birds. Since human-made environmental changes are considered to be responsible for the different occurrences of ARB in the world (SKURNIK et al., 2016). Besides contaminating waterways and food resources in fields and human-related environments through faeces (COLE et al., 2005; BAQUERO et al., 2008), wild birds also provide a biological transportation service for ARB and AMR genes (ALLEN et al., 2010). Several authors implied the important role that the ecology and behaviour of wildlife, especially wild

birds, play in the distribution of AMR into the environment (ARNOLD et al., 2016; DOLEJSKA & LITERAK, 2019). For example, studies implied that the omnivorous feeding pattern of generalist birds might predispose them to ARB of agricultural origin (ATTERBY et al., 2017). Also, the migratory nature of some wild birds may explain the introduction of AMR in some of the most remote areas in the world (SJÖLUND et al., 2008; HERNANDEZ et al., 2010; GUENTHER et al., 2012). The spread of pathogens through migratory wild birds was previously demonstrated with the dissemination of West Nile virus in the US (LADEAU et al., 2011). Given that birds can fly longer distances in shorter periods of time, they can easily act as spreaders of ARB. In a study on the arctic, SJÖLUND et al. (2008) highlight the ability of birds to act as long-distance vectors of AMR. As resistant *E. coli* were isolated in arctic birds from Siberia, Alaska, and Greenland, lower frequencies were seen in wild mammals from similar environments.

2.6. Objectives

Detailed ecological and microbiological analysis of urbanisation processes are necessary for both scientific research and management efforts of urban wildlife. The purpose of this study was to investigate the urbanisation of the Hooded Crow in Hungary by using ecological and microbiological data collected from rural and urban areas and comparing the outcomes between the two distinct habitat types.

This study aimed first to explore if and how the urban environment in our cities would affect the continuously increasing Hooded Crow community by examining the presence of any morphological and physical differences between individuals from rural and urban areas and thus exploring any phenotypic responses from the crows to urbanisation. We speculate to find some differences between individuals from the different habitats because of their adaptation to the highly urbanised environment, which could further explain the adaptation process of these birds in cities.

Secondly, this study aims at examining the presence of multi-resistant bacteria in the Hooded Crow within the city of Debrecen and the neighbouring rural areas, as well as in Budapest, and the link between ecological factors and the carriage of these multi-resistant bacteria. We speculate that the Hooded Crow might harbour multi-resistant bacteria, and since this species is thriving in different cities around Europe, including the cities of Budapest and Debrecen in Hungary. We assume that urban crows carry higher bacterial diversity and abundance than their rural counterparts. Therefore, our aim is to

identify multi-resistant GNB present in the faeces of Hooded Crows from two distinct habitats. To do so, birds from different urban and rural crow populations have been sampled and the isolates recovered on selective media. We are primarily interested in the presence of human and veterinary-related resistant microorganisms in the Hooded Crow in our study areas. Since these wild, synanthropic birds are not exposed to any kind of treatment, we believe that they are more likely to acquire human-related resistant bacteria from different anthropogenic food sources and would eventually contaminate human-associated settlements. Our focus is primarily directed towards ESBL producing Enterobacterales, such as *E. coli*. This type of bacteria is of interest to our study due to (1) their importance as pathogens and widespread worldwide; (2) their being ubiquitous microorganisms in nature, normally found in the gastro-intestinal tract of humans and animals, including birds; (3) their being a good indicator for faecal contamination; and (4) their being widely used for AMR surveillance. The other investigated resistant bacteria was VRE. We chose these for two important reasons: (1) enterococci are commensal bacteria normally found in the human gastric tract and often found in the environment that have the ability to acquire resistance in a short period of time; and (2) these bacteria can cause serious health issues.

3. MATERIAL AND METHODS

3.1. Area of study

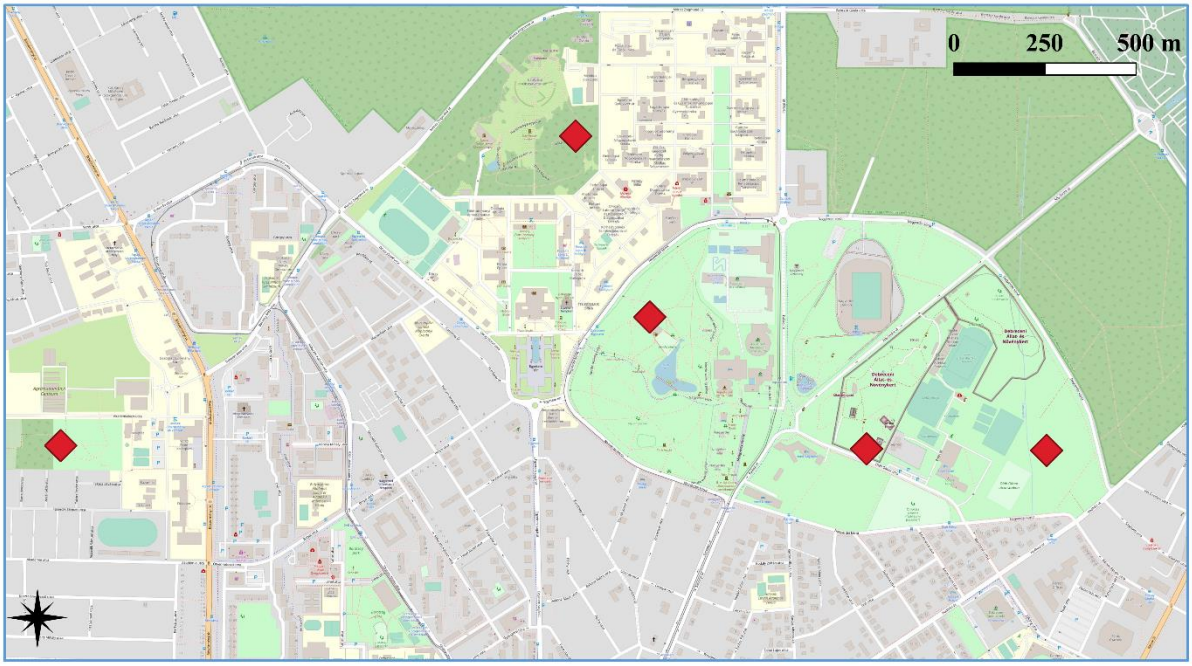
This project was carried out in the city of Debrecen (47°52′27.7″N; 21°63′91.6″E) (Figure 1), the second largest city of Hungary after the capital Budapest (47°29′33″N; 19°03′05″E), located at the centre of the Northern Great Plain region and situated nearby the Hortobágy National Park. We operated four separate traps in the city for our urban sample collection. Additional urban samples were collected from wild crows trapped at the Budapest Zoo. 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 1).

Figure 1: **Maps showing the different study areas; urban study site in Debrecen (a); rural study site in Szakoly (b); urban study site in Budapest (c) and rural study site in Balmazújváros (d). 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 Isma Benmazouz and Dr. László Kövér, where traps were visited exclusively after sunset during the entire study period. 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 a had fixed structure and were open continuously during the trapping occasions.

Figure 2: **Map showing the locations of the traps within the city of Debrecen. Red coloured dots = Trap sites during the study period. Green colour = natural landscapes, Grey colour = urban infrastructure (e.g. buildings and roads)**



3.2. Species of study

The Hooded Crow (*Corvus cornix*) is an Eurasian bird species of the *Corvus* genus, found across Northern, Eastern, and Southeastern Europe, as well as parts of the Middle East. The Hooded Crow is an ashy grey bird with a black head, throat, wings, tail, and thigh feathers, as well as a black bill, eyes, and feet. Their length varies from 48 to 52 centimetres. Crows often inhabit fields, woodlands, and forests, as well as cities and towns. They are especially social animals, curious, great learners, and problem solvers (CORNELL LAB OF ORNITHOLOGY, 2015).

Initially, the Hooded Crow lived exclusively in rural habitats (KÖVÉR et al., 2015). However, since the 1960s, this species has shown a rapid adjustment to urban environments. Throughout the years, various authors documented the urban colonisation of Hooded Crows and its continuous population increase in different regions of Europe

(VUORISALO et al., 2003; KÖVÉR et al., 2015). For instance, Hooded Crows were first recorded in Debrecen, our study area, in 1959 at the university's botanical garden. The Hooded Crow reappeared as a nesting species in 1972 and then in 1979 (KÖVÉR et al., 2015). By 2013, the crow's urban population exceeded 100 nesting pairs (and 4 nests/km^2) and is still increasing linearly, with no sign of reaching carrying capacity (KÖVÉR et al., 2015).

Sedentary by nature, Hooded Crows usually stay in or near their territory all year. Yet, some northern populations move south or southwest in winter, reaching the west of France, while some flocks form communal roosts. As MCIVOR and HEALRY (2017) describe crows living within and nearby the city of Orkney, Scotland, as sedentary and partially migratory around the city, moving for a few kilometres in proximity of their initial habitat. Like any other *Corvidae*, the Hooded Crow is an omnivorous and opportunistic forager and feeder.

Diet: A constant scavenger, a crow feeds on a wide variety of food such as grains, seeds, nuts, fruits, a variety of worms, mice, insects, fish, turtles, frogs, clams, birds, bird eggs and nestlings, carrion, litter, and garbage (CORNELL LAB OF ORNITHOLOGY, 2015).

Breeding: The Hooded Crow is a monogamous species, and the pair breeds for many successive seasons (CORNELL LAB OF ORNITHOLOGY, 2015). A female lays around 3 to 6 eggs in a nest. The incubation lasts for 18-19 days, and both adults tend to the hatchlings, which leave the nest about 28 days later. The young become fully independent after 3 to 5 weeks.

Nesting: In crows, nesting varies depending on the region; in colder regions like Northwest Russia or the Faroe Islands, nesting occurs around mid-May to mid-June and in late February in the Persian Gulf region. In warmer parts of Europe, the clutch is laid in April (CORNELL LAB OF ORNITHOLOGY, 2015; MCIVOR & HEALRY, 2017). Generally, the crows start their nesting and breeding earlier than their rural counterparts do. In urban environments, their nesting is asynchronous (KÖVÉR et al., 2015). In addition, corvids exhibit selective preferences for nesting sites; in urban areas, Hooded Crows were observed choosing nesting trees based on tree species, tree height, and distance from human-occupied buildings, and the availability of older, suitable nests (MCIVOR, & HEALEY 2017). In Hungary, Hooded Crows in urban areas were found to

prefer to nest on oak, pine (since they are the tallest tree species), and poplars (KÖVÉR et al., 2015). Nest re-use is not common in Hooded Crows. Yet, MCIVOR & HEALEY (2017) observed about two-thirds of crow pairings reusing nests from previous seasons, and the pairs using old nests appeared to be more successful.

Audio Calls: Crows are vocal animals, using calls and sometimes songs for social bonding and communication (HAUSER & CAFFREY, 1994). Numerous bird species, including wild crows (*Corvus brachyrhynchos hesperisis*), have various sensory mechanisms that they developed to detect predators and communicate within the group (HAUSER & CAFFREY, 1994).

Hooded Crows' increasing occurrence in urban areas can be a source of human-related concerns or conflicts. This species is often a source of nuisance, from disturbing noises and faecal droppings to garbage scattering; this bird can even be aggressive to humans and pets as well as be vectors of diseases (VUORISALO et al., 2003). Crows also cause damage to various agricultural fields, from crop yields to livestock in rural areas, and can damage different infrastructures (SZALA et al., 2020). For instance, in Hungary, based on a survey conducted in 2021, numerous people have disclosed unpleasant experiences with Hooded Crows residing in the city, calling for rigorous management efforts (KÖVÉR et al., 2022). Therefore, all the increasing nuisances raise the need for the control and management of the Hooded Crow, especially in urban settlements. Often referred to as problem species, corvids have been subject to different management efforts for decades (BENMAZOUZ et al., 2021). However, the current status of these species in our cities reflects the complex nature of wildlife management in urban areas. Indeed, urbanisation provides an interface where humans interact with urbanised wildlife regularly. An interface that calls for the involvement of all interactive parties, including citizens from the concerned area, especially for better management of human-wildlife conflicts in urban areas.

3.3. Crow trapping and sample collection

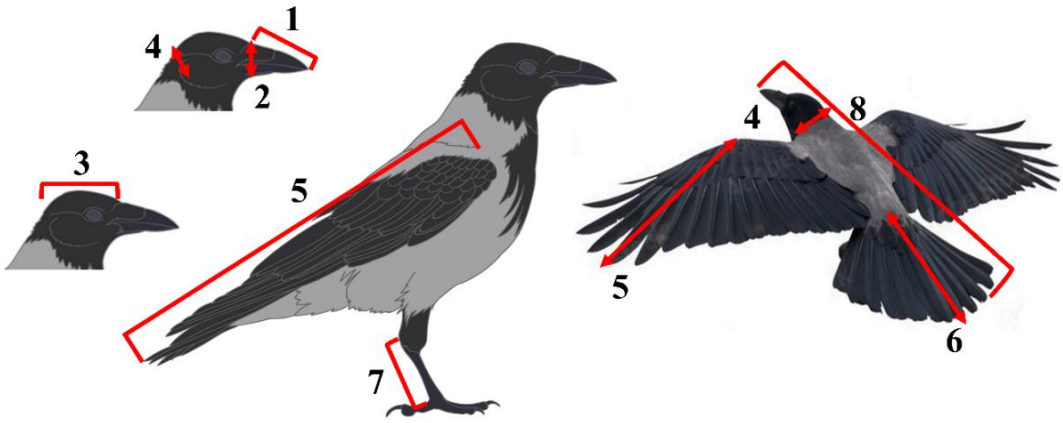
Wild Hooded Crows are caught using a 'Ladder entrance trap' (Figure 3) in both urban and rural environments continuously throughout the years (KÖVÉR et al., 2018). Originally from Scandinavia, first it was developed for decreasing the populations of Rooks, but this kind of trap has been successfully 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 3: **A ladder entrance trap used for Hooded Crow capture**

The trap used looks like a large flight cage due to its size (2 m width, 2 m length, 2.5 m height) and has a ladder-like door on the top side with a 0.2 m opening where crows attracted to the bait can jump in. Live crows from previous catches were kept to be used as decoys. The traps are always checked after sunset. Since the start of the project, the traps have been visited at least twice a week, and food ─bread, dry and wet cat food - and water are supplied for the decoy birds as well as used as bait. The management and good maintenance of the traps are very critical for the success of our research. 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 were measured for each captured and sampled individual from all areas (Figure 4, Appendix 1).

Figure 4: **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 (Figure 5). The swabs are transferred to the medical microbiology laboratory at the University of Debrecen, where they will be immediately cultured. We have already obtained permissions for the trapping.

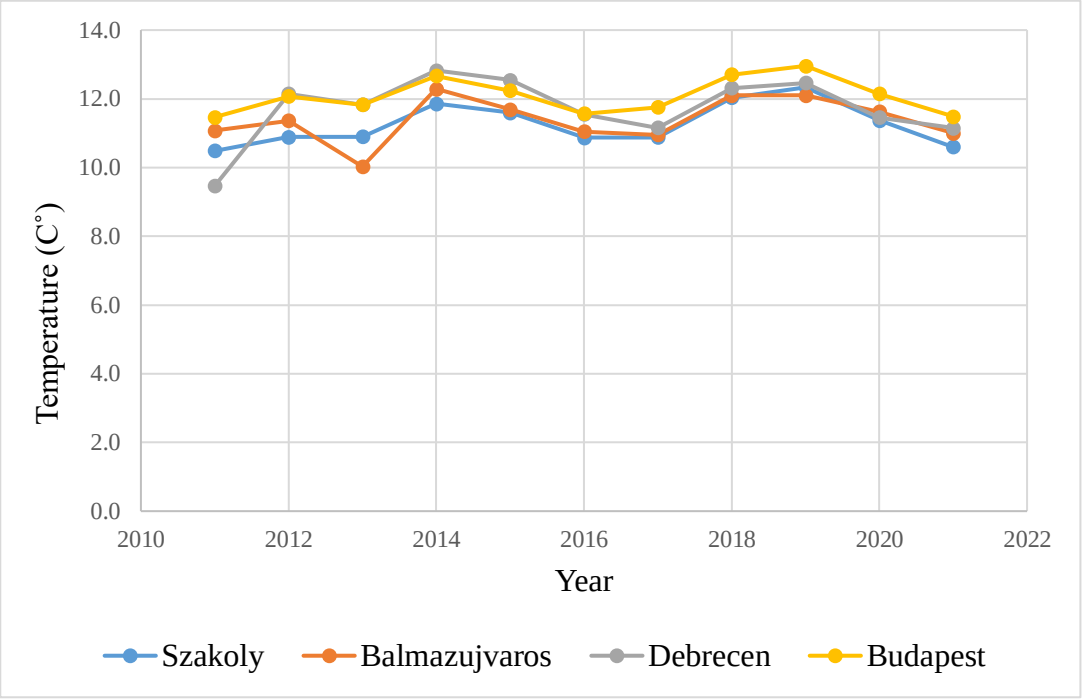
Figure 5: **Faecal sample collection from a wild Hooded Crow**

3.4. 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. Additional randomly selected 200-m circles were also created in order to have five circles in all study sites (Appendix 2).

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 from all study sites (Figure 6) were obtained from the meteorological station in close proximity to the area of study (Appendix 3).

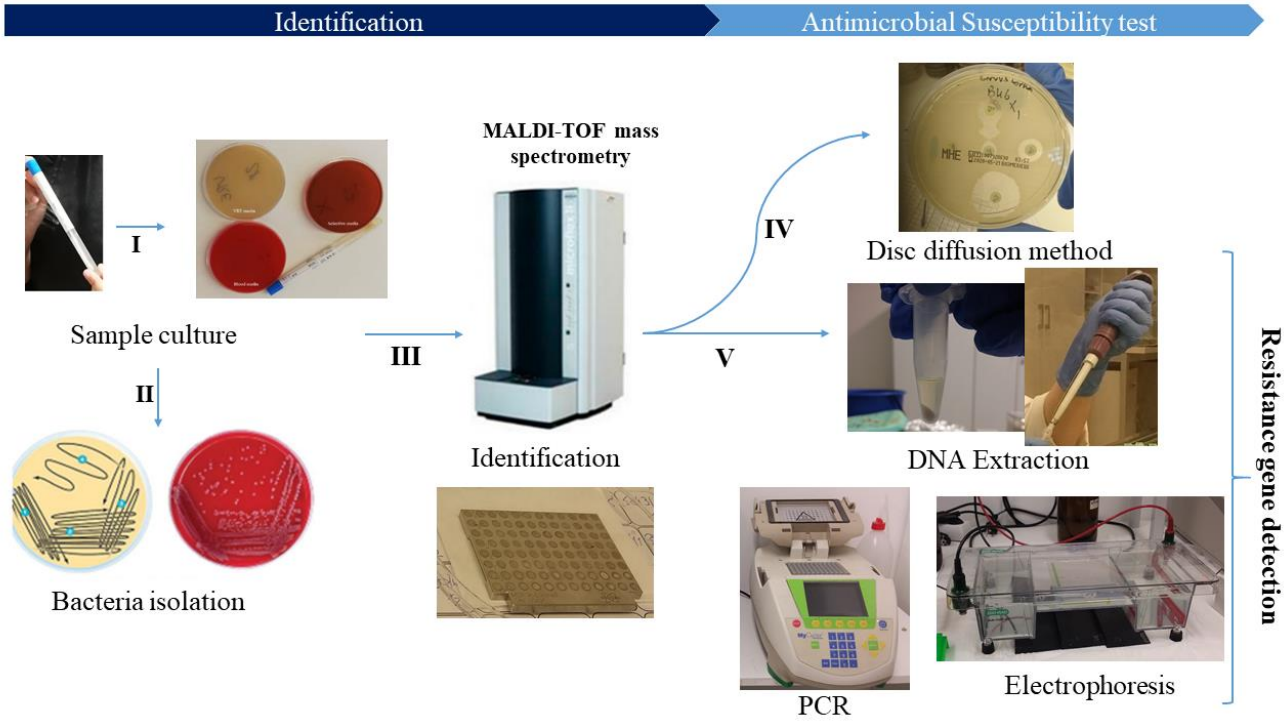
Figure 6: **The annual average of daily temperature over 10 years (2011-2021) from all capture areas**



3.5. Microbiological analysis

At the lab, our samples are analysed as follows (Figure7):

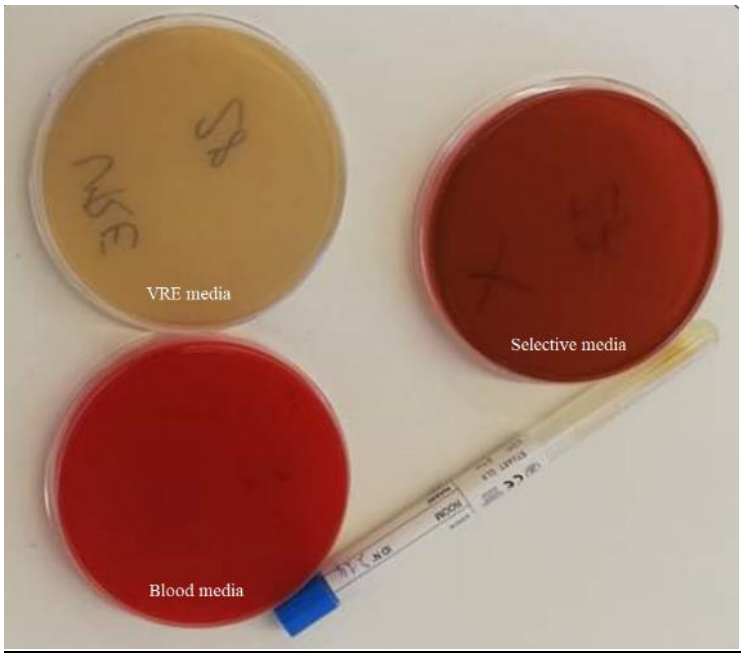
Figure 7: **Summary of the laboratory methods used for the analysis of AMR in Hooded Crow faecal samples. MALDI-TOF: matrix-associated laser desorption/ ionization — time of flight. DNA: deoxyribonucleic acid. PCR: polymerase chain reaction**



Step 1: Samples are cultured directly on solid blood and selective media, prepared by adding 2 mg/l of cefotaxime to eosin methylene blue agar media, allowing the isolation of third-generation cephalosporin-resistant bacteria, as well as a bile esculin azide (BEA) agar with vancomycin, specific and selective for the detection of VRE (Figure 8). All media were prepared at the medical microbiology laboratory at the University of Debrecen (Appendix 5). At this point in the analysis, blood agar is solely used to ensure bacterial growth, and thus making sure that our samples are useful.

Step 2: After incubation for 24 hours at 37 ºC, each phenotypically different bacterial colony growing in the selective media and each colony showing brown to black discoloration in the surrounding medium in the VRE screening plates were considered of interest and were all transferred onto separate blood media plates.

Figure 8: **Three different culture media used to isolate the bacteria from collected samples: VRE media (a Bile Esculin Azide (BEA) agar supplemented with vancomycin), selective media (Eosin methylene blue supplemented with 2 mg/l of cefotaxime), and blood media**



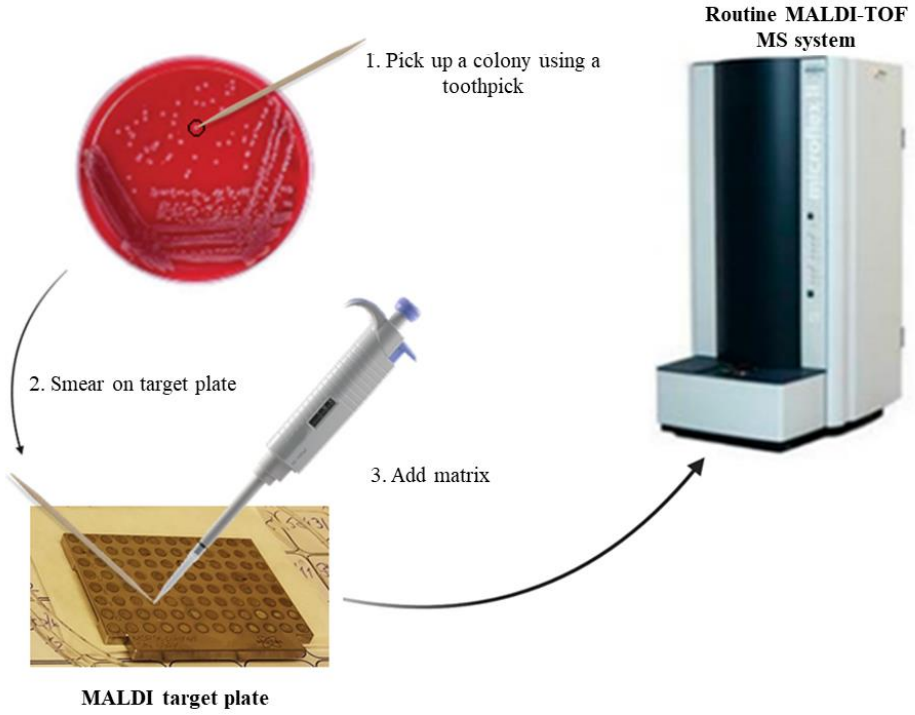
Step 3: Bacteria identification

After incubation for 24 hours, suspect colonies were identified by mass spectrometry (Matrix-Assisted Laser Desorption/Ionization-Time Of Flight) using the MALDI Biotyper® developed by Bruker Daltonics (a microbial identification system based on MALDI-TOF mass spectrometry that allows the identification of microorganisms within a few minutes down to the species level). Numerous studies reveal that the identification rates at the genus level are very high (97–99%) and vary from 85% to 97% at the level of species (ALBY et al., 2013).

A small part of the colony of isolated strains was picked up using a toothpick and spread on the MALDI target plate. We applied 0.5 µL of the MALDI matrix solution (**alpha-cyano-4-hydroxycinnamic acid, CHCA, or HCCA**) to each spot and then let the sample dry at room temperature for MALDI-TOF analysis (Figure 9). Bruker LT Microflex MALDI-TOF MS (Bruker, Daltonics, Germany) and Bruker Biotyper 2.3 system software were adopted to read the target plates. After analysis with Microflex LT, Biotyper software calculated a similarity score. Several scores per spot could be obtained, ranging from a higher to a lower probability of valid identification. According to the

manufacturer's instructions, a score ≥2.000 indicates identification at the species level; a score between 1.700 and 1.999 indicates identification at the genus level; and a score <1.700 indicates no reliable identification.

Figure 9: **MALDI-TOF mass spectrometry bacterial identification workflow. Bacterial colonies from a standard culture in a blood agar plate are applied directly on MALDI target plate via the standard smear method and overlaid with a matrix**



Once identified, we selected only the bacteria of interest, mainly *Echerichia coli* and other Enterobacterales such as *Klebsiella, Proteus, Morganella spp.*, etc. from the third-generation cephalosporins' investigation and *Enterococcus faecalis or Enterococcus facium* for the VRE investigation. A given identification was accepted with a high confidence identification score of 2.00-3.00.

Step 4: AMR susceptibility testing

Once the bacteria were identified and selected, an AMR susceptibility test by disc diffusion was performed following the European Committee on Antimicrobial Susceptibility Testing (EUCAST) recommendations. Thus, using Muller-Hinton media we tested the resistance to the following antibiotics: amoxicillin/clavulanic acid,

ertapenem, cefepime, ceftazidime, cefotaxime, amikacin, gentamicin, tobramycin, ciprofloxacin, and trimethoprim/sulfamethoxazole (Table 1). Vancomycin resistance was examined using the determination of the vancomycin and teicoplanin minimum inhibitory concentrations (MIC) using MIC test strips (Liofilchem, Roseto degli Abruzzi, Italy).

Table 1

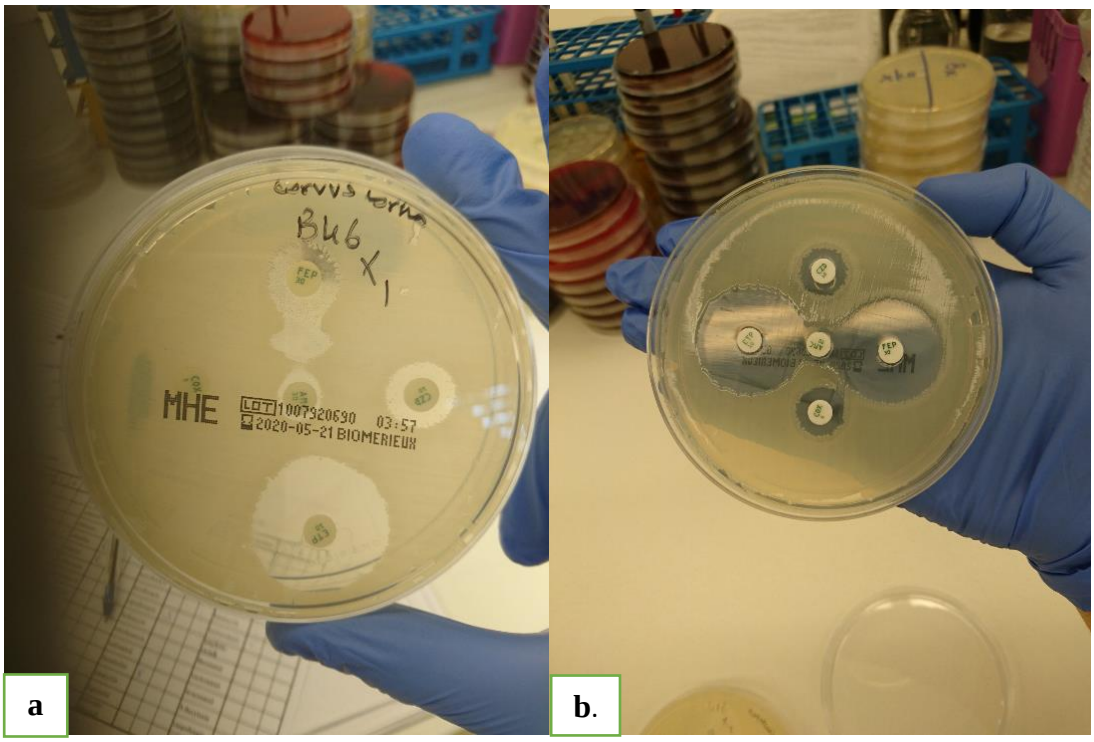
List of antimicrobial discs (manufactured by BIO-RAD) containing different concentrations used for susceptibility testing of Enterobacterales isolated from Hooded Crows.

Antimicrobial	Abbreviation	Disk concentration (µg)
amoxicillin + clavulanic acid	AMC30	20/10
Ceftazidime	CZD10	10
Cefepime	FEP10	10
Cefotaxime	COX5	5
trimethoprim + sulfamethoxazole	SXT25	1.25/23.75
Ertapenem	ETP10	10
Tobramycin	TMN10	10
Ciprofloxacin	CIP	5
Gentamicin	GMN10	10
Amikacin	AKN30	30

Following the standard Kirby-Bauer testing, a small inoculum of bacteria was prepared by using a McFarland Standard solution (prepared from the mixture of 0.05 ml (or 50 µl) BaCl2 in 9.95 ml of 1% H2SO4 solution) standard. By using a cotton swab, a well-isolated bacterial colony is picked, suspended in the solution, and mixed to match a 0.5-McFarland turbidity. The density of the suspension was adjusted to the required standard using a photometric device to measure the turbidity. Then bacteria from the

adjusted suspension streaked across a Mueller-Hinton media plate, dried over night at room temperature, to form a bacterial lawn using a cotton swab. The inoculum is thus spread over the surface of the media by swabbing in different directions in order to cover the entire surface evenly. The antibiotic discs were removed from the fridge and carefully put on the inoculated plates in a way that allowed the formation of disc synergy in case of an ESBL producer (Figure 10). While the MIC test stripes were carefully laid down on the agar, in opposite directions. These phenotypic procedures require around 18–24 hours before reporting any resistance information, which is inconvenient for prompt medical intervention (JORGENSEN & TURNIDGE, 2015). The test results allow us to identify and phenotypically characterise our bacteria, both ESBL producing Enterobacterlaes (Figure 10) and VREs. The prevalence of AMR was expressed as the percentage of samples from which resistant strains were isolated.

Figure 10: **Double disc synergy test. (a) shows phenotypic screening and confirmation of extended-spectrum β-lactamases (ESBL) production, (b) shows phenotypic screening of an AmpC producer isolate**



Susceptibility to antibiotics in our isolates was determined based on the inhibition zone diameter interpretation criteria determined by the EUCAST (EUCAST, 2020) (Table 2). As the Muller-Hinton plates are put against a dark background, and the inhibition zones are measured to the nearest millimetre with a ruler.

Table 2

The EUCAST breakpoint inhibition-zone diameters of antimicrobials used for disk diffusion test (https://www.eucast.org/clinical_breakpoints).

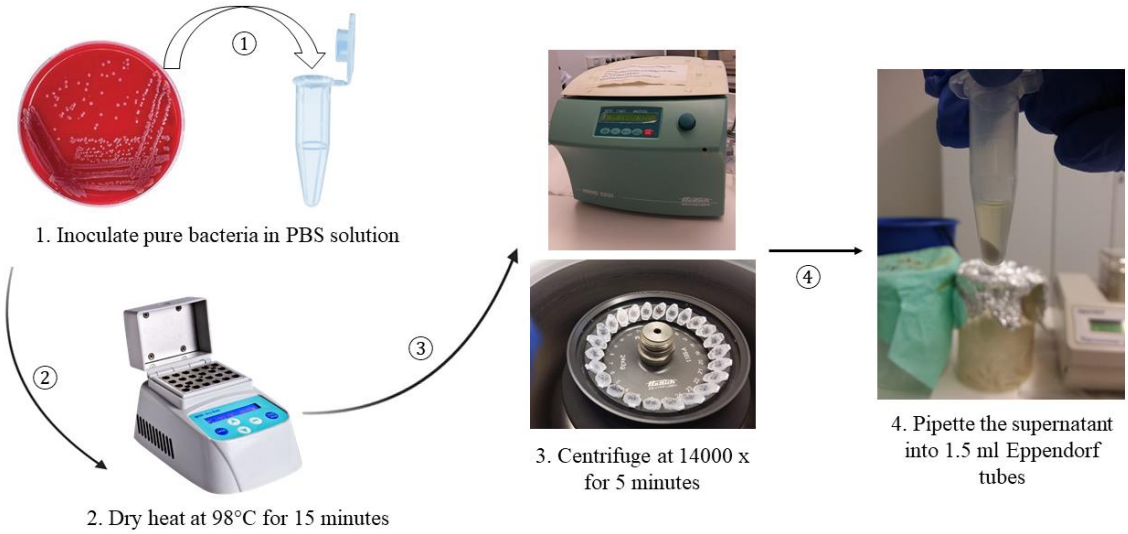
Abbreviation	Antimicrobial	Disk content (µg)	EUCAST Inhibition Zone Diameter (IZD) (mm)	
			Susceptible	**Resistant**
AMC30	amoxicillin + clavulanic acid	20/10	≥ 19	<19
FEP10	Cefepime	10	≥ 27	<24
COX5	Cefotaxime	5	≥ 20	< 20
CZD10	Ceftazidime	10	≥ 22	<19
ETP10	Ertapenem	10	≥ 25	< 25
CIP	Ciprofloxacin	5	≥ 24	< 22
AKN30	Amikacin	30	≥ 18	< 18
TMN10	Tobramycin	10	≥ 16	< 16
GMN10	Gentamicin	10	≥ 17	< 17
SXT25	trimethoprim + sulfamethoxazole	1.25/23.75	≥ 14	< 11

A multidrug resistant (MDR) isolate was defined as the one that was resistant to three or more of the seven classes of antibiotics tested.

Step 5: Bacterial DNA isolation

Since all the identified bacteria were frozen, DNA was isolated after the re-culture of the frozen isolates on blood agar. Grown colonies were picked, immersed in a phosphate-buffered saline (PBS) solution, incubated at 98 °C for 15 min, then centrifuged at 14000x for 5 min. The supernatant (the final lysate) was pipetted and stored in a 1.5 ml Eppendorf tube at -20°C (Figure 11).

Figure 11: **Bacterial DNA isolation workflow. All bacterial colonies from a standard blood agar are inoculated into PBS solution. Dry heated at 98°C for 15 min. Centrifuge at 14000x for 5 min. Pipette the produced supernatant**



Step 6: Resistance related gene detection using PCR and gel electrophoresis.

The main gene groups investigated are *bla*$_{CTX\text{-}M\text{-}1}$, *bla*$_{CTX\text{-}M\text{-}2}$, *bla*$_{CTX\text{-}M\text{-}9}$, *bla*$_{CTX\text{-}M\text{-}8}$ and *bla*$_{SHV}$ (Table 3).

Table 3

The oligonucleotide sequences used in this study.

Oligo name	Sequence (5'-3')
***bla*-SHV-F**	CGCCGGGTTATTCTTATTTGTCGC
***bla*-SHV-R**	TCTTTCCGATGCCGCCGCCAGTCA
***bla*-CTX-M-1-F**	ATGGTTAAAAAATCACTGCG
***bla*-CTX-M-1-R**	CAGCGCTTTTGCCGTCTAAG
***bla*-CTX-M-2-F**	GCGACCTGGTTAACTACAATCC
***bla*-CTX-M-2-R**	CGGTAGTATTGCCCTTAAGCC
***bla*-CTX-M-8-F**	CGCTTTGCCATGTGCAGCACC
***bla*-CTX-M-8-R**	GCTCAGTACGATCGAGCC
***bla*-CTX-M-9-F**	ATGGTGACAAAGAGAGTGCA
***bla*-CTX-M-9-R**	CCCTTCGGCATGATTCTC

The PCR master mix is always prepared according to the method assigned for different genes, as shown in Table 4. Differences in these mixtures are optimised for the genes examined. The thermal cycling programmes used for each reaction mix are shown in Table 5.

Table 4

Master Mix compositions for PCR amplification used in this study.

	1 Volume (2.5 ml) - PCR mix					
Groups	CTX-M-1	CTX-M-8	CTX-M-9	CTX-M-2	TEM	SHV
Water	16.4			15,4		
Mg (25Mm-os):	2			3		
Buffer (10 and 2Mm-os):	2.5			2,5		
dNTP (1mg/ml and 10mg/ml):	0.5			0,5		
Primers:	0.5			0,5-0,5		
5U/µl Enzyme:	0.1			0,1		

Table 5

Thermal cycling programs for PCR using the primers shown in Table 3.

GENE GROUPS	CTX-M-1 CTX-M-2 CTX-M-8		CTX-M-9		**GENE GROUPS**	SHV		TEM	
PROGRAM	Temp	Time	Temp	Time	**Program**	Temp	Time	Temp	Time
INITIAL	94 °C	5 min	95 °C	5 min	**Initial**	95 °C	5 min	95 °C	5 min
X 30 CYCLES	94 °C	1 min	95 °C	1 min	**x 35 cycles**	95 °C	1 min	95 °C	1 min
	55 °C	1 min	57 °C	1 min		56 °C	1 min	52 °C	1 min
	72 °C	1 min	72 °C	1 min		72 °C	1 min	72 °C	1 min
FINAL	72 °C	5 min	72 °C	5 min	**Final**	72 °C	5 min	72 °C	5 min
HOLD	4 °C	Infinity	4 °C	Infinity	**Hold**	4 °C	infinity	4 °C	infinity

After amplification, the amplimers were analysed by horizontal gel electrophoresis. A 1.5% agarose gel was prepared by adding 100 ml of 5% TBE buffer to 1.5g of agarose (Sigma). The mixture was microwaved and mixed simultaneously until

we had a homogenous mix, then we added 3 µl of SYBR™ Safe DNA Gel Stain (Thermo Fisher) to the mixture and poured it into a gel tray (with well combs), then left it to solidify for 20-30 min. Wells were loaded with 20 µl of PCR reaction product previously mixed with DNA loading dye (Thermo Fisher Agarose Gel Loading Dye), 7µl of a 100 base pair (bp) ladder in the first well, and positive and negative controls in the rightmost wells. Then we ran the electrophoresis for 60 min at 100 V. 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.

Step 7: Whole genome sequencing.

All ESBL-producing *E. coli* (197) isolates were subjected to whole genome sequencing (WGS) for the characterisation of the different ESBL strains carried by free-ranging Hooded Crows. 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.

To prepare Illumina-specific libraries, we used 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). We diluted the DNA samples to 0.2 ng/µL in nuclease-free water (Promega, Madison, WI, USA) to obtain a final volume of 2.5 µL. For the tagmentation reaction, five microliters of Tagment DNA buffer with 2.5 µL of Amplicon Tagment Mix were used. The samples were incubated for 6 min at 55 ℃ using the GeneAmp PCR System 9700 (Applied Biosystems/Thermo Fisher Scientific, Foster City, CA, USA) during tagmentation. The samples were left to cool to 10 ℃, and then 2.5 µL of the Neutralising tagment buffer were added. At room temperature, neutralisation was performed for 5 min. Then, we added 7.5 µL of the Nextera PCR Master Mix and 2.5 µL of the i5 and i7 index primers to the tagmented DNA samples. The index primers were incorporated into library DNA via 25 PCR cycles (each cycle consisted of the following steps: 95 ℃ for 10 s, 55 ℃ for 30 s, followed by 72 °C for 30 s). Following the PCR cycles, the samples were held at 72 ℃ for 5 min and then at 10 °C. Libraries were purified using the Gel/PCR DNA Fragments Extraction Kit (Geneaid Biotech Ltd., Taipei, Taiwan). The size selection of DNA libraries was performed by E-Gel™ SizeSelect™ II Agarose Gels, 2%. The concentration of the purified libraries was measured, and then the libraries were pooled and denatured. The denatured library pool

at a final concentration of 1.5 pM was loaded onto a NextSeq 500/550 High-Output flow cell and sequenced using an Illumina® NextSeq 500 sequencer (Illumina, San Diego, CA, USA).

FastQ files were quality trimmed using FastQC and filtered using FastQP, then errors were corrected using Bloocoo (a kmer-spectrum-based read error corrector). 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). 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 realised using the Enterobacterales_odb10 core gene database. The quality of the genome of our isolates was analysed using CeckM (PARKS et al., 2015) and Kraken2. The purity of isolates was analysed with checkm and kraken2 using the k2_pluspfp database (WOOD & LANGMEAD, 2019).

The resistomes were 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 (MultiLocus Sequencing Typing) (https://github.com/tseemann/mlst). Then, cgMLSTFinder 1.2 (CLAUSEN et al., 2018; ZHOU et al., 2020), VirulenceFinder 2.0 (CAMACHO et al., 2009; JOENSEN et al., 2014; MALBERG et al., 2020), and PlasmidFinder 2.1 (CAMACHO et al., 2009; CARATTOLI et al., 2014) tools available at the Centre for Genomic Epidemiology were used to identify genomic sequence types, virulence factors, and plasmid replicon types. Phylogenetic groups were identified using the Clermontyping web interface (http://clermontyping.iame-research.center/; BEGHAIN et al., 2018; CLERMONT et al., 2019).

3.6. Statistical analysis

All statistical analyses were performed using R 4.2.1 software (R Core Team 2022) or PAST software (Paleontological Statistics Software Package) version 4.03 (HAMMER et al., 2001). Data from each area (urban and rural) were pooled for the entire study period.

To enable the exploration of morphological divergence in Hooded Crows and the possible effect of urbanisation in the process, 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 (Zoo vs. open-air theatre, Sport Complex, and Agriculture Faculty Campus (Agrár Campus)).

We tested for divergence in functional morphological traits that are related to feeding ecology (i.e., bill morphology, body mass, and condition) as well as those related to the locomotor performances (i.e., wing, tarsus, and tail morphology, body mass, and condition) of Hooded Crows from rural and urban areas. Since the effect of urbanisation on Hooded Crows' body size and condition can be age-dependent, adult and juvenile individuals were considered separately.

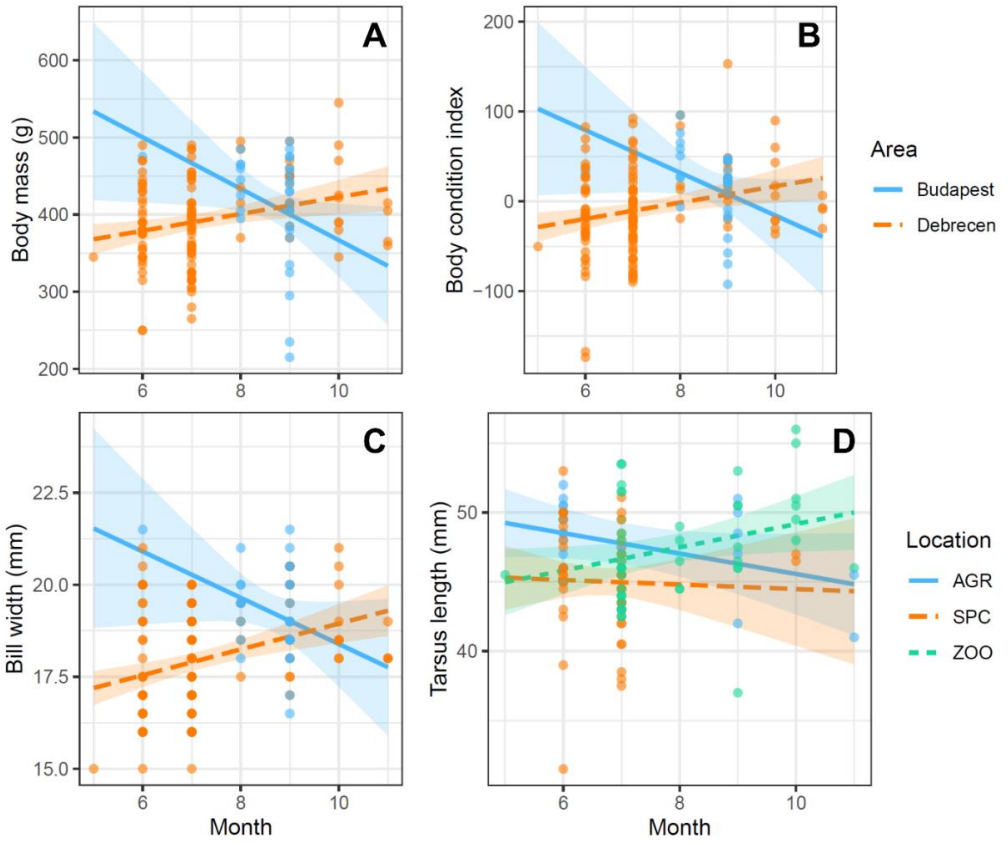
All morphological data were combined into a PCA analysis. Accordingly, PCAs and the correlation matrix between principal components and the original body size variables (Appendix 4) were calculated in PAST version 4.03 (HAMMER et al., 2001). Additionally, as an index to body condition, we calculated a regression residual between body mass and a tarsus length following the literature (LABOCHA & HAVES, 2012; DULISZ et al., 2016).

We tested the differences in the nine original morphological traits, the first four principal components obtained by a principal component analysis (PCA) conducted to reduce the original nine body size variables into four independent, non-correlating variables, and an index of body condition.

Additionally, as this study was conducted between the months of April and November, we evaluated any possible monthly variations in our variables. First, we tested whether the month of capture had an effect on all variables in simple linear and quadratic regression models. Although we did not find any significant monthly effect on adult crows' body measurements, the month of capture significantly influenced several variables in juvenile individuals, possibly because young birds continue to grow post-fledging. Following these results, we aimed to control the monthly variation in our variable of juvenile samples but not that of adults.

We constructed linear models with the month and a factor of interest as the two main effects. In the first analysis, the factor of interest was habitat type (urban vs. rural); in the second analysis, it was the city (Budapest vs. Debrecen); and in the third analysis, it was location (the capture site within the city of Debrecen). We also tested the interaction between the month and the factor of interest to test for changes in the opposite direction (Figure 12).

Figure 12: **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 (Agrár Campus of University of Debrecen), SPC (Sport Complex), and Zoo) in Debrecen (D)**



Depending on the significance of the interaction term, we proceeded accordingly:

1. In case of a non-significant interaction term ($p < 0.05$), we removed the interaction and accepted the model only with the month and the factor of interest.

2. In case of a significant effect of “month of capture” or “the factor of interest” ($p < 0.05$), we accepted the linear model as final.

3. In case of a non-significant effect of “month of capture” but a significant effect of “factor of interest”, we used Wilcoxon rank-sum tests to test differences driven by the factor of interest in Analysis 1 (urban/rural) and 2 (Debrecen/Budapest) and Kruskal-Wallis tests in Analysis 3 (between capture locations within Debrecen).

4. In case of adults’ measurements, for which different tested variables were not expected to change between months, we used Wilcoxon rank-sum tests (Analyses 1 and 2) or Kruskal-Wallis tests (Analysis 3).

5. for post-hoc testing:

- In case of a significant effect of “factor of interest” in the linear models, we compared mean values and contrasts between them using the ‘emmeans’ function in R (in Analyses 1 and 2) and reported the t-statistic and its associated p-value.
- In case the Kruskal-Wallis test was significant (Analysis 3), we compared group medians using the post-hoc Dunn test, as implemented in the ‘dunnTest’ function in R, and reported the z statistic and its associated p-value.

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, we used the False Discovery Rate method (BENJAMINI & HOCHBERG 1995) in all statistical tests presented in order to adjust the p-values. Other than the PCA, all calculations and statistical testing were conducted in R version 4.2.2. (R Core Team 2022).

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.

4. RESULTS

4.1. Interspecies morphological divergence in the Hooded Crow

4.1.1. Morphological differences between Hooded Crows 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, and found an important age effect on several morphological traits measured, and decided to divide our data into to subsets based on age. 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). We were able to explore phenotypic variation within both age groups at a city level, between large-scale (Budapest) and smaller-scale urban settings (Debrecen), as well as between juvenile individuals from different sampling locations within the same city (Debrecen) (Table 6).

The first, second, third, and fourth PCA axes had values of 45.193, 26.221, 10.945, and 8.903, respectively (Table 7). Therefore, we used the first four axes in our analysis, as they cumulatively explain 91.3% of the variation in morphology. The PC1 axis correlated positively with bill length and bill width, effectively describing bill size. The PC2 axis correlated positively with tarsus length, skull length, and body length and less strongly with wing length, tail length, skull width, and bill width, describing linear body size measurements or ‘lengthiness’. The PC3 axis correlated positively with body length and skull width and negatively with tarsus length, mostly describing body length. Lastly, PC4 was correlated positively with skull width, describing head and skull size (Table 8).

Differences in morphological traits were tested within both age groups from different habitats, despite the low numbers of juvenile measurements from rural areas. At a city level, between a large-scale (Budapest) and a smaller-scale city (Debrecen), as well as between individuals from different sampling locations within the same city (Debrecen) (Table 9).

Table 6

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 (at habitat level (urban and rural habitats), at city (Debrecen and Budapest) level, and at capture location level in the city of Debrecen (three locations (juveniles) or 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	AGR	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**	-

	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**
Adult	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**

Table 7

Eigenvalues and explanatory power of PCA axes.

PCA axis	Eigenvalue	% Variance explained
PC 1	38.479	45.193
PC 2	22.326	26.221
PC 3	9.319	10.945
PC 4	7.581	8.903
PC 5	3.408	4.003
PC 6	1.775	2.084
PC 7	1.494	1.754
PC 8	0.764	0.898

Table 8

Pearson correlation coefficients between PCA axes 1 to 4 and the original body size variables.

Variable	PC1	PC2	PC3	PC4
Body length	0.183	0.641***	0.636***	-0.373
Wing length	0.232	0.386	0.179	-0.215
Tail length	0.191	0.369	0.263	-0.053
Skull length	-0.048	0.671***	0.152	-0.067
Skull width	0.249	0.351	0.406***	0.828***
Bill length	0.986***	-0.011	0.018	0.049
Bill width	0.410***	0.379	0.191	-0.070
Tarsus length	0.251	0.890***	-0.431	0.029
Interpretation	‘Bill size’	‘Lengthiness’	‘Body length’	‘Skull width’

*** Bonferroni-adjusted $p < 0.0001$

Table 9

Results of statistical tests for the difference in body size variables of Hooded Crows between urban and rural habitats, between cities (Debrecen vs. Budapest), and between capture locations 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	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		
	Bill width	F = 3.677	0.099		**F = 21.252**	**0.0005**	**Budapest > Debrecen**	**F = 6.045**	**0.009**	**Zoo > SPC**	**0.085**
Adults	Body mass	W = 960	0.934		W = 157.5	0.815		H = 0.976	0.807		
	Fat reserves	W = 633	0.351		W = 130	0.815		H = 6.959	0.228		
	PC1 'bill size'	W = 1211	0.214		W = 190	0.913		H = 4.093	0.441		
	PC2 'lengthiness'	**W = 1352**	**0.015**	**Rural > Urban**	W = 222	0.815		H = 11.849	0.068		
	PC3 'body length'	W = 911	0.665		W = 207	0.885		H = 2.389	0.572		
	PC4 'skull width'	W = 911	0.665		W = 221	0.815		H = 1.861	0.645		
	Body condition	W = 794	0.236		W = 125	0.638		H = 2.711	0.548		
	Body length	W = 1246.5	0.135		W = 214.5	0.865		H = 6.869	0.228		
	Wing length	W = 921.5	0.665		W = 24.5	0.39		H = 11.494	0.068		
	Tarsus length	**W = 1353.5**	**0.015**	**Rural > Urban**	W = 220	0.815		H = 10.369	0.08		
	Tail length	W = 1099.5	0.236		W = 179.5	0.885		H = 4.432	0.441		
	Skull length	W = 1124	0.351		W = 224	0.815		H = 3.717	0.441		
	Skull width	W = 1191.5	0.236		W = 238.5	0.815		H = 3.039	0.526		
	Bill length	W = 1170	0.236		W = 183	0.885		H = 3.856	0.441		
	Bill width	W = 975.5	0.973		W = 158	0.815		H = 5.511	0.345		

In this study, urban and rural Hooded Crows differ in some of their morphological traits, with morphological divergence only being statistically significant for some traits. On a large scale (urban vs. rural comparison), we found that in juvenile Hooded Crows, 'bill size', and bill length were larger in urban birds than rural ones, in contrast; 'lengthiness', 'skull width', tarsus length, and skull length were found to be larger in rural birds than urban ones. Additionally, the month effect was statistically significant for different morphological traits in juvenile crows (Figure 12), including body mass (slope B = 9.506 ± S.E. 3.354; $F_{1,158} = 8.035$, p = 0.005), PC1 (0.837 ± 0.278; $F_{1,157} = 13.508$, p = 0.0003), body condition (9.034 ± 2.807; $F_{1,156} = 10.356$, p = 0.002), bill length (0.015 ± 0.005; $F_{1,157} = 9.589$, p = 0.002), and bill width (0.022 ± 0.005; $F_{1,157} = 24.205$, p = 0.0001).

This demonstrates that rural juvenile crows were bigger in size than urban ones, but the latter had stronger bills. In adult Hooded Crows, only 'lengthiness' and tarsus length were greater for rural birds than urban ones, indicating smaller adult crows in urban areas. No additional differences were significant. This higher variation in juvenile birds is probably due to the fact that these young birds can still develop during their post-fledging season, after the young leave the nest. This was supported by our results, in which the month effect was statistically significant for different morphological traits in juvenile crows (Figure 12), while no such effect was observed in adult crows.

Our findings are in accordance with previous studies, where locomotor traits such as tarsus length and tail length were smaller in urban birds than in their rural conspecifics (DULISZ et al., 2016; CAIZERGUES et al., 2018). Additionally, MEILLÈRE et al. (2015) previously showed that free-ranging House Sparrows (*Passer domesticus*) from urban areas exhibited shorter tarsi in response to urbanisation, while no other differences between urban and rural subjects were reported, a pattern also described by BÓKONY et al. (2012) in Hungary's sparrow populations. Several studies have described some inconsistencies regarding urbanisation's impact on birds' body size and condition, ranging from a negative or positive effect to no effect (FOLTZ et al., 2015; GIRAUDEAU et al., 2014; GRUNST et al., 2014). We found no difference in other body measurements assessed, such as body condition and wing length, in adult Hooded Crows. Still, urban juveniles had larger/longer bills but were smaller (shorter tarsi and smaller skulls) than rural ones. Although such findings are similar to previous studies (BADYAEV et al.,

2008; EVANS et al., 2009; AMIOT et al., 2022), due to the low sample size of rural juveniles (n = 3), our results are not conclusive, and further investigation is necessary.

At the regional scale (Budapest vs. Debrecen), Hooded Crows exhibited inter-city differences. The direction of the effect of the area seemed age-dependent. No differences were found in any of the tested variables between adult crows from the two cities. On the contrary, juvenile crows from the city of Debrecen displayed weaker body conditions and smaller bill forms (smaller 'bill size', bill length, and bill width) than individuals from Budapest. 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).

Juveniles had longer and thicker bills and better body condition in the larger city of Budapest than in the smaller city of Debrecen. This is noteworthy since previously, a trade-off between bill length and width was generally reported, with stubbier bills (deeper and wider bills with a higher ratio of bill width/length) either in rural birds (SHOCHAT, 2004; SHOCHAT et al., 2006; SERESS & LIKER, 2015) or in urban birds (EVANS et al., 2009; AMIOT et al., 2022). Longer bills might facilitate the access of hardly obtainable foodstuffs to juvenile crows, allowing thus the retrieval of food from hard-to-reach places such as garbage cans as well as ensuring greater biting force to break up hardly accessible foods such as hard-shell fruits or bones. Such changes in bill forms may also arise from urbanisation-induced changes in food resources (SHOCHAT, 2004; SHOCHAT et al., 2006; SERESS & LIKER, 2015). By changing their food preferences and feeding strategies, birds seemed to exhibit different bill formation, as bill size and shape in corvids were strongly correlated with their frequency of food searching (KULEMEYER et al., 2009). Thus, if longer and thicker bills contribute to the success of food finding and retrieval, individuals from Budapest may feed on less accessible food during their development than individuals from Debrecen. Such access to a wider range of food items could explain the better condition observed in birds from Budapest. Yet again, the higher availability of anthropogenic food sources in Budapest may also explain the differences observed in body condition. However, the significant month-city

interaction observed, demonstrating an increase in body mass or condition in Debrecen and a decrease in Budapest during the study period, may be due to the fact that most of the captures in Budapest happened in August and September, when late-summer droughts may have caused a temporary decrease in food availability, leading to a temporary decline in body mass or condition. In contrast, the seasonal increase in body mass and condition in Debrecen probably better reflects real-world tendencies, considering that measurements were more evenly distributed in the season. Nonetheless, the lower body condition of juveniles in Debrecen may also be linked to a poorer health status caused by diseases and parasites (PAP et al., 2011; GIRAUDEAU et al., 2014), high predation (MACLEOD et al., 2006), and low predictability of food sources in space and time, leading to reduced body reserves (SHOCHAT, 2004; BRODIN, 2007).

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 condition, more fat reserves, and a wider skull and bill than those caught elsewhere, especially those captured at the Sport Complex (SPC). What is more interesting is that such a high 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).

As the first nesting site of Hooded Crows' in the city of Debrecen, the Zoo has been the centre of these birds' colonisation process ever since (KÖVÉR et al., 2015). Essentially, the Zoo provides year-round high availability of easily accessible food in open-top enclosures. Crows were regularly seen in open-air enclosures where animals were regularly fed daily with nutrient-rich food (KÖVÉR et al., 2019). Additional anthropogenic foods are accessible to crows from trash bins, near open-air restaurants, and food stands. Whereas, levels of natural food sources are not as important since the Zoo in the city of Debrecen is mostly a forested area and has no open space, allowing any foraging chances (KÖVÉR et al., 2019). In contrast, the Sport Complex (SPC) is characterised by artificial green and grey surfaces, mainly regularly mowed lawns that likely provide suboptimal food to crows compared to the Zoo, dirt tracks, and hard concrete courts. Although the difference in food availability between locations could explain the differences observed, other explanations are also possible. For instance, since the Zoo is nesting site number one in the city, it is possible that juveniles caught there are the progeny of high-quality individuals, which makes them more likely to be larger-

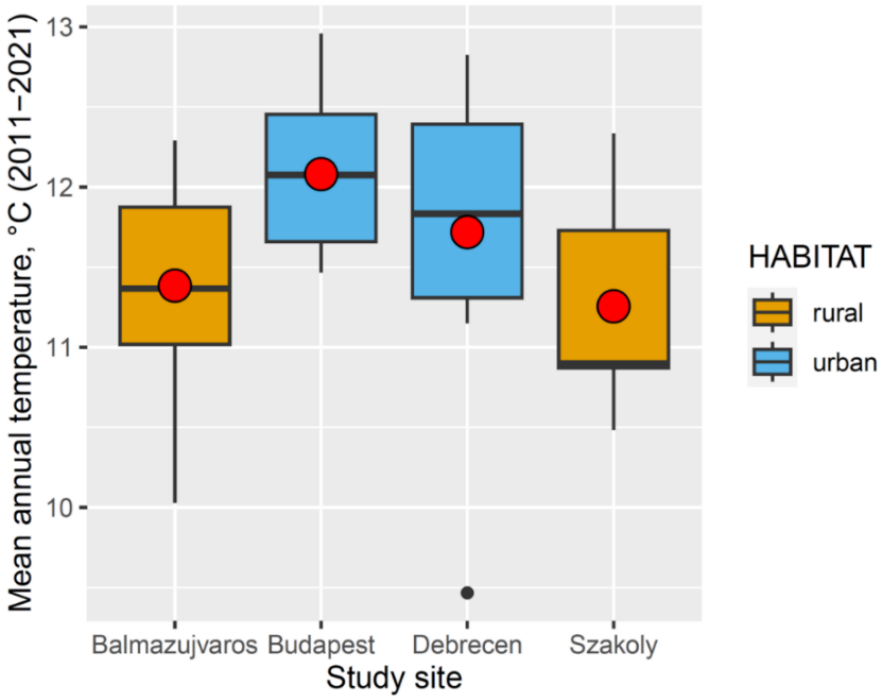
bodied than juveniles from other locations. Nevertheless, our findings at a local, within city-scale suggest that local environmental factors and population genetic mechanisms may attenuate or exacerbate the large-scale impact of the urban-rural gradient on the morphology of Hooded Crows.

4.1.2. Environmental factors: temperature and anthropogenic food availability

4.1.2.1. Local temperature

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 13). 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 13).

Figure 13: **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 significant difference between the annual average temperature between urban and rural study areas is unsurprising, since a warmer environment in urban habitat was expected, and it is consistent with the literature, which generally describes a warmer

climate in urban settings (HEISS et al., 2009; KRAUSMAN et al., 2014). The indifference observed in the annual average temperature between cities 200 km away also demonstrates the warmer environment resulting from increasing urbanisation. Ultimately, our study supports the notion that urban habitats do offer warmer environments than more natural habitats.

4.1.2.2. Anthropogenic food sources

The availability of anthropogenic food sources, such as restaurants and café shops, was greater in urban than in rural study areas (Table 10).

Table 10

Number of anthropogenic food sources within a 200 m radius circle in trapping and random sites in different study areas, shown in Appendix 2.

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

Based on the mapping of different food establishments in all four-study areas, it is clear that urban habitats contain anthropogenic food sources. Additionally, based on our survey, we can see that the presence of Hooded Crows in urban areas evidently overlaps with much more food sources within a 200-m range than in rural areas, where the presence of such sources is almost null. Moreover, the frequency of such sources seems to be higher in Budapest compared to the city of Debrecen, and therefore, Hooded Crows are presented with a greater chance of easily accessed food in Budapest. Our observation relates to the higher availability of artificial food sources in urban areas than in rural ones. This is in accordance with CEREGHETTI et al., (2019)'s records that the daily availability of anthropogenic food was superior within the urbanised area, thereby illustrating greater food abundance and predictability in urban areas (SHOCHAT, 2004; SALLEH HUDIN et al., 2016).

Presently, investigative research on the role of anthropogenic food sources on wildlife is limited. However, urban settlements offer affluence through easily accessed food sources, manifested almost exclusively in the form of non-natural food (SHOCHAT, 2004; SALLEH HUDIN et al., 2016). These sources can be a consistent food supply for urban wildlife, including omnivorous species with generalist diets like Hooded Crows (SHOCHAT et al., 2006; BALTENSPERGER et al., 2013; KÖVÉR et al., 2019).

4.1.2.3. Environmental factors and morphology

By comparing urban and rural environments, we confirmed that urban settings were warmer and had a higher availability of anthropogenic food sources than rural ones. Urban environments are generally described as being warmer than rural areas (HEISS et al., 2009; BALTENSPERGER et al., 2013; KRAUSMAN et al., 2014). Indeed, the resistant surfaces found in urban areas, such as asphalt and concrete, and the limited presence of tree cover in cities may support warmer environments throughout the year (EU SCIENCE HUB, 2022). Additionally, higher levels of solar energy and radiation commonly reflect warmer atmospheres, and cities are believed to have more solar energy thanks to their different anthropogenic structures and their ability to absorb sunlight and thus increase the heat levels in the area (ZITER et al., 2019). However, a recent study on urban-rural surface temperature variations found that in cities like Budapest, urban parks, green zones, and bodies of water such as the Danube River could reduce the urban heat island effect (EU SCIENCE HUB, 2022).

Higher temperatures have previously been associated with variability in animal morphology (BROMMER et al., 2015; CAIZERGUES et al., 2018; MAINWARING & STREET, 2021). Even small changes in response to warmer environments can trigger a series of trade-offs affecting foraging, reproduction, nest selection, food provisioning efforts, and migration (VAN DE VEN et al., 2019). For instance, in higher temperatures, birds may not be able to acquire enough food and water from their environment, and some birds may even reduce foraging activity, which can result in lower body mass and/or condition. This might explain why birds in urban environments can be smaller than their rural conspecifics. Our results are partly in agreement with Bergman's rule, suggesting that individuals of the same species living in warmer conditions are smaller than their conspecifics living in colder conditions. Our results also agree with several previous reports demonstrating that wild birds have been found to decrease their body size in

response to warmer climates (YOM-TOV et al., 2006; BROMMER et al., 2015; CAIZERGUES et al., 2018), especially sedentary ones (MAINWARING & STREET, 2021).

Higher availability of anthropogenic food sources in urban than rural environments has also often been reported (TRYJANOWSKI et al., 2015; CEREGHETTI et al., 2019). Anthropogenic food sources such as street food stands, restaurants, garbage cans, city dumps, etc. provide year-round availability of food, which is augmented also by bird-feeding activities by humans, which often provide high food availability all year long, with a higher intensity during the winter season (GALBRAITH et al., 2014; CEREGHETTI et al., 2019). Illustrating thus a greater food abundance and predictability in urban areas (SHOCHAT, 2004; SALLEH HUDIN et al., 2016).

In our study, the presence of the Hooded Crow in the urban environment most likely correlates with the abundance of easily accessible anthropogenic food, whereas in rural areas, where the capture happened exclusively in hunting areas, there was little human presence and no anthropogenic food sources. Such a gradient can have far-reaching consequences; for example, BROWN et al. (2022) showed that human-related food sources, particularly restaurants, could decrease wild bird species diversity yet increase the abundance of some species, especially omnivorous ones. The high availability of food may attract more generalist species with higher population densities, which in turn can drive competition for anthropogenic food resources, resulting in specialisation and even faster adaptation of urban species to their environment over time (SHOCHAT et al., 2006; BALTENSPERGER et al., 2013).

Considering the increasing availability of easily accessible food in cities, one would think that urban environments would benefit wild species able to tolerate anthropogenic environments and that such species would have a larger body size and display an overall better body condition. However, various reports indicate the contrary, i.e., birds in cities are often smaller and/or in a worse body condition than their rural conspecifics (EVANS et al., 2009; MÈILLERE et al., 2015). Higher food availability can increase the reliance of urban birds on specific food items, which can explain their lower body mass, and condition, and fat reserves (MÈILLERE et al., 2015). According to BRODIN's (2007) adaptive mass regulation hypothesis, the high predictability of food in urban areas can lead to lower body mass and a decreased body condition since storing fat

reserves is mostly required during food shortages, which urban birds rarely experience. Moreover, it is highly likely that not only the quantity but also the quality of anthropogenic food also influence the body size and condition of the birds. Anthropogenic food is often low in proteins and vitamins and rich in fats, carbohydrates, and salt, which may lead to lower breeding success (CHAMBERLAIN et al., 2009; MEYRIER et al., 2017; CAIZERGUES et al., 2018) and slower young development (PEACH et al., 2008; SERESS et al., 2012) in birds inhabiting urban areas. In addition, the lack of certain nutrients can impose direct costs on bird development. For example, vitamin B12 is central to carbohydrate and fat metabolism and affects weight gain, food intake, and feather development (VAN GROUW, 2018). Body size and tarsus length in particular are determined during the growth of individuals (ANDERSON, 2006), associating shorter structural size in birds with poor development conditions (PEACH et al., 2008; SERESS et al., 2012), supporting the idea that urban environments may limit bird development, which may be the case in the Hooded Crow in Hungary. Our results on shorter tarsi and structural size ('lengthiness') in urban than rural adult Hooded Crows agree with this mechanism and support the idea that urban environments may slow down or limit bird development. For instance, RICHNER et al. (1989) found that urban Carrion Crow (*Corvus corone*), a closely related species to the Hooded Crow, also exhibited smaller body size and shorter tarsi, which also resulted in smaller fledglings, and these findings were confirmed experimentally, where the potential effect of the availability of accessible food was tested (RICHNER et al., 1989).

While smaller body size is often related to poor conditions of development, it can also reflect ongoing adaptations to novel environments, ultimately allowing birds to survive and reproduce in urban environments. Accordingly, environmental changes observed in cities can induce adaptations at different levels (LIKER, 2020). For example, birds can change their food preferences and feeding strategies (SHOCHAT, 2004), but they can also exhibit morphological changes (BADYAEV et al., 2008; BOSSE et al., 2017). The availability of anthropogenic food may therefore be a driving factor in wild bird adaptation to novel environments.

In case the urban and rural crow populations were genetically related, one would not find morphological differences between individuals living in the different habitats. Same, earlier studies have detected gene flow between urban and rural populations (BJÖRKLUND et al., 2016; CARLEN & MUNSHI-SOUTH, 2021), while others did not

(TANG et al., 2016; MARKOWSKI et al., 2021). Unfortunately, molecular data is lacking to consider this possibility.

4.2. Occurrence of AMR in Hooded Crows in Hungary

4.2.1. Occurrence of ESBL producing Enterobacterales in Hooded Crows

The overall prevalence ESBL-producing Enterobacterales in Hooded Crow samples was 51% (134/ 264). ESBL prevalence in samples from the urban areas was 61% (130/212; with 108/171 and 20/41 isolates from Debrecen and Budapest, respectively), which was significantly higher than the prevalence of resistance in samples from rural areas (7.7%; 4/52; exclusively from Balmazújváros as no ESBL isolates were found in samples collected from Szakoly; chi square $p<0.0001$). With the overwhelming dominance of *E. coli* (2/4 and 122/130 in rural and urban positive birds, respectively), however, between urban sites, the prevalence was always comparable (Budapest: 48.8%; Debrecen: 63.2%, chi square $p = 0.10$).

A total of 221 isolated ESBL-producing Enterobacterales were isolated and further analysed. Overall, 82% (183/221) of the ESBL-producing Enterobacterales isolated from the Hooded Crow were MDR. All resistant bacteria isolated from rural areas were multidrug-resistant strains, while the prevalence of MDR in samples from urban areas is 92% (122/130). Isolated *E. coli* (84%, 167/197 isolates) were as likely to be MDR as other Enterobacterales (100%; n = 24 isolates).

AMR was common among *E. coli* isolated from free-ranging Hooded Crows studied, with clear patterns in resistance, considering that more than 60% of all crows carrying ESBL-producing bacteria were found in urban areas, in the proximity of different anthropogenic settings that can be possible sources for this observation. Indeed, previous studies described such occurrences (VITTECOQ et al., 2016; LARSSON et al., 2018).

Previous studies reported the use or misuse of antibiotics in human and veterinary medicine as well as the use of antibiotics in other agricultural activities to be an important cause for this frequent incidence of AMR (SIMOES et al., 2010; SMITH et al., 2014). Nowadays, following several reports of the emergence and spread of these organisms into the environment as well as wildlife (RADHOUANI et al., 2014; and WANG et al., 2017), the WHO has called for the use of a “One Health approach” when investigating ESBL-producing *E. coli* (WHO, 2019). Moreover, reports revealed similarities between ESBL-

encoding genes identified in wildlife and those identified in human and veterinary medicine, suggesting a possible gene transfer between humans, pets, and wild animals (ARNOLD et al., 2016; DOLEJSKA & LITERAK, 2019).

Although the prevalence of AMR in remote areas (SJÖLUND et al., 2008; SANTOS et al., 2013) demonstrates the presence of potential non-anthropogenic factors influencing AMR occurrence in wildlife, the wide distribution of antimicrobial organisms into the environment is yet to be fully understood. Most of the existing literature describes a close connection between the dissemination of AMR and proximity to anthropogenic factors (LANTHIER et al., 2010; ALROY & ELLIS, 2011; HASAN et al., 2015; ZURFLUH et al., 2016). Accordingly, easily contaminated anthropogenic establishments such as landfills and wastewater plants seem to be very important sources of human-related AMR observed in wildlife, especially urbanised birds (ALROY & ELLIS, 2011; ATTERBY et al., 2017; AHLSTROM et al., 2018; MARCELINO et al., 2019). For instance, ALROY & ELLIS (2011) found that the AMR strains of *E. coli* found in Yellow-legged Gulls (*Larus michahellis*) and Herring Gulls (*Larus argentatus*) are very much like those from human wastewater in the US and clinical isolates from France.

Thus, birds in contact with these sources could therefore be at increased risk of acquiring resistant isolates, although empirical data to support such an idea are scarce (VITTECOQ et al., 2016). Several studies have established the possible acquisition and, subsequently, sharing of AMR bacteria between different ecosystems (ATTERBY et al., 2017; WANG et al., 2017; AHLSTROM et al., 2018), as MARCELINO et al. (2019) articulated the possible re-introduction of acquired resistance by wildlife back to the environment.

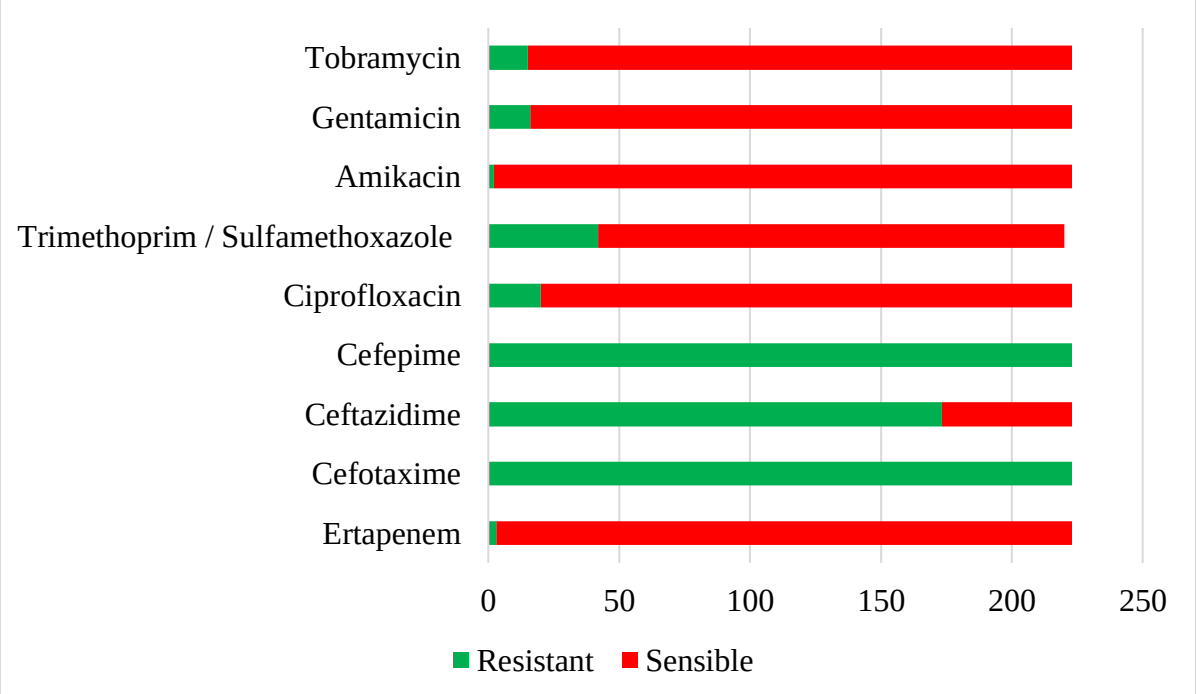
Our results fully support the notion that the occurrence of AMR in the wild Hooded Crow is undeniably related to their proximity to anthropogenic sources. In fact, the lower levels of AMR in rural Hooded Crows suggest that wildlife living in areas with high levels of human density are more likely to acquire AMR than those living in more remote areas. Nonetheless, the emergence of AMR in remote areas is noteworthy, highlighting the complexity of AMR dissemination in wildlife. Such findings suggest that ARB in wild crows might not be solely driven by anthropogenic factors. For instance, Hooded Crows could have picked up resistant isolates from a direct or indirect interaction with other wild birds possibly harbouring ARB, like wintering rooks migrating from

Russia and Western Asia (MAGDE et al., 2020), implying the role of birds' migratory behaviour on the one hand and the infinite influence of humans on the planet on the other.

4.2.2. Characteristics of ESBL producing Enterobacterales in Hooded Crows

Overall, 221 ESBL producers were recovered, 197 and 24 of which were *E. coli* and other Enterobacterales, respectively (90% vs. 10%). The *bla*$_{CTX\text{-}M\text{-}1}$ group genes were predominant in the Hooded Crow samples (n = 217/221), followed by *bla*$_{SHV}$ and *bla*$_{CTX\text{-}M\text{-}9}$, found in two samples each. With 71% of all isolates showed resistance to ceftazidime (158/217 urban vs. rural 4/4; 139/197 isolated *E. coli* vs. 19/24 Enterobacterales), and resistance against fluoroquinolones (9%) was also observed. Additionally, fewer isolates showed resistance to aminoglycosides (1-7%). Only 1.4% of ESBL-producing isolates displayed resistance to ertapenem; three exclusively urban isolates were found (Figure 14, Appendix 6).

Figure 14: **Multi drug resistance phenotypic profile amongst ESBL-producing Enterobacterales isolated from Hooded Crows.**



Resistance to third-generation cephalosporins has been frequently reported among *E. coli* (MAEYAMA et al., 2018; VOUNBA et al., 2019). Here, we found a high prevalence of ESBL-producing bacteria (51% of collected samples) resistant to third-generation cephalosporins, carried by free-ranging Hooded Crows almost exclusively

from urban habitats. These results are similar to previous reports (COSTA et al., 2006; WANG et al., 2017) and even higher than other reported incidences of ESBL strains in wild birds (VELDMAN et al., 2013; GÜNTHER, 2015; ALCALÁ et al., 2016), which may be due to the dominance of urbanised birds among our samples.

Although our study focused on Enterobacterales resistant to third-generation cephalosporins, the resistance of ESBL isolates to other antimicrobial agents, listed as essential medicines by the WHO, was also tested. Amongst these ESBL isolates, which were resistant to cefotaxime, ceftazidime, and cefepime, 68 strains (31%) showed no other resistance. Yet, some isolates were resistant to trimethoprim/sulfamethoxazole, quinolones (ciprofloxacin), and aminoglycosides (gentamicin and tobramycin mainly), consistent with findings in birds of prey (GUENTHER et al., 2012) and wild birds in general (VELDMAN et al., 2013; GÜNTHER, 2015). Accordingly, the ECDC reported that significant co-resistance to multiple antimicrobial groups in *E. coli* in human clinical isolates is common in the EU/EEA region (ECDC, 2022). Resistance to fluoroquinolones, third-generation cephalosporins, and aminoglycosides was 5-7% in all surveyed *E. coli* strains isolated from patients during the period 2016–2020 in the region, however, with high variation by country (ECDC, 2022).

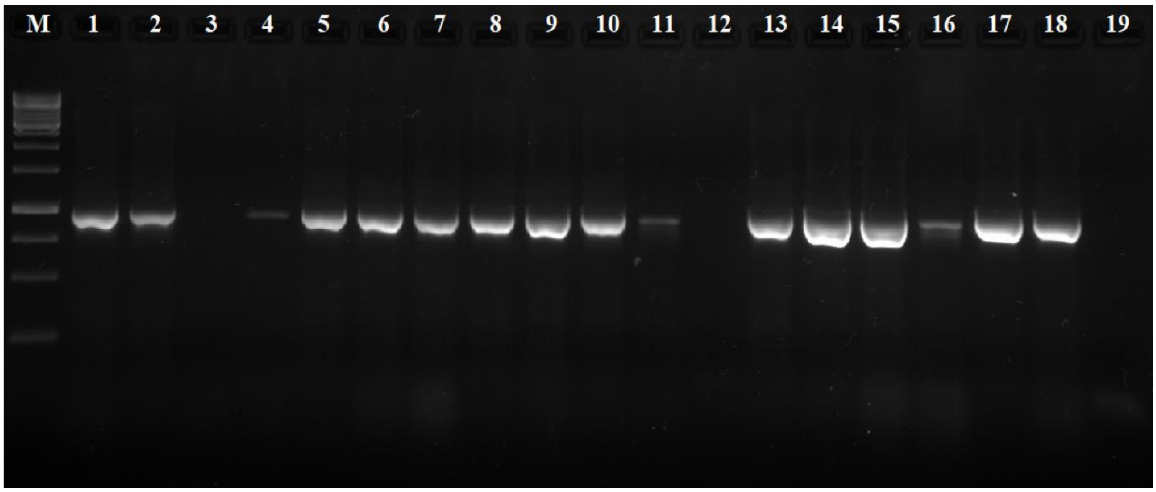
Traditionally, ESBL-producing bacteria display resistance to other antimicrobial classes, such as aminoglycosides, tetracycline, and sulphonamides, as well as fluoroquinolones (CANTÓN & RUIZ-GARBAJOSA, 2011). For instance, VALAT et al. (2012) reported high rates of such co-resistance in all ESBL-producing *E. coli* they isolated from livestock, ranging from 69-90% in different antimicrobial classes.

Such resistance reflects the co-existence of several resistance mechanisms to different antimicrobial classes in the same bacterial strain, which can be either the result of chromosomal mutations or the acquisition of resistance genes by horizontal transfer (TACÃO et al., 2014). Considering their strong ability to acquire other elements for resistance encoded by genetic materials such as plasmids, transposons, and integrons, ESBLs often carry genes responsible for resistance to antimicrobials other than beta-lactams, which makes them of particular importance.

4.2.3. Genetic characterisation of ESBL-producing Enterobacterales in Hooded Crows

In the present study, the group CTX-M-1 was predominant in the Hooded Crow (Figure 15). In addition to wild birds in Europe (BONNEDAHL & JÄRHULT, 2014; RADHOUANI et al., 2014; WANG et al., 2017), *bla*$_{CTX\text{-}M\text{-}1}$ was also predominant in livestock, including poultry (GÜNTHER, 2015), as well as in humans (ECDC, 2022).

Figure 15: **Agarose gel image showing the electrophoresis of CTX-M-1 gene group PCR; lane M: A 100 Bp. ladder; lane 1,2,4,5,6,7,8,9,10,11,13,14,15,16,17: positive samples; lanes 3, 12: negative samples; lane 18: positive control; lane 19: negative control**



Among the several genotypes responsible for ESBL production, CTX-M became the most common type in GNB, especially in Enterobacterales (CANTON et al., 2008). The spread of CTX-M enzymes was reported not only in clinical samples but also in domestic animals, livestock (ARNOLD et al., 2016; DOLEJSKA & LITERAK, 2019), and even wildlife (RADHOUANI et al., 2014; WANG et al., 2017). Furthermore, these strains of CTX-M-producing Enterobacterales are highly likely to be resistant to other families of antibiotics, leading to more limited treatment options.

First found by KNOTHE in 1983 and originating in species of the genus *Kluyvera* (reported by CANTÓN et al., 2012), the widely distributed CTX-M-type ESBLs have higher levels of prevalence than other ESBL variants such as SHV- or TEM-types worldwide (CANTÓN et al., 2012). This global dissemination of CTX-Ms was described as the “CTX-M pandemic” (CANTÓN et al., 2012).

The first ESBL-producing bacterium harbouring CTX-M-type enzymes was an *E. coli* isolated from dog faeces by Matsumoto in 1986 (CANTÓN et al., 2012). A few years later, more global reports of cefotaxime-resistant *E. coli* were published, and several variants of CTX-M types were recorded (CANTÓN et al., 2012). Subsequently, a worldwide emergence of a rapidly growing family of CTX-M enzyme-producing ESBLs among a wide range of clinical bacteria, especially the Enterobacterales, has been observed. Today, *E. coli* is still the principal host of these enzymes (CANTÓN et al., 2008). However, the prevalence of these enzymes in other Enterobacterales has increasingly been reported (CANTÓN et al., 2012).

Currently, there are more than 150 CTX-M beta-lactamases (www.lahey.org/studies/other.asp) classified into the following subgroups: CTX-M-1, CTX-M-3, CTX-M-8, CTX-M-9, and CTX-M-25 (CANTÓN et al., 2012).

The most frequently found genes in wild birds are $bla_{CTX\text{-}M\text{-}1}$ and $bla_{CTX\text{-}M\text{-}15}$ (WANG et al., 2017; ZURFLUH et al., 2019; ATHANASAKOPOULOU et al., 2022). Similarly, $bla_{CTX\text{-}M\text{-}1}$ and $bla_{CTX\text{-}M\text{-}15}$ were previously dominant in rooks wintering in Europe (LONCARIC et al., 2013; JAMBOROVA et al., 2015); more recently, the prevalence of $bla_{CTX\text{-}M\text{-}55}$ and $bla_{CTX\text{-}M\text{-}24}$ was also reported in wintering rooks (JAMBOROVA et al., 2015; SÖDERLUND et al., 2019; NAGY et al., 2021). Curiously, rooks (*Corvus frugilegus*) wintering in the same city predominantly carried different $bla_{CTX\text{-}M}$ genes (NAGY et al., 2021), highlighting the role of bird migration in the introduction of novel genes to an area.

Extensively described as a cause of human infections, the prevalence of CTX-M-1 and CTX-M-15-encoded beta-lactamases points to not only the zoonotic potential of *E. coli* but also to the possible transfer of ESBL-encoding genetic elements between humans, livestock, and wild birds (BONNEDAHL & JÄRHULT, 2014).

4.2.4. Molecular epidemiology of ESBL-producing *E. coli* isolates

4.2.4.1. Serotyping, Phylogenetic grouping and Multi Locus Sequence Typing

In this study, the 197 sequenced ESBL producing *E. coli* belong to 22 sequence types (ST) (Table 11). The most prevalent ST was ST58, found in 34 isolates. While core-genome (cg) MLST revealed that these ESBL isolates belong to 33 distinct cgSTs (Table 11).

Table 11

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 urban study areas (Budapest and Debrecen)

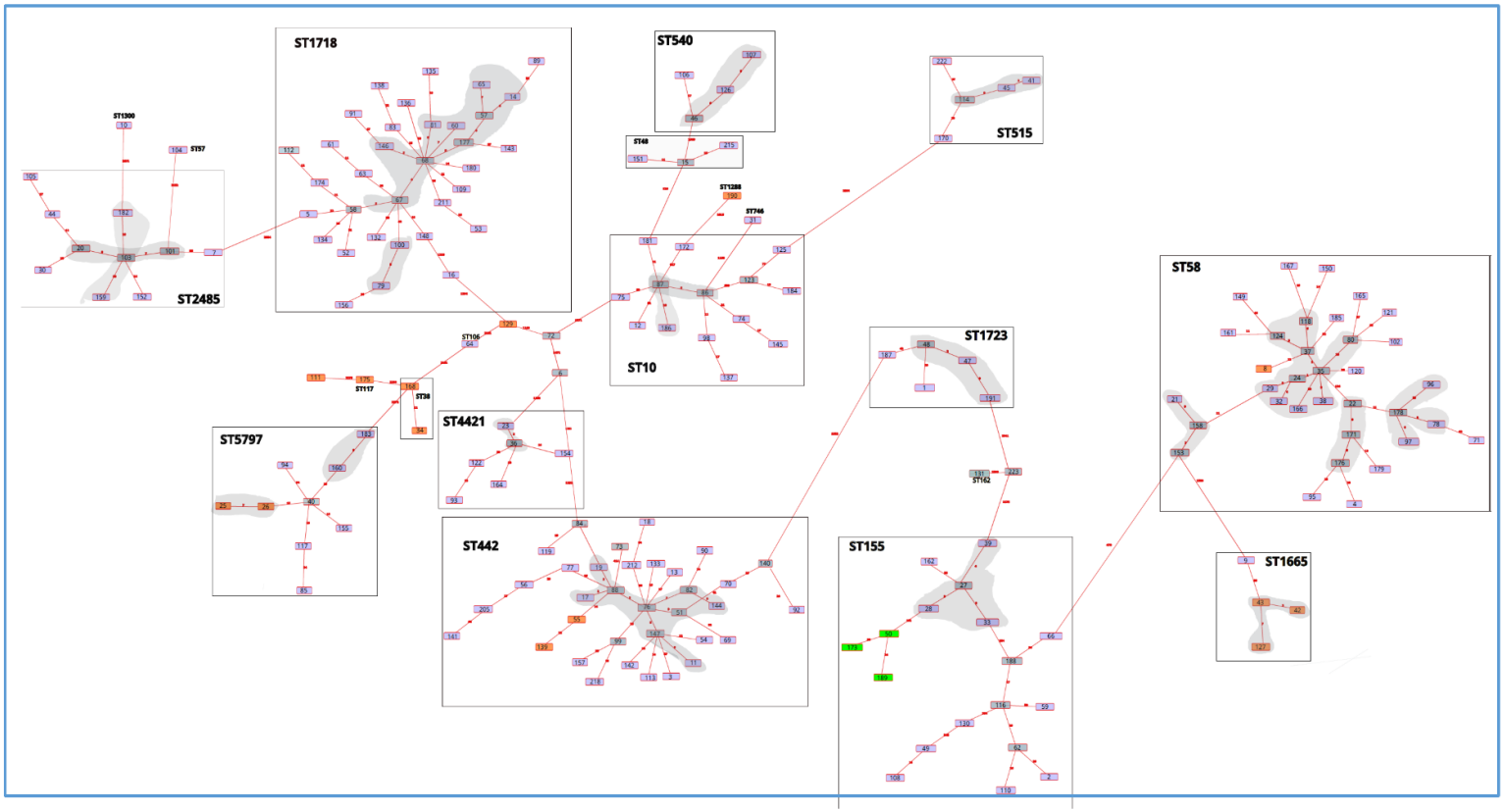
ST	cgST	Adult (%)	Juvenile (%)	Budapest (%)	Debrecen (%)
10		0 (0,0%)	12 (7,1%)	0 (0,0%)	12 (6,6%)
	196882	0 (0,0%)	9 (5,4%)	0 (0,0%)	9 (5,0%)
58		1 (3,4%)	33 (19,6%)	1 (8,3%)	33 (18,2%)
	45951	0 (0,0%)	11 (6,5%)	0 (0,0%)	11 (6,1%)
	46030	1 (3,4%)	22 (13,1%)	1 (8,3%)	22 (12,2%)
155		7 (24,1%)	10 (6,0%)	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%)

Most of the STs found have been previously described in human and animal isolates, such as 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).

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 11, Figure 16), and 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.

Figure 16: **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, 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)). 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. Moreover, overall, 21 different serotypes were predicted for the 197 analysed ESBL strains. Eighty strains had non-detectable O types and 14 different H types. For six isolates, the serotype was not determined (Table 12).

Table 12

Different MLST (STs) and core- genome MLST (cgMLST) profiles, phylogenetic groups and associated serotypes among 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

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 see that Hooded Crows host ESBL strains of primary importance. For instance, most isolates belonging to ST58 were affiliated with phylogenetic group B1, this ESBL-producing *E. coli* is found worldwide, previously isolated from a wide range of hosts, particularly avian ones, but not associated with serious diseases in humans and animals (MCKINNON et al., 2018). Such reports highlight the potential spread of commensal multi-resistant bacteria and the diffusion of resistance carried by these commensal strains to other possibly pathogenic ones (ZHUGE et al., 2019). Similarly, ST10 and ST155 represent important strains previously associated with the transmission of ESBL genes from animals to humans (SALIM et al., 2019). Another example of a widely distributed *E. coli* strain previously reported to cause extra-intestinal pathogenic *E. coli* (ExPEC) infection is ST38, also isolated in the Hooded Crow here. Our ST38 carried $bla_{CTX\text{-}M\text{-}15}$ and belonged to phylogenetic group D. Interestingly, only one isolate was identified as ST162, a major type sequence amongst wintering rooks in Europe (NAGY et al., 2021); nonetheless, this isolate is of great importance considering that ST162 was previously reported causing infection in livestock and other farm animals (ZHUGE et al., 2021). The occurrence of CTX-M-55 carrying ST162 highlights the recent spread of CTX-M-55 in Europe, as sedentary birds like the Hooded Crow are more likely to have acquired this strain from contaminated environments. It is noteworthy that the second most prevalent sequence type ST1718 (33/197) was reported only once in the available literature, a single isolate previously found in soil in farmland in Portugal (serotype O9:H31) (Jones-Dias et al., 2017). A survey of such strains in the surrounding agricultural setting in our study areas might provide insight into this occurrence.

These results highlight the potential threat that commensal *E. coli* can present to the healthcare system. The STs found have a global distribution and a range of hosts that build the human-animal-environment interface. Thus, the presence of ESBL-producing *E. coli* strains found circulating at such interfaces in Hooded Crows might be the result of their exposure to antibiotic residues, ARB, or resistance genes, considering their close encounters with different potential sources. Subsequently, the presence of these ESBL in

the faecal matter of these highly urbanised birds can be transferred to other potential hosts as well.

4.2.4.2. Resistance Gene Identification

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 (*aph(6)-Id* (54/197, 27.6%), *aph(3'')-Ib* (54/197, 27.6%), *aac(3)-IId* (12/197, 6.1%), *ant(3'')-IIa* (11/197, 5.6%), *ant(3'')-Ia* (9/197, 4.6%), and *aac(6')-II* (1 isolate)), fluoroquinolones (*QnrS1* (32/197, 16.3%), and *QnrB5* (1 isolate)), trimethoprim–sulfamethoxazole (*dfrA5* (10.2%, 20/197), *dfrA12* (4.6% 9/197), *dfrA14* (2.5%, 5/197), and *dfrA1* (1 isolate)), Sulphonamides (*Sul1* (5.1%, 10/197), and *Sul2* (21.9%, 43/197)), and tetracycline (*tet(A)* (14.7%, 29/197), and *tet(B)* (1%, 2/197)) (Table 13). All this genetic material might help the *bla*$_{CTX\text{-}M\text{-}}$ genes face co-selection pressures. Carbapenem resistance was not detected in these isolates.

Table 13

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

ESBL genes	Other resistance genes	Number of isolates
bla$_{CTX\text{-}M\text{-}1}$	-	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; tet(A); dfrA12; sul1	1
	APH(3'')-Ib;APH(6)-Id; QnrS1; sul2	1
bla$_{CTX\text{-}M\text{-}1}$/*bla*$_{TEM\text{-}1}$	-	24
bla$_{CTX\text{-}M\text{-}55}$	*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

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 importance to both humans and animals. Our findings are in accordance with previous reports of such ESBL types in wildlife, particularly wild birds (WANG et al., 2017; ZURFLUH et al., 2019; BLANCO et al., 2020; ATHANASAKOPOULOU et al., 2022). Hooded Crow isolates showed interesting co-resistance rates to non-β-lactam antibiotics. For instance, in contrast to rook isolates (NAGY et al., 2021), Hooded Crow-derived isolates carrying *bla*$_{CTX-M-15}$ showed resistance to aminoglycosides, fluoroquinolones, and trimethoprim–sulfamethoxazole. This is not surprising, as MDR strains carrying the CTX-M-1 group tend to co-carry genes encoding resistance to non-β-lactams, particularly *bla*$_{CTX-M-15}$. Similarly, isolates carrying *bla*$_{CTX-M-55}$ displayed higher levels of resistance to non-β-lactam antibiotics.

The prevalence of *bla*$_{CTX-M-55}$ in our Hooded Crows is noteworthy, considering its infrequent presence in Europe. Nonetheless, recently, *bla*$_{CTX-M-55}$ was reported in wintering rooks in Hungary (NAGY et al., 2021), which might explain *bla*$_{CTX-M-55}$ 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.

Concomitantly, we found that our disc diffusion method showed high resistance to different aminoglycosides, including gentamicin, amikacin, and tobramycin, in 21 isolates, which expressed various aminoglycoside resistance genes, including *AAC(3)-IId, ANT(3'')-IIa, APH(3'')-Ib, APH(6)-Id*, and *aadA2* genes. However, 38 isolates tested susceptible to aminoglycosides were found to harbour different aminoglycoside resistance genes, including *APH(3'')-Ib, APH(6)-Id, a*nd *aadA2*. It is worth noting that only three aminoglycoside drugs were tested in this study. The fact that we did not test for susceptibility to other aminoglycosides such as streptomycin, kanamycin, and netilmicin might explain our findings.

Whilst, not all ESBL strains manifesting phenotypic resistance to ciprofloxacin during the disc diffusion tests harboured *qnr* genes, as 15 of these resistant isolates did not carry *qnr* genes, a number of isolates carrying *qnr* genes (30/197) were identified amongst susceptible ESBL isolates. The acquisition of *qnr* genes by quinolone-susceptible ESBL producers might lead to a selection of cephalosporin and ciprofloxacin strains (JACOBY et al., 2014). However, the absence of the *qnr* gene in resistant strains suggests the presence of different quinolone-resistance mechanisms, such as gyrase (*gyr*A) mutations (JACOBY et al., 2014; REDGRAVE et al., 2014).

Previous studies have demonstrated the co-occurrence of *qnr* genes with ESBL-encoding genes in both human and wildlife isolates (MOHSIN et al., 2017; SALAH et al., 2019); we also found that in 14 strains, *qnr* genes have been associated with *bla*TEM/CTX-M combinations, similar to previous reports (SALAH et al., 2019). Such association is usually facilitated by multi-resistance plasmids linking different resistance determinants (JACOBY et al., 2014; REDGRAVE et al., 2014; SALAH et al., 2019).

Thirty isolates found to be resistant to trimethoprim-sulfamethoxazole, based on the susceptibility test, harboured one of the *dfr*A genes identified using the WGS. Only four phenotypically resistant isolates did not harbour any *dfr* gene. Similarly, of the 30 isolates that harboured *tet* genes, we found that 11 strains harboured *tet (A*) in association with $bla_{CTX\text{-}M\text{-}1}$ and 2 others harboured the tet *(A)* gene with $bla_{CTX\text{-}M\text{-}15}$. The *tet (A) genes* were also carried with a CTX-M-55/TEM-150 and a CTX-M-15/TEM-1 combination in 10 and two isolates, respectively. *Tet (B)* was found in two isolates carrying $bla_{CTX\text{-}M\text{-}14}$.

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 characterisation 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.

4.2.4.3. Plasmids in *E. coli* strains

In this study, dominant resistant genes $bla_{CTX\text{-}M\text{-}1}$, $bla_{CTX\text{-}M\text{-}55}$, and $bla_{CTX\text{-}M\text{-}15}$ were associated with a wide range of plasmid replicons (Table 14). And some of these strains also harbour aminoglycoside modifying genes (mainly APH (30 isolates)), sulphonamide resistance genes (*sul1* (9 isolates), *sul2* (23 isolates)), tetracycline resistance gene (*tet (A)*, 8 isolates), and quinolone resistance gene (*QnrS1* (2 isolates)). 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 14

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*.

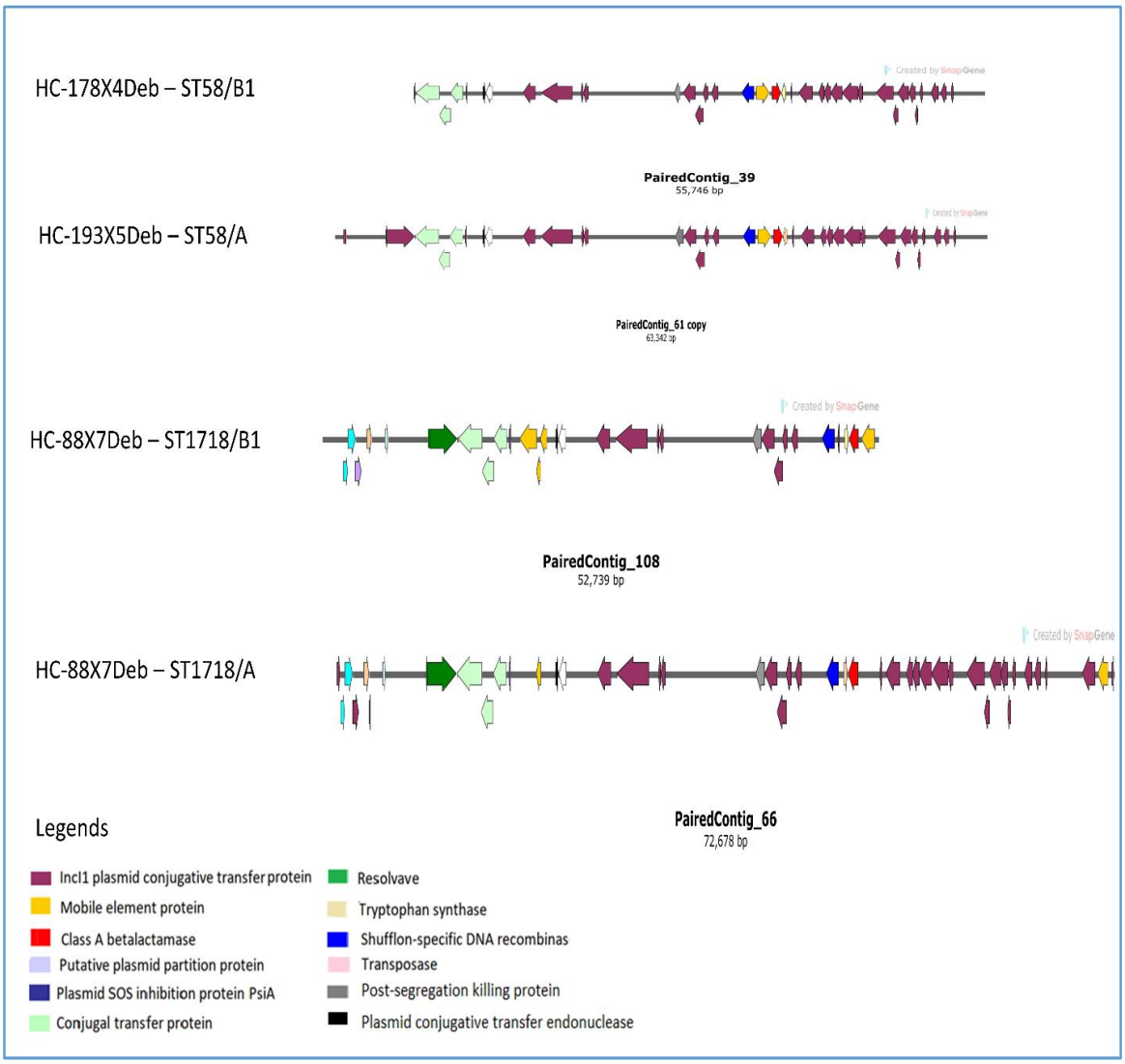
ESBL genes	Other resistance genes	Associated plasmids	Number of isolates
CTX-M-1		col156 / IncY	1
		IncFIB / INCI1-I	5
		IncFIC / INC1I-I	26
		IncFIA / FIC / INC1I-I	8
		IncFIA /FIB / FIC / INC1I-I	24
		IncFIB / INCI1-I / IncX1	6
		IncFIB / IncFIC/ IncI1-I	9
	APH(3'')-Ib;APH(6)-Id; QnrS1; sul2	IncFIB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

Furthermore, our findings revealed that *bla*$_{CTX-M-}$ containing contigs share a high degree of similarity among the strains with the same STs. Plasmid prediction revealed that genes encoding for mobile elements, transposases and plasmid conjugative transfer proteins were located near the *bla*$_{CTX-M-}$ genes in most of the strains (Figure17), suggesting the possible transfer of these ESBL genes via plasmids.

Figure 17: **Linear view of the genetic environment of *bla*$_{CTX-M-}$ 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/)**



The most frequent predicted plasmid type in our isolates, *IncI1*, was associated with most isolates carrying *bla*$_{\text{CTX-M-1}}$ (130 isolates) *and* *bla*$_{TEM-1}$ (20 isolates) in combination with *bla*$_{\text{CTX-M-1}}$. Previously associated with the *bla*$_{CTX-M-1}$ ESBL gene variant (CARATTOLI et al., 2021; ROZWANDOWICZ et al., 2018), *IncI1* is the most common plasmid in Enterobacterales from human and animal origins (SMITH et al., 2014) and the first plasmid associated with ESBL and AmpC gene transmission in farm animals (CARATTOLI et al., 2021), which is in accordance with our findings.

IncF plasmids are the second most frequent in our strains, not only associated with *bla*$_{CTX-M-1}$ but also with *bla*$_{CTX-M-14}$ and *bla*$_{CTX-M-32}$. IncF has variants, i.e., several replicons, such as FII, FIA, and FIB, which are often carried together in one multi-replicon plasmid (VILLA et al., 2010; ROZWANDOWICZ et al., 2018) and can co-exist with IncI1 and IncN (FROEHLICH et al., 2005; ROZWANDOWICZ et al., 2018).

CTX-M-55, the second most dominant ESBL type found in our isolates, was mostly associated with the IncN/IncF association, with only two of our isolates carrying *bla*$_{CTX-M-55}$ being linked to the IncI2 replicon. Although previously associated with IncI2 (LV et al., 2013), like IncF plasmids, IncN has been found to carry different resistance genes, which is the case in this study, as several strains harbouring CTX-M, APH, *QnrS*, *dfrA*, and *sul* genes were associated with IncN, either solely or in association with IncF plasmids. Whereas, the *bla*$_{CTX-M-15}$ was largely associated with col156/IncY and IncFIB/IncY. This frequent ESBL type in human clinical *E. coli* isolates in most EU countries is often in association with IncF plasmids (ROZWANDOWICZ et al., 2018). Nonetheless, IncY, a rarely reported plasmid, was found in 11 Hooded Crow isolates, carrying *bla*$_{CTX-M-1}$ (6 isolates), CTX-M-15/TEM-1 combination (four isolates), or *bla*$_{CTX-M-15}$ (1 isolate). Although multireplicon plasmid col156/IncY has not been reported yet, IncFIB/IncY was previously reported in dog faeces collected from local gardens in Denmark (DAMBORG et al., 2015). Additionally, col156 plasmids, a specific col-like replicon recognised as important transmitters of resistance genes, were reported to have a wide distribution (KONDRATYEVA et al., 2020). Such findings suggest that either Hooded Crows carrying these strains were exposed to CTX-M-producing *E. coli* or plasmid recombination occurred.

From a One Health perspective, the presence of these genes must be monitored, especially in bacteria with zoonotic potential. Here, we found that ESBL-producing *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 of large animal and human occurrences. Additionally, the potential presence and coexistence of mobile plasmids and their association with CTX-M genes highlight the possible transmission of such resistance between different bacteria and the introduction of different ARB and AMR genes into the wild (BLANCO et al., 2020; ATHANASAKOPOULOU et al., 2022), which may lead to higher risks to human and animal health.

4.2.4.4. Virulence characteristics

Based on the WGS results, *E. coli* isolated from Hooded Crow had a high prevalence of several virulence factors (VFs), including *hlyE, hlyF, iss, iroN, gad, cib, cvaC, ompT, mch B,C,F, IpfA, iucC, iutA,* and *afaD*. Amongst the 197 isolates, the prevalence of VF genes 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).

Since VFs are unequally distributed amongst *E. coli* strains (CLERMONT et al., 2000), there is no basic virulence profile to clearly identify a given strain as a commensal or an ExPEC or distinguish which pathogenic strain group it may belong to. Although most Hooded Crow-isolated *E. coli* belonged to phylogenetic groups B1 and A, a number of these isolates seemed to harbour both avian pathogenic (APEC) and ExPEC virulence genes.

Previous studies have shown that APEC and ExPEC strains are phylogenetically close and share some VFs, suggesting the potential role of APEC as a reservoir of virulence genes for ExPEC. Previous studies also demonstrated the potential of commensal *E. coli* to 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 (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).

The presence of isolates harbouring several key virulence genes (*iss, iroN, hlyF, ompT, and iutA*), such as ST10 and ST38, a globally disseminated extra-intestinal pathogenic *E. coli* (ExEPEC) with increasing importance in human health (BOJESEN et al., 2022), suggests that the Hooded Crow-derived isolates might be reservoirs of APEC virulence genes. And considering the frequent reports of these virulence genes in the human ExPEC strain (ZHUGE et al., 2019) a zoonotic transmission of such virulence from avifauna to humans is foreseeable. Thus, crows carrying these strains might pose a threat to both animal and human health.

In summary, this study provides insight on ESBL-producers' carriage by Hooded Crows in Hungary. The prevalence of $bla_{CTX\text{-}M}$ along with resistance genes of other important antimicrobial classes highlights the potential role of these free-ranging birds as reservoirs for MDR bacteria. Moreover, the ability to harbour multiple plasmids might facilitate the transmission and dissemination of ARB and resistance material, emphasising the need for more surveillance studies to demonstrate AMR occurrence, investigate its origin, and fully demonstrate the role of wild birds as reservoirs, vectors, and indicators of AMR in the environment, especially in urban habitats.

4.2.5. 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 carried VRE strains. Enterococci were detected on nonselective media, which were not vancomycin-resistant, demonstrating that these crows carry enterococci in general but not VRE strains.

VRE is an increasing global health problem. In addition to their increasing occurrence in medical establishments, the presence of VREs in domestic animals like dogs (RAMOS et al., 2012; WADA et al., 2019) and their isolation from wildlife (ORAVCOVA et al., 2016) is of growing concern. The emergence of VRE outside health institutes in Europe, especially in farm animals, has been attributed to the use of avoparcin

as a growth promoter (KRUSE et al., 1999); however, despite the ban on avoparcin by the EU in 1997, VRE still occurs years later (NILSSON et al., 2009). In contrast, VRE is rarely found in livestock in North America, where avoparcin has never been approved for use in animals (LANTHIER et al., 2010). Nevertheless, a few isolates were detected in the faeces of the American crow in the USA (ORAVCOVA et al., 2013). Demonstrating that the origin of these VREs in wild birds may be anthropogenic, such as wastewater, landfills, or other urban environments where birds are in close contact with potentially human-contaminated objects or foods. In this manner, VRE seems to behave similarly to the findings presented concerning ESBL producing *E. coli*.

Previous findings of VRE among birds of the genus *Corvus* (ORAVCOVA et al., 2013, 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; AYOBAMI et al., 2020; WERNER et al., 2020). 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., 2013, 2016), a potential environmental spread of VRE should be foreseeable, warranting monitoring of these urban bird populations.

4.2.6. Role of Hooded Crows in the dissemination of AMR

Present data and those reported in previous works clearly show that wild birds play a significant role in the dynamics of AMR. They can be reservoirs of antimicrobial-resistant bacteria and resistance genes, considering the high prevalence of AMR found over the years. Thus, even in the absence of direct antibiotic pressure, resistance levels can be due, in large part, to the bird's feeding behaviour. Most of them search for food in agricultural and urban areas, sewage, and garbage dumps that are potentially contaminated with human-derived bacteria, which facilitates the acquisition of such

resistant bacteria from human and livestock sources. This is underlined by the vast difference between the prevalence of AMR in rural and urban birds.

In the case of Hooded Crows, their flexibility as an omnivorous, highly urbanised bird and proximity to human settlements can favour the accumulation of resistant bacteria derived from their diet and contact with anthropogenic sources. There are various studies supporting the idea of the transmission of resistances from anthropogenic sources to wild birds (HASAN et al., 2015; SKURNIK et al., 2016; SWIFT et al., 2019; TOUCHON et al., 2020). Our findings in Hooded Crows, especially birds living in urban areas (especially isolates harbouring CTX-M genes), might indicate the possibility of AMR transmission between humans, livestocks, and wildlife. The particularly high proportion of commensal ESBL-producing *E. coli* carrying the CTX-M-1 group reported in this study may be due to the spread of one or various successful plasmids or the predominance in avian species of specific clones (such as ST58/B1 and ST58/A) carrying this enzyme. While the prevalence of CTX-M-55 reported as the second most predominant in these Hooded Crows may be due to their contact with wintering Rooks, previously found to harbour mainly this type of beta-lactamase-encoding gene (NAGY et al., 2021).

Additionally, some studies have been focusing on the epidemiology of zoonosis involving wildlife (KRUSE et al., 2004); the role of the latter in the ecology and evolution of AMR is poorly addressed, especially in highly active (mobile) animals like birds. Little is known about the evolution of AMR in nature, and the mechanisms of AMR acquisition are still ambiguous; is this process caused by the direct transfer of ARB, or by the horizontal transmission of related genetic materials, or most probably, both.

We believe that wild birds, particularly in urban areas, may be an important source of information for AMR surveys. Wild birds are the most studied hosts, where $bla_{CTX\text{-}M\text{-}1}$ and $bla_{CTX\text{-}M\text{-}15}$ were the most frequently found genes (WANG et al., 2017). Birds may serve as long-distance vectors of strains or genes of human origin. In Chile, BÁEZ et al. (2015) found that Franklin's Gulls (*Leucophaeus pipixcan*) carried widespread ST131/CTX-M-15-producing strains, which are exceedingly prevalent in humans in the United States but rarely found in South America. Similarly, this pandemic lineage was also found in wintering Rooks in Hungary (NAGY et al., 2021). Although ST131 was not detected in our samples, strains previously associated with human and animal health (e.g., ST10, ST38, ST48, ST155, and ST442) were found.

The occurrence of ARB in wild animals is increasingly reported across different areas. These wild animals act like a biological transportation service for AMR bacteria and AMR genes (ALLEN et al., 2010). Moreover, several authors have implied that the migratory nature of some wild birds may explain the introduction of AMR in some of the most remote areas in the world (SJÖLUND et al., 2008; HERNANDEZ et al., 2010). In the case of the Hooded Crow, a semi-migratory wild bird, more or less sedentary in Hungary and very successful in urban environments, with its increasing direct and indirect contact with humans, the prevalence of AMR implies the ability of these birds to acquire resistance from contaminated surroundings, making them a typical model for AMR surveillance in the environment. Their carriage of ESBL-producing GNB is an example of their important role in such surveillance. The finding of $bla_{CTXM\text{-}1/55/15}$, along with genes conferring resistance to other classes of antimicrobials in crows, highlights the wide spread of MDR Enterobacterales in the environment and the need for an integrated surveillance scheme.

4.2.7. Role of urbanisation in AMR emergence

Urban environments offer high chances of spatial overlaps between humans, animals, and urbanised wildlife and play a key role in different exchanges between these subjects. For instance, wildlife in urban areas can pick up pathogens of human and veterinary origin from different anthropogenic sources. With the increasing occurrence of wildlife in anthropogenic settings, the human-animal-wildlife interfaces outline critical points for pathogen emergence and cross-species transmission (OSTFELD et al., 2018). Subsequently, associating urbanisation with the prevalence of ARB in free-ranging wildlife is feasible. The dynamic of AMR emergence can be determined by changes in population abundance and contact rate between different groups, thus influencing the risk of cross-species ARB transmission.

Here, we assessed whether ARB isolated from wild Hooded Crows had a higher prevalence in individuals from urban study areas. A statistically significant difference between the frequency of isolated ESBL-producing Enterobacterales in urban and rural areas was found, with frequencies of 60% and 7% of carriers in urban and rural settings, respectively. Moreover, the high prevalence of globally spread STs such as ST10, ST38, and ST58 circulating at the human-animal-environment interface in the Hooded Crow suggests that urban free-ranging crows may be more exposed to AMR of human and

animal origin, and they are more inclined to acquire AMR genes from urban environments. Moreover, the presence of ARB and AMR genetic materials in the faecal matter of Hooded Crows may itself be a source of contamination, resulting in the transfer and reintroduction of these elements into the environment.

5. CONCLUSIONS, RECOMMENDATIONS

5.1. Conclusions

Hooded Crow is an ideal model for investigating the effect of urbanisation as well as a suitable host species to investigate and monitor AMR bacteria in Hungary. Urbanisation is a process that provides a clear interface where humans, animals, and the environment interconnect, creating a complex system of great interest. Our data show the importance of the interface not only for the well-being of wildlife but also of humans, emphasising the One Health concept.

Urban environments distinctly differ from natural ones, offering a new selective force that is changing the composition of wild bird communities tremendously. Likely to affect different life traits in wildlife, urbanisation may have long-term consequences for urban residents. Additionally, the changes observed in different traits in urban individuals are attributes of the response of these bird species to manmade habitat alterations. Moreover, human-made habitats are generally characterised by high food availability and a warmer climate. Urbanised birds, such as corvid species, respond to these novel ecological factors by modifying different traits, including morphometric ones.

In the present study, we found that urban Hooded Crows were smaller and had shorter tarsi than their rural conspecifics. In urban areas, adult crows showed no significant difference. While juveniles from Budapest (a larger-scale city) had longer and thicker bills and better body condition than individuals living in Debrecen (a smaller, less built-up city). Between capture locations within the city of Debrecen, birds living around the Zoo area were bigger, heavier, had better body condition and more fat reserves, and had wider skulls and bills than in other locations. Overall, these intraspecific phenotypic divergence observed between individuals from different habitats demonstrated the adaptation potential of the Hooded Crows to urban habitats. Additionally, the indifferences observed in the body condition and body mass of Hooded Crows from different habitats here indicated that these birds have the ability to sustain their nutritional

needs in an urbanised environment, most likely due to the high availability of easily accessed food. However, morphological differences observed over larger and smaller scales suggest the occurrence of rapid responses to local factors and provide insights that could be critical for the ecology and evolution of species thriving in human-made environments and the possible consequences unfolding. Nonetheless, here, urbanisation seems to drive adaptation in this Hooded Crow.

On the other hand, the high prevalence of clinically important ARB, manifested in the occurrence of third-generation cephalosporin-resistant ESBL-producing Enterobacterales in urban Hooded Crows, underlines the importance of the interconnections between human health, and animal heath, as well as their shared environment, and highlights in the process the role of urbanisation in facilitating these interconnections and their foreseeable consequences. Our findings provide a first look into a so-far poorly explored part of the global environment. The presence of other resistance genes against several antibiotics, including sulphonamides, trimethoprim, quinolones, and aminoglycosides, with several virulence agents, as well as the presence and possible cohabitation of transferable plasmids, in Hooded Crows illustrates the potential value of this wild bird as a sentinel of AMR and zoonotic agents in the environment. Additionally, our results provide a comprehensive phenotypic and genotypic characterisation of ESBL-producing *E. coli* isolated for free-ranging Hooded Crows in Hungary. Our data shows that not only phylogenetic groups B2 and D are of priority, considering the predominance of commensal ESBL producers found. While the molecular analysis of our data revealed the presence of globally disseminated strains, such as ST10, ST442, and ST155, displaying a rapid increase and spread in a range of hosts, of which the Hooded Crow seems to be a reservoir. Specific actions under the One Health approach to assess the potential implications of these findings for the human population and/or for environmental health are necessary. AMR surveillance schemes targeting wildlife in urban settings, where a new wildlife–human interface is growing, are needed. Since wild species, like the Hooded Crow, may become infected and/or acquire resistant bacteria through the consumption of anthropogenic food sources in public spaces in cities, further strategies preventing contact between these wild species and humans should be further developed. Besides, the current absence of VRE bacteria in these ESBL-carrying crows must be stated, demonstrating that Hooded Crows do not carry VRE strains despite previous findings amongst the genus *Corvus* in Europe (ORAVCOVA et al., 2013, 2016).

Still, taking into account the multi-resistance observed in hospital-isolated strains. A potential environmental spread of VRE should be foreseeable, warranting monitoring of urban bird populations like the Hooded Crows in urban settlements.

5.2. Recommendations

The morphological differences observed in the Hooded Crows are attributed to the differential responses of this free-ranging bird species to habitat alterations caused by urbanisation, suggesting further inquiries on the role of the different environmental factors in urban areas. Indeed, considering the presumed effect of anthropogenic food sources on urban wildlife, spatial and temporal analyses by urban Hooded Crows should be considered for better understanding the adaptation of these wild birds to urban settlements. Further studies involving multiple scales, multiple sites, and factors other than food availability and microclimate will be appropriate to explore inter-site variability in morphology and may result in a more robust perception of the effect of urbanisation on wildlife. Additionally, associating morphological and behavioural studies should be considered since urban corvids were previously found to be bolder than rural ones (BJÖRKLUND et al., 2010; MATSYURA et al., 2015), which might influence their foraging patterns and consequently their growth. Finally, investigating morphological changes at a molecular level is also recommended, as future studies to determine the adaptive nature of these morphological variations and to test their potential plastic or genetic origin are required. Consequently, worldwide cooperation for collecting data over multiple urban areas might promote the recognition of global designs and essential standards of urban wildlife ecology.

Likewise, understanding the role of urban environments in the emergence and spread of ARB is crucial for a better comprehension and adequate management responses. Accordingly, a continuous investigation into the frequency of ARB as well as regular surveillance of such microorganisms in wildlife in both anthropogenic and natural environments are essential to face future public, animal, and environmental health challenges. Furthermore, we call for inter-disciplinary cooperation that allows the examination of human-animal-wildlife interactions, especially in urban areas, in order to characterise the potential high-risk interfaces and identify possible routes for the potential cross-species transmission of AMR. We believe that such a concept would consolidate the current knowledge on the dynamics of this phenomenon and the development of appropriate prevention approaches that can be used to reduce transmission risks.

6. 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).

7. 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.

8. SUMMARY

Urbanisation is a complex process that creates an interconnected interface that includes humans, wildlife, and the environment, where interdependent exchanges happen between these different parties, affecting all. Thus, in order to understand this interface, we aim to study how this phenomenon influenced wildlife populations and the consequences behind the success of wild birds like the Hooded Crow (*Corvus cornix*) in cities from the One Health point of view. Urban habitats commonly display different ecological features than more natural ones. Therefore, we first explored the morphological differences between urban and rural crows in order to explore crows' responses to urbanisation. We then investigated the presence of ARB of health importance in these free-ranging wild birds, focusing primarily on the prevalence of third-generation cephalosporin-resistant ESBL-producing Enterobacterales.

Accordingly, we studied the effect of urbanisation on the morphological characteristics of the Hooded Crow in Hungary. We collected data from 249 Hooded Crows, from urban and rural areas. We found individuals living in urban sites to be smaller than their rural conspecifics, displaying shorter tarsi, which supports the conception that the urban environment might influence bird development in their early lives. However, no differences were detected in body mass or body condition. Due to plenty of anthropogenic food sources, we believe that urban adult birds are able to maintain the same body mass as their rural counterparts. Our results also showed that juveniles from urban habitats have longer and larger bills but smaller body sizes, exhibiting shorter tarsi and smaller skulls than their rural conspecifics. However, it is noteworthy that, due to the low sample size of rural juveniles (n = 3), these findings are not conclusive and further inquiries are necessary.

Moreover, our results also showed that differences displayed by individuals from different urban areas seemed to be age-dependent and may be related to the availability of food sources. Young crows from a larger urban area (Budapest) have longer and thicker bills and better body condition than those from a smaller city (Debrecen). However, lower body condition in juveniles from Debrecen may also be explained by other factors such as poor health status (GIRAUDEAU et al., 2014), predation (MACLEOD et al., 2006), and low food predictability (BRODIN, 2007). These findings demonstrate that factors other than anthropogenic food sources can also explain the effect of urbanisation on wild

birds. Interestingly, some traits also seemed influenced by the location of capture in the city of Debrecen. Individuals captured at the city Zoo were generally bigger in size, with higher body mass, better body condition, more fat reserves, longer bodies, and wider skulls and bills than individuals caught elsewhere, especially at the Sports Complex, suggesting that local factors may attenuate or exacerbate the habitat type effect on Hooded Crows' growth. Intra-species divergence in morphological traits between birds inhabiting different habitats varies and may be subjected to various factors that need to be explored. Consequently, different scales and multi-site studies exploring factors other than anthropogenic food availability and warmer microclimates are recommended to explore inter-site variabilities and subsequently provide robust models regarding the effect of urbanisation on wild birds' phenotypic traits. Moreover, it may be fruitful to combine morphological and biological studies to investigate more adequately the adaptation process of birds like the Hooded Crow to urban development; likewise, molecular studies investigating the genetic basis of the observed variations between rural and urban populations should also be considered.

Urban habitats allow for unprecedented levels of direct and indirect interactions between Hooded Crows and humans, establishing important wildlife-animal-human interfaces and outlining critical points for bi-directional exchanges of possible pathogens, including antimicrobial-resistant bacteria. We assessed the prevalence of third-generation cephalosporin-resistant ESBL-producing Enterobacterales and VREs in urban and rural Hooded Crow. In total, 264 fresh faecal samples were collected between January and August 2020 and analysed for AMR bacteria. Bacteria were cultured on selective media, the eosin methylene blue agar supplemented with 2 mg/l cefotaxime media, and the Bile Esculin Azide (BEA) agar supplemented with vancomycin. Isolates were identified using the MALDI TOF. The susceptibility test of Enterobacterales was performed using the disc diffusion method by following EUCAST recommendations, while vancomycin resistance was examined using the determination of the vancomycin and teicoplanin MICs using MIC test strips. ESBL isolates were identified per the double-disc synergy method. ESBL genes were identified using PCR, and isolates were characterised by whole genome sequencing. Four of the sampled rural Hooded Crows and 125 urban ones (7% vs. 60%, chi-square $p<0.0001$) yielded ESBL-producing Enterobacterales with the overwhelming dominance of *E. coli* (2/4 and 105/125 in rural and urban positive birds, respectively). The $bla_{CTX\text{-}M\text{-}1}$ group ESBLs were predominant in both groups. None of the

samples from both habitat types yielded VRE. A WGS analysis was then performed on ESBL-producing *E. coli* isolates. In addition to the presence of ESBL-encoding genes, particularly $bla_{CTX\text{-}M\text{-}1/55/15}$, genes encoding resistance to other antimicrobial classes such as macrolides, sulphonamides, quinolones, trimethoprim/sulfamethoxazole, and tetracycline were also detected, as well as various virulence factors, in association with several plasmids, mainly IncI-1 and IncF plasmid groups. IncN plasmids and Col156 were also detected. Most ESBL isolates belonged to the B1 and A phylogenetic groups. Fewer strains belonged to phylogenetic groups E, F, and D, suggesting that Hooded Crows carry commensal ESBL producers and might be reservoirs of ESBL-encoding genes. Overall, 22 sequence types (STs) were defined. Core-genome (cg) MLST revealed that these ESBL isolates belong to 33 distinct cgSTs. The most prevalent ST was ST58, found in 34 strains. Most of the STs found, such as ST10, S38, ST155, and ST442, have been previously described in human and animal isolates, including wildlife. However, some STs described in our crow samples, such as ST4412 and ST5798, are yet to be reported in humans and/or animals (MELLMANN et al., 2009; EWERS et al., 2021).

Thus, the high frequency of ESBL producers carried by the urban population of the Hooded Crow points out the role of anthropogenic sources in the origin of ESBL producers. At the same time, it seems that Hooded Crows from Hungary do not necessarily constitute a reservoir of VREs. Nonetheless, the prevalence of $bla_{CTX\text{-}M}$, along with resistance genes of other antimicrobial classes, strengthens the notion that even free-ranging wild birds, with increasing contact with humans, pose health-related threats. Strongly emphasising the need for more surveillance studies to investigate AMR occurrence and to fully demonstrate the role of wild birds as reservoirs, vectors, and indicators of AMR in the environment, especially in urban habitats. While continuous surveillance of VRE would also be judicious.

In summary, here we demonstrated the possible link between the carriages of AMR by the Hooded Crow and its urban adaptation. Suggesting the importance of ecological information for the study of AMR in wildlife, particularly in urban habitats. Moreover, urbanisation clearly provides a meeting ground where wildlife, domestic animals and/or livestock, and humans interconnect in an intensifying manner, and such contact facilitates the emergence and transmission of various pathogens between different host populations. Thus, understanding the ecological parameters related to the successful co-existence in urban settings of certain wildlife, like Hooded Crows, might be an

essential element in AMR investigations in the wild. To do so, complex multidisciplinary studies are required to get a more comprehensive view on the effect of urbanisation on wildlife and, subsequently, its role as an interface from a health point of view. We believe that understanding what influences the success of wild birds in urban habitats might mitigate the surveillance of AMR emergence in such hosts and potentially help identify appropriate actions.

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Doctoral School: Doctoral School of Animal Husbandry
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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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2. **Benmazouz, I.**, Kövér, L., Kardos, G.: Does the Hooded Crow (Corvus cornix) harbour vancomycin-resistant enterococci in Hungary?
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3. **Benmazouz, I.**, Jokimäki, J., Juhász, L., Kaisanlahti, J. M. L., Paládi, P., Kardos, G., Lengyel, S., Kövér, L.: Morphological changes in hooded crows (Corvus cornix) related to urbanization.
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6. **Benmazouz, I.**, Kövér, L., Kardos, G.: Absence of Vancomycin resistant enterococci among Urban and Rural Hooded Crows in Hungary.
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7. **Benmazouz, I.**, Nagy, J. B., Balázs, B., Kövér, L., Kardos, G.: Occurrence of ESBL producing Enterobacterales in Hooded crows (Corvus cornix) in urban and rural areas of Hungary.
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11. Emami-Khoyi, A., Agnew, T. W., Adair, M. G., Murphy, E. C., **Benmazouz, I.**, Monsanto, D. M., Parbhu, S. P., Main, D. C., Le Roux, R., Golla, T. R., Schnelle, C., Alizadeh, H., Csányi, S., Heltai, M., Jansen van, V. B., Paterson, A. M., Teske, P. R., Ross, J. G.: A New Non-invasive Method for Collecting DNA From Small Mammals in the Field, and Its Application in Simultaneous Vector and Disease Monitoring in Brushtail Possums.
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14. Kövér, L., Paládi, P., **Benmazouz, I.**, Tóth, N., Varga, S. Z., Juhász, L., Lengyel, S.: A városi vadgazdálkodás rejtélyei - avagy hogyan csaljuk csapdába a varjat?
In: 2. Urbanizációs Ökológia Konferencia : Absztrakfüzet, [s.n.], Győr, 28, 2021.

Total IF of journals (all publications): 24,158
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07 February, 2024

DEBRECENI EGYETEM * Egyetemi és Nemzeti Könyvtár *

11. STATEMENTS

STATEMENT

I wrote this thesis in the framework of the University of Debrecen Doctoral School of Animal Science for the purpose of obtaining a doctoral degree (Ph.D.) at the University of Debrecen.

Debrecen, 20.....

.....

PhD candidate

STATEMENT

I hereby certify that the doctoral candidate **Isma Benmazouz** has carried out his/her work under my/our supervision within the framework of the above-mentioned Doctoral School between 2019-2023 The candidate has made a decisive contribution to the results of the thesis through his/her independent creative work, and the thesis is the candidate's independent work. I/we recommend that the thesis be accepted.

Debrecen, 20.....

…… …….

László Kövér, PhD. Gábor Kardos, PhD.

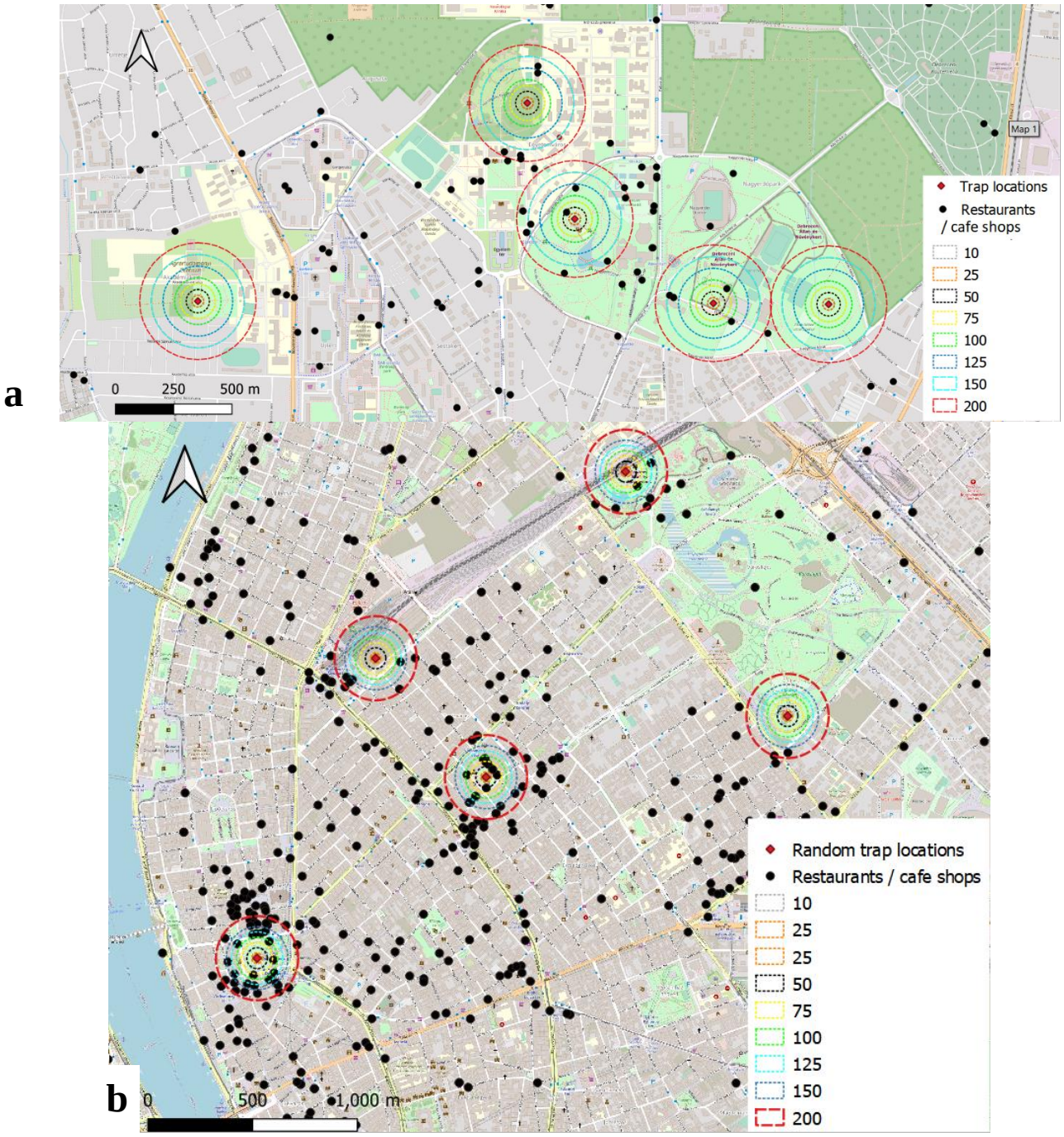
12. ANNEXES

Appendix 1: Capture and Morphological traits data sheet

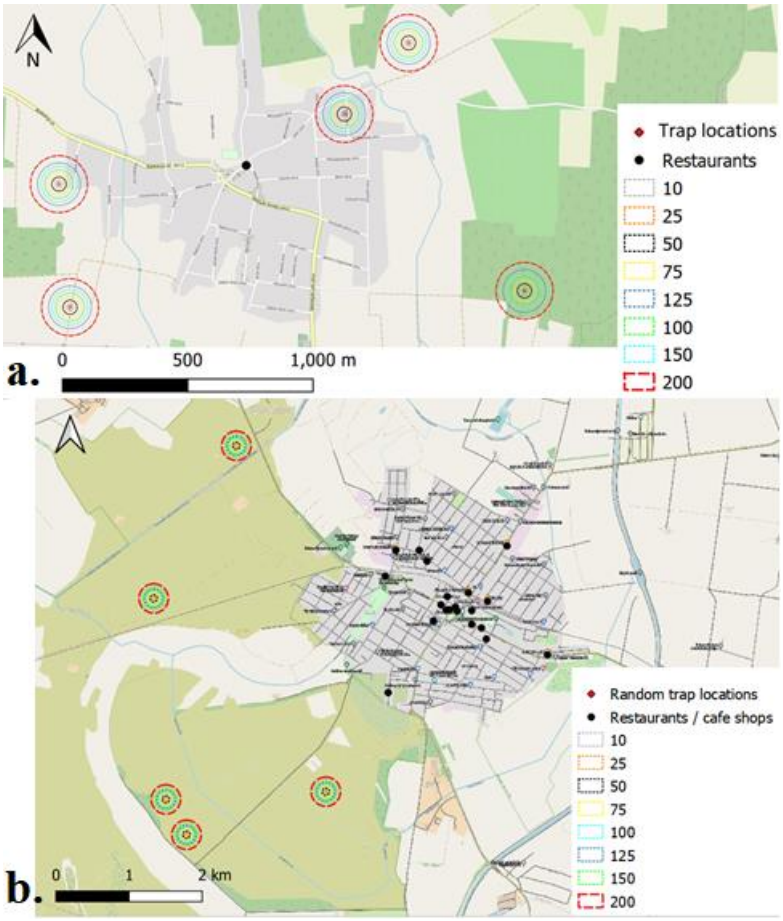
NR	SPEC_NI	N	YEAI	MONTI	DAY	AREA	HABITAT	LOCATION	METHOD	AGI	AGE	AGE	SEX	GRIN	BMAS	FATR	PC1	PC2	PC3	BM_PI	BODYC	ODYL	WINGI	TAILL	HEADI	KULL	KULL	EAKI	AKTI	ARSI	NOT
1	U1	1	2020	1	30	Debrecen	urban	Békás-lake	net-gun	2	1	1																			1
2	U2	1	2020	3	23	Debrecen	urban	ZOO	ladder-trap	2+	2	1																			2
3	R1	1	2020	3	30	Szakoly	rural	hunting area	ladder-trap	2	1	1																			3
4	R2	1	2020	3	30	Szakoly	rural	hunting area	ladder-trap	2+	2	1																			4
5	U3	1	2020	4	2	Debrecen	urban	open stage theatre	ladder-trap	2	1	1		G01																	5
6	R3	1	2020	4	5	Szakoly	rural	hunting area	ladder-trap	2+	2	1																			6
7	R4	1	2020	4	5	Szakoly	rural	hunting area	ladder-trap	2	1	1																			7
8	R5	1	2020	4	5	Szakoly	rural	hunting area	ladder-trap	2	1	1																			8
9	R6	1	2020	4	5	Szakoly	rural	hunting area	ladder-trap	2	1	1																			9
10	R7	1	2020	4	5	Szakoly	rural	hunting area	ladder-trap	2+	2	1																			10
11	R8	1	2020	4	5	Szakoly	rural	hunting area	ladder-trap	2	1	1																			11
12	R9	1	2020	4	5	Szakoly	rural	hunting area	ladder-trap	2+	2	1																			12
13	U4	1	2020	4	14	Debrecen	urban	Agrar campus	ladder-trap	2	1	1			405		0.5698	-1.3012	-1.7634	411.07	-6.0719	41	29.5	18.5	91.5	39.5	35.5	52	17.5	48	13
14	R10	1	2020	4	16	Szakoly	rural	hunting area	ladder-trap	2+	2	1			390		5.7638	6.0237	0.25026	439.39	-49.386	49	31	19.5	95	41.5	36	53.5	18.5	52.5	14
15	R11	1	2020	4	16	Szakoly	rural	hunting area	ladder-trap	2	1	1			390		0.22378	5.1198	-4.1577	448.82	-58.824	45	29	17.5	91	41	35	50	17	54	15
16	R12	1	2020	4	16	Szakoly	rural	hunting area	ladder-trap	2	1	1			365		-1.8559	1.1764	-0.0963	411.07	-46.072	45	31	19.5	89.5	40	34.5	49.5	19	48	16
17	R13	1	2020	4	16	Szakoly	rural	hunting area	ladder-trap	2	1	1			365		-4.3864	-0.2618	0.28516	398.49	-33.488	42	27.5	17.5	89.5	42	35	47.5	18	46	17
18	R14	1	2020	4	16	Szakoly	rural	hunting area	ladder-trap	2	1	1			430		8.5607	7.2537	-0.1383	448.82	-18.824	48.5	32	20	97	42	36.5	55	20.5	54	18
19	R15	1	2020	4	16	Szakoly	rural	hunting area	ladder-trap	2	1	1			355		-0.9008	0.88498	-2.7183	423.66	-68.656	43	28.5	19	90	39.5	35	50.5	17.5	50	19
20	U5	1	2020	4	16	Debrecen	urban	open stage theatre	ladder-trap	2	1	1			400		1.8471	-2.4412	1.5034	392.2	7.804	43.5	28	17.5	93	40	34	53	20	45	20
21	U6	1	2020	4	16	Debrecen	urban	open stage theatre	ladder-trap	2+	2	1			470		10.336	3.9207	5.4291	411.07	58.928	48	29.5	19.5	100	43.5	39	56.5	20.5	48	21
22	U7	1	2020	4	16	Debrecen	urban	open stage theatre	ladder-trap	2	1	1			380		-3.2814	-1.2482	2.467	385.9	-5.904	43	29	17	90.5	42	35.5	48.5	17	44	22
23	U8	1	2020	4	16	Debrecen	urban	Botanic garden	ladder-trap	2+	2	1		W11	480		7.5562	0.71059	1.6149	411.07	68.928	44	32	20	97	41	36	56	20	48	23
24	U9	1	2020	4	20	Debrecen	urban	Agrar campus	ladder-trap	2	1	1			425		0.71816	-0.8446	-0.3015	407.93	17.074	42.5	29	19	91	39	37	52	20	47.5	24
25	U10	1	2020	4	23	Debrecen	urban	open stage theatre	ladder-trap	2	1	1			355	2	-0.7915	-4.2419	-1.1455	395.34	-40.342	40.5	25	17.5	90.5	38.5	35	52	18.5	45.5	25
26	U11	1	2020	4	23	Debrecen	urban	open stage theatre	ladder-trap	2	1	1			520	2	7.1679	9.3851	-1.3426	458.26	61.738	45	30	19	98	45.5	37.5	52.5	21.5	55.5	26
27	U12	1	2020	4	26	Debrecen	urban	open stage theatre	ladder-trap	2	1	1			395	1	-4.1333	-0.8842	-1.8727	407.93	-12.926	41.5	29	18	88	39.5	35	48.5	18.5	47.5	27
28	U13	1	2020	4	30	Debrecen	urban	open stage theatre	ladder-trap	2	1	1			530	3	6.4598	5.8775	-2.8082	448.82	81.176	44	31	18	97	43.5	33.5	53.5	20.5	54	28
29	U14	1	2020	4	30	Debrecen	urban	Botanic garden	ladder-trap	2+	2	1	F		365	2	0.20715	0.35797	0.72724	404.78	-39.78	45	30	20	92	41	33	51	17.5	47	29
30	U15	1	2020	4	30	Debrecen	urban	Botanic garden	ladder-trap	2+	2	1	M		495	2	7.0488	4.4617	-2.1635	442.53	52.468	44	33	10.5	97	42.5	36	54.5	19.5	53	30
31	U16	1	2020	4	30	Debrecen	urban	open stage theatre	ladder-trap	2+	2	1	M		470	2	5.4653	2.6774	2.7216	414.22	55.782	47	29.5	19	95	41	38	54	21	48.5	31
32	U17	1	2020	4	30	Debrecen	urban	open stage theatre	ladder-trap	2	1	1			395	1	1.0428	-0.4934	5.4966	379.61	15.388	45	29	18	94	43	36	51	20.5	43	32
33	U18	1	2020	4	30	Debrecen	urban	open stage theatre	ladder-trap	2	1	1			430	2	4.2773	-2.6404	6.1962	373.32	56.68	46	30.5	19.5	95	40.5	36	54.5	20	42	33
34	U19	1	2020	5	4	Debrecen	urban	ZOO	ladder-trap	2	1	1		VR09544	435	3	1.8765	5.229	-6.0595	455.12	-20.116	40	31	18.5	93.5	43	33.5	50.5	20	55	34
35	U20	1	2020	5	4	Debrecen	urban	ZOO	ladder-trap	2+	2	1	M?		515	3	5.9015	1.3658	3.6021	404.78	110.22	49	33	19.5	95	40	35	55	19	47	35
36	U21	1	2020	5	4	Debrecen	urban	Botanic garden	ladder-trap	2	1	1	F?		385	2	3.9701	-3.6176	-3.189	420.51	-35.51	41	29	18	90	33.5	42	56.5	17	49.5	36
37	R16	1	2020	5	8	Balmazújváros	rural	hunting area	ladder-trap	2+	2	1	M?		480	2	10.117	5.037	1.7042	429.95	50.052	45	32	20	100	44	37	56	20.5	51	37
38	R17	1	2020	5	8	Balmazújváros	rural	hunting area	ladder-trap	2	1	1	F?		405	1	4.8272	1.1427	1.6671	411.07	-6.0719	45	28.5	19.5	95	41	37	54	19	48	38
39	R18	1	2020	5	8	Balmazújváros	rural	hunting area	ladder-trap	2+	2	1	M?		470	2	8.9872	4.0199		420.51	49.49	48	32	21	98	42	37	56	19.5	49.5	39
40	R19	1	2020	5	8	Balmazújváros	rural	hunting area	ladder-trap	2+	2	1			490	2	2.2187	3.6142	-0.8467	429.95	60.052	44	31	19	92.5	41	38	51.5	19.5	51	40
41	R20	1	2020	5	8	Balmazújváros	rural	hunting area	ladder-trap	2+	2	1			430	2	3.4991	0.31143	4.3979	392.2	37.804	46	31.5	20	94.5	41.5	36	53	20	45	41
42	R21	1	2020	5	8	Balmazújváros	rural	hunting area	ladder-trap	2	1	1			430	2	5.7207	5.415	8.9744	404.78	25.22	45	29	19.5	95	42.5	57	52.5	20	47	42
43	R22	1	2020	5	8	Balmazújváros	rural	hunting area	ladder-trap	2	1	1			355	2	0.06122	-1.0246	-0.4249	407.93	-52.926	45	29	19	90	38	35	52	18	47.5	43
44	R23	1	2020	5	8	Balmazújváros	rural	hunting area	ladder-trap	2	1	1			380	2	-3.1671	-0.5994	3.2881	385.9	-5.904	44.5	30	18	90	41.5	36	48.5	18	44	44

45	R24	1	2020	5	8	Balmazújváros	rural	hunting area	ladder-trap	2+	2	1	F		480	3	2.2098	2.2723	3.1083	404.78	75.22	46	32	20	93.5	42	36.5	51.5	19.5	47			45
46	R25	1	2020	5	8	Balmazújváros	rural	hunting area	ladder-trap	2+	2	1	M		510	2	6.5304	9.4706	0.435	451.97	58.03	48.5	34	21	96	43.5	37.5	52.5	21.5	54.5			46
47	R26	1	2020	5	8	Balmazújváros	rural	hunting area	ladder-trap	2+	2	1	M?		485	2	11.84	6.2389	3.6152	429.95	55.052	48.5	32	20	101	44	38	57	19	51			47
48	R27	1	2020	5	8	Balmazújváros	rural	hunting area	ladder-trap	2	1	1			380	2	-2.4001	-0.155	1.8128	395.34	-15.342	44	28	18	90.5	41.5	35.5	49	18.5	45.5			48
49	R28	1	2020	5	8	Balmazújváros	rural	hunting area	ladder-trap	2+	2	1			485	3	2.9908	4.2494	0.97942	423.66	61.344	45.5	31	18.5	94	42.5	37	51.5	20	50			49
50	R29	1	2020	5	8	Balmazújváros	rural	hunting area	ladder-trap	2	1	1			440	2	7.3775	3.9125	2.1889	420.51	19.49	46	30	19	98	43.5	36	54.5	20	49.5			50
51	R30	1	2020	5	8	Balmazújváros	rural	hunting area	ladder-trap	2+	2	1	M?		435	2	7.6476	1.1468	3.7845	404.78	30.22	47	31	20	97	41	37	56	18.5	47			51
52	R31	1	2020	5	8	Balmazújváros	rural	hunting area	ladder-trap	2	1	1			400	2	-0.8938	0.43162	1.1726	401.63	-1.6339	44.5	30	18.5	91.5	41.5	34	50	18	46.5			52
53	U22	1	2020	5	11	Debrecen	urban	Agrar campus	ladder-trap	2+	2	1			430	3	-0.4276	-1.1487	1.6115	395.34	34.658	44	29	18	91	40	35.5	51	20	45.5			53
54	U23	1	2020	5	23	Debrecen	urban	ZOO	ladder-trap	2	1	1			500	3	8.8687	6.8121	-2.6631	458.26	41.738	46	33	19.5	97	41.5	36	55.5	20	55.5			54
55	U24	1	2020	5	23	Debrecen	urban	ZOO	ladder-trap	2+	2	1	F		390	2	-0.4775	9.6696	-1.9808	451.97	-61.97	46	32	20	93	46	34	47	17.5	54.5	feathers		55
57	U26	1	2020	5	27	Debrecen	urban	ZOO	ladder-trap	2+	2	1	F		395	2	0.61715	1.9255	0.91959	411.07	-16.072	44	30	18.5	92.5	42	36	50.5	19.5	48	feathers		57
58	U27	1	2020	5	27	Debrecen	urban	ZOO	ladder-trap	2+	2	1	F		420	3	-2.3795	1.9231	-0.0629	414.22	5.7821	46	31	19.5	89	40	35	49	17.5	48.5	feathers		58
59	U28	1	2020	5	27	Debrecen	urban	ZOO	ladder-trap	2+	2	1	M		475	2	11.681	9.1593	-2.0214	467.7	7.3002	47.5	31.5	19	100	43.5	36.5	56.5	19	57	feathers		59
60	U29	1	2020	5	27	Debrecen	urban	ZOO	ladder-trap	2	1	1	F		400	2	0.02907	3.1148	-1.395	426.8	-26.802	43.5	30	18	91.5	41.5	36.5	50	18.5	50.5	feathers		60
61	U30	1	2020	5	27	Debrecen	urban	Agrar campus	ladder-trap	2+	2	1	M		440	2	-3.6246	-0.6475	2.7432	385.9	54.096	43	31.5	19	90	42	35	48	19	44	feathers		61
62	U31	1	2020	5	27	Debrecen	urban	Agrar campus	ladder-trap	2	1	1	F		410	2	-3.3618	-2.6912	3.2562	376.47	33.534	43	30	19	90	41	35	49	17.5	42.5	feathers		62
63	U32	1	2020	5	27	Debrecen	urban	Agrar campus	ladder-trap	2+	2	1	M		420	2	6.5916	-0.8468	5.3253	385.9	34.096	46	32	21	97	41.5	35	55.5	20	44	feathers		63
64	U33	1	2020	6	1	Debrecen	urban	Sport center	ladder-trap	2	1	1	F		410	3	-7.2007	-3.7725	3.9661	363.88	46.118	42.5	31	19	87	40.5	34.5	46.5	20	40.5	feathers		64
65	U34	1	2020	6	1	Debrecen	urban	Sport center	ladder-trap	2	1	1	F		370	3	-2.6228	-3.3116	-0.4086	392.2	-22.196	39.5	28.5	17.5	90	40	36	50	17	45	feathers		65
66	U35	1	2020	6	1	Debrecen	urban	Sport center	ladder-trap	2+	2	1	M		520	3	6.1641	8.9339	-3.7573	467.7	52.3	44	32.5	20.5	96	43.5	37.5	52.5	19	57	feathers		66
68	R33	1	2020	6	2	Szakoly	rural	hunting area	larsen	2	1	1	F		395	2	1.7288	1.2131	-2.1097	420.51	-25.51	41	30.5	19	94	42.5	33	51.5	17.5	49.5	feathers		68
69	R34	1	2020	6	2	Szakoly	rural	hunting area	ladder-trap	2+	2	1	M		495	2	9.4179	6.9549	-1.572	451.97	43.03	43	32.5		99.5	44.5	38	55	18.5	54.5	feathers		69
70	R35	1	2020	6	2	Szakoly	rural	hunting area	ladder-trap	2	1	1	F?		365	2	1.9086	2.6105	-5.4834	445.68	-80.678	41.5	28	19	92	40	34.5	52	19	53.5	feathers		70
71	R36	1	2020	6	2	Szakoly	rural	hunting area	ladder-trap	2	1	1	M		380	3	2.1944	1.8843	-3.2067	433.09	-53.094	40.5	27.5	17.5	93	41	38	52	18	51.5	feathers		71
72	R37	1	2020	6	2	Szakoly	rural	hunting area	ladder-trap	2	1	1	F		300	1	0.69541	0.91264	-1.5061	417.36	-117.36	41.5	29.5	18	92.5	41.5	35	51	19	49	feathers		72
73	R38	1	2020	6	2	Szakoly	rural	hunting area	ladder-trap	2+	2	1	M		430	2	9.3839	1.5684	-0.9777	429.95	0.052146	44	31	19.5	97.5	40	36	57.5	19	51	feathers		73
74	R39	1	2020	6	2	Szakoly	rural	hunting area	ladder-trap	2	1	1	M		365	1	6.6429	5.9983	0.59584	436.24	-71.24	46	31.5	19.5	97	43.5	37	53.5	19	52	feathers		74
75	R40	1	2020	6	2	Szakoly	rural	hunting area	ladder-trap	2+	2	1	M		400	2	4.2933	1.8085	-1.0399	423.66	-23.656	43.5	30.5	19.5	94.5	41	34.5	53.5	20	50	feathers		75
77	U37	1	2020	6	5	Debrecen	urban	Agrar campus	ladder-trap	2	1	1	F		370	2	-6.4912	0.17817	-2.9113	411.07	-41.072	39	31.5		87.5	41.5	34	46	18	48	feathers		77
84	U44	1	2020	6	10	Debrecen	urban	Sport center	ladder-trap	2	1	1	F		350	2	-3.0891	-5.4644	0.99551	373.32	-23.32	39	29.5	18	90.5	40.5	32.5	50	19	42	feathers		84
86	U46	1	2020	6	10	Debrecen	urban	Sport center	ladder-trap	2	1	1	M		410	2	-2.5749	1.5408	2.0306	398.49	11.512	44	31.5	19	91	43	34	48	20	46	feathers		86
89	R41	1	2020	6	16	Balmazújváros	rural	hunting area	ladder-trap	2	1	1	F		370	2	-0.0611	1.7042	-2.0698	423.66	-53.656	43	29	17.5	91.5	41	35	50.5	17.5	50	feathers		89
90	R42	1	2020	6	16	Balmazújváros	rural	hunting area	ladder-trap	2+	2	1	M		490	2	-34.772	13.966	-4.9536	448.82	41.176	49.5	33	21	65	43	34.5	22	20	54	feathers		90
92	R44	1	2020	6	16	Balmazújváros	rural	hunting area	ladder-trap	2+	2	1	F		470	3	1.7593	-3.7249	-11.577	429.95	40.052	42.5	31	17.5	95	41	3	54	21	51	feathers		92
94	R46	1	2020	6	16	Balmazújváros	rural	hunting area	ladder-trap	2+	2	1	M		485	3	5.8053	5.3355	-0.6043	439.39	45.614	46	32	18	95.5	42	37	53.5	18.5	52.5	feathers		94
95	R47	1	2020	6	16	Balmazújváros	rural	hunting area	ladder-trap	2	1	1	F		400	2	3.7881	1.2868	-0.6546	420.51	-20.51	44.5	30	19	94	40.5	35	53.5	17	49.5	feathers		95
96	R48	1	2020	6	16	Balmazújváros	rural	hunting area	ladder-trap	2	1	1	M		360	1	7.2864	3.0188	-0.6013	429.95	-69.948	44	27.5	18	97.5	42.5	35.5	55	20	51			96
97	R49	1	2020	6	16	Balmazújváros	rural	hunting area	ladder-trap	2+	2	1	M		450	1	8.502	3.2429	1.4385	423.66	26.344	46.5	33	19.5	97.5	41.5	35.5	56	20	50			97
98	R50	1	2020	6	16	Balmazújváros	rural	hunting area	ladder-trap	2	1	1	F		450	3	-0.5985	3.4411	-1.3533	426.8	23.198	44.5	30	19	91	41.5	35	49.5	19	50.5			98
99	R51	1	2020	6	16	Balmazújváros	rural	hunting area	ladder-trap	2	1	1	F		410	2	-2.5727	1.8005	-0.5888	414.22	-4.2179	43	32	18.5	89.5	41	36	48.5	19	48.5			99
100	R52	1	2020	6	16	Balmazújváros	rural	hunting area	ladder-trap	2+	2	1	M		400	1	8.8112	3.3372	-0.19	433.09	-33.094	46	32	19.5	97	40.5	36	56.5	20	51.5			100
103	Bd2	1	2020	6	22	Budapest	urban	ZOO	ladder-trap	2	1	1			430	2	0.93441	4.0631	0.06988	423.66	6.3441	44.5		18	93	43	36	50	19	50	feathers		103
104	Bd3	1	2020	6	22	Budapest	urban	ZOO	ladder-trap	2	1	1	M		435	2	2.489	2.8114	3.2842	404.78	30.22	45	28	19	95.5	44.5	36.5	51	18	47	feathers		104
105	Bd4	1	2020	6	22	Budapest	urban	ZOO	ladder-trap	2	1	1	M		420	2	2.2826	-4.6211	2.4504	379.61	40.388	42		18.5	93	39	35	54	19.5	43	feathers		105
106	Bd5	1	2020	6	22	Budapest	urban	ZOO	ladder-trap	2	1	1	M		430	2	6.464	4.9125	0.70638	429.95	0.052146	45	29	19.5	97.5	44	36	53.5	20	51	feathers		106
107	Bd6	1	2020	6	22	Budapest	urban	ZOO	ladder-trap	2	1	1	M		400	2	-1.4867	2.0949	-3.4489	429.95	-29.948	42			90	40.5	35	49.5	17.5	51	feathers		107
108	Bd7	1	2020	6	22	Budapest	urban	ZOO	ladder-trap	2	1	1	F		355	2	-0.8861	-2.8174	1.4838	389.05	-34.05	44		19	90	38.5	34.5	51.5	19	44.5	feathers		108
109	Bd8	1	2020	6	22	Budapest	urban	ZOO	ladder-trap	2	1	1	M		355	2	-5.0187	2.541	-2.0557	420.51	-65.51	42.5		18	88	41.5	35	46.5	19	49.5	feathers		109
110	Bd9	1	2020	6	22	Budapest	urban	ZOO	ladder-trap	2+	2	1	F		380	2	0.61276	-1.2721	2.9906	389.05	-9.05	44	29	18.5	92.5	41	36	51.5	19	44.5	feathers		110
111	Bd10	1	2020	6	22	Budapest	urban	ZOO	ladder-trap	2	1	1	F		410	2	1.5267	2.6928	2.1095	411.07	-1.0719	46		18.5	93	42	37	51	18	48	feathers		111
112	Bd11	1	2020	6	22	Budapest	urban	ZOO	ladder-trap	2	1	1	M		420	2	5.7492	2.9908	0.52601	423.66	-3.6559	45	29.5	20	96	42	36	54	19	50	feathers		112
116	U54	1	2020	6	25	Debrecen	urban	Sport center	ladder-trap	2+	2	1	M		450	3	1.3648	0.03382	0.90661	404.78	45.22	44	33	19.5	92	40	35	52	19.5	47	feathers		116
117	U55	1	2020	6	25	Debrecen	urban	Sport center	ladder-trap	2	1	1	F		420	2	-1.1884	-1.418	2.3214	389.05	30.95	44.5	31.5	19.5	90.5	40	34	50.5	20	44.5	feathers		117
253	U191	1	2020	10	9	Debrecen	urban	Agrar campus	caught by hand	2	1	1			355	1	-0.2108	-3.7092	-4.3657	414.22	-59.218	40	30.5	18	89	36	34.5	53	17.5	48.5	feathers		253
263	U201	1	2020	11	22	Debrecen	urban	Agrar campus	ladder-trap	2	1	1			420	2	-2.9844	-2.9354	3.1797	376.47	43.534	44	30	17.5	90	40.5	34	49.5	18	42.5	feathers		263
264	U202	1	2020	11	22	Debrecen	urban	Agrar campus	ladder-trap	2+	2	1			420	2	0.55281	-4.309	5.8702	360.74	59.264	44	31	17	93.5	41.5	33.5	52	19.5	40	feathers		264

Appendix 2. QGIS maps for anthropogenic food sources



Appendix 2a: (a) Distribution of different anthropogenic food sources along the study area in the city of Debrecen. (b) Distribution of different anthropogenic food sources along the study area in the city of Budapest.



Appendix 2b: (a) Distribution of different anthropogenic food sources along the study area in Szakoly. (b) Distribution of different anthropogenic food sources along the study area in Balmazújváros.

Appendix 3: Location of meteorological stations for annual average temperature collection and their distance from urban study areas.

Area of study	Meteorology station, i.d.	Distance from study area
Budapest	EW3429 Budapest HU, i.d.: E3429	6 km from city centre.
Debrecen	DEBRECEN HU, i.d.: 12882099999	5 km from city centre.
Balmazújváros	POROSZLO, HU, i.d.: 12866099999	121.1 km from Budapest 74.7 km from Debrecen
Szakoly	NYIREGYHAZA, HU, i.d.: 12892099999	207.5 km from Budapest 47.4 km from Debrecen

Appendix 4: Principal component analysis' correlation with morphological variables.

	PC1	PC2	PC3	Body length	Wing length	Tail length	Head length	Skull length	Skull width	Bill length	Bill width	Tarsus length
PC1		1	1	0.00012538	3.0917E-05	0.0013326	6.3223E-150	0.094437	0.00016436	2.2017E-101	9.3684E-14	6.9871E-08
PC2	6.1053E-06		1	1.1714E-27	1.9045E-08	1.1922E-07	1.6576E-05	2.4701E-46	5.2061E-06	4.7378E-07	1.7708E-07	2.899E-68
PC3	-0.0082295	-0.005219		1.9077E-11	0.82769	0.068781	0.0060719	0.20196	9.4757E-06	1	0.13911	9.1196E-21
Body length	0.29657	0.63468	0.44089		2.3916E-07	1.0103E-07	0.00021584	4.6971E-10	0.094897	1	2.5948E-07	2.9726E-05
Wing length	0.31715	0.39106	0.16028	0.36782		6.9435E-12	4.4581E-06	2.3246E-06	1	0.51208	0.0017699	0.0031552
Tail length	0.26816	0.37154	0.20783	0.37307	0.45564		0.00054662	5.6624E-05	0.095353	1	0.0067177	0.038098
Head length	0.96886	0.31979	0.24527	0.28998	0.33843	0.27994		1.3806E-08	0.0023914	7.2565E-71	1.6549E-13	3.5678E-06
Skull length	0.201	0.76014	0.18697	0.41662	0.34524	0.30772	0.38871		0.099103	1	0.00014122	7.6332E-12
Skull width	0.2933	0.33225	0.32587	0.20091	0.059769	0.20203	0.25858	0.20014		0.58931	0.23668	0.18829
Bill length	0.91988	-0.35506	-0.10049	0.077596	0.17077	0.12748	0.85588	-0.14382	0.16539		1.828E-06	1
Bill width	0.47733	0.36575	0.19397	0.36216	0.2664	0.24524	0.47363	0.29514	0.18392	0.34304		0.00058123
Tarsus length	0.37433	0.84809	-0.56705	0.31329	0.25827	0.21793	0.33619	0.44748	0.1883	0.10991	0.27751	

Appendix 5: culture media composition and preparation metod

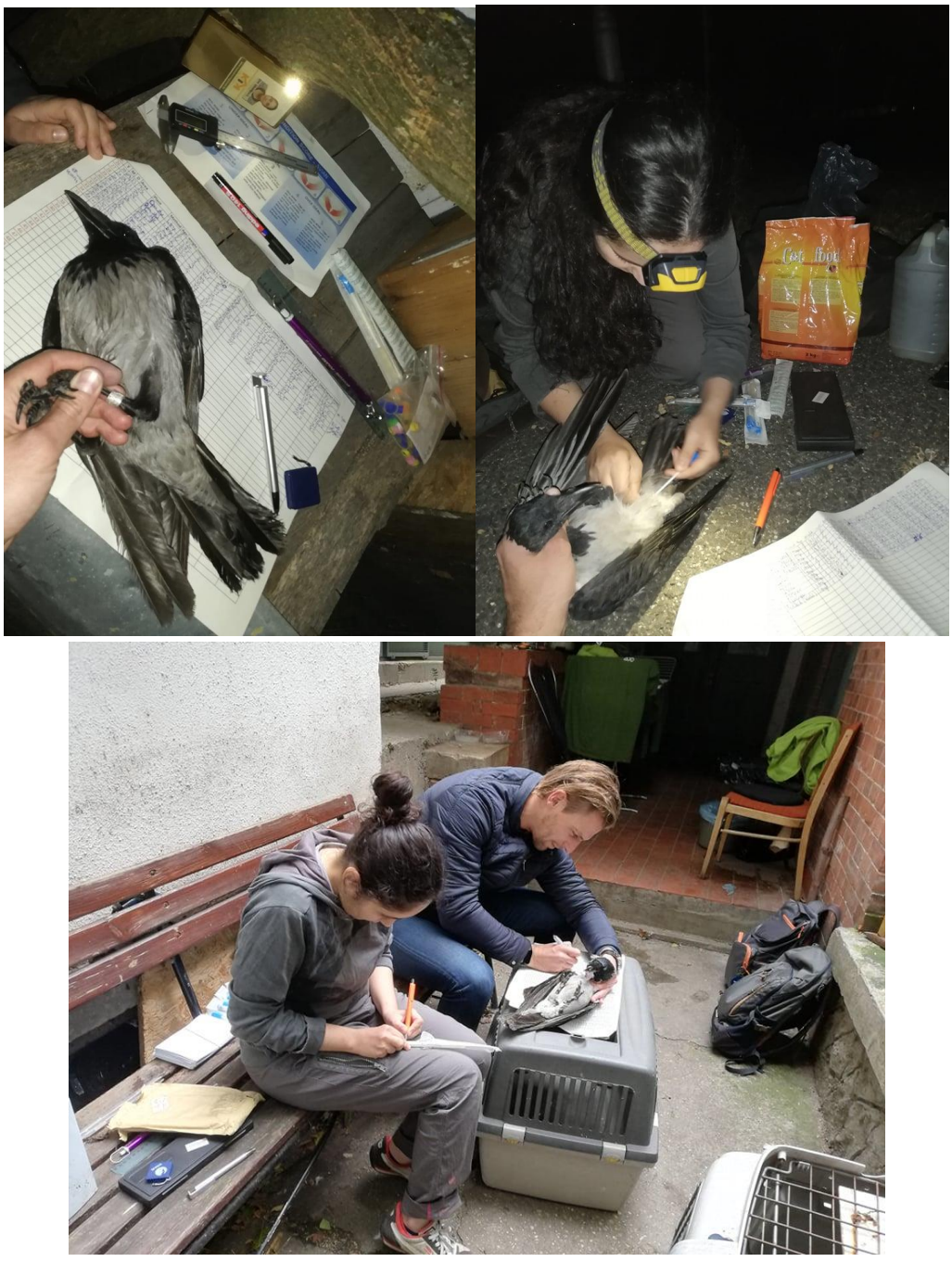
media	Composition		Preparation method
	Components	Concentration	
Blood agar	Peptospetial (enzymatic digest of animal protein) Starch Agar NaCl Distilled water **Sheep or horse blood**	23.0 g/L 1.0 g/L 14.0 g/L 5.0 g/L 1L **50 ml**	1. Suspend 43.0g of Columbia Agar Base (liofilmchem$_{srl}$, Italy) powder in 1L of distilled water. 2. Heat the mixture while stirring to fully dissolve all components. 3. Autoclave the dissolved mixture at 121°C for 15 min. 4. Cool to 50 °C. 5. Aseptically add 50 ml defibrinated horse or sheep blood previously warmed to room temperature and mix gently. 6. Avoid air bubbles. 7. Dispense preparation into sterile Petri plates. 8. Final pH should be 7.3 ± 0.2 at 25 ºC.
Selective media: Eosine Methylene Blue agar supplemented with 2mg/l cefotaxime	Peptone Dispotassium phosphate Lactose Eosine – Y Methylene blue Agar **Cefotaxime**	10.0 g/L 2.0 g/L 10.0 g/L 0.4 g/L 0.065 g/L 15.0 g/L **2 mg/L**	1. Suspend 37.5 g of E.M.B. Levine Agar (liofilchem$_{srl}$, Italy) in 1L of distilled water. 2. Mix until uniform then heat to boiling in order to fully dissolved all components. 3. Autoclave the dissolved mixture at 121°C for 15 min. 4. Cool to 50 °C. 5. Add 2mg/L cefotaxime. 6. Shake the medium to oxidize the methylene blue and to suspend the flocculent precipitate. 7. Avoid air bubbles. 8. Dispense preparation into sterile Petri plates. 9. Final pH should be 7.1 ± 0.2 at 25 °C.
VRE screening media: Bile Esculin agar supplemented with 6mg/l vancomyxcin	Peptone Tryptone Yeast extract Aesculin Sodium chloride Ox bile	3.0 g/L 17.0 g/L 5.0 g/L 1.0 g/L 5.0 g/L 10.0 g/L	1. Suspend 57.0 g of VRE Agar Base (liofilchem$_{srl}$, Italy) in 1L distilled water. 2. Heat to boiling to dissolve the medium completely. 3. Autoclave at 121°C for 15 min. 4. Cool to 50 °C. 5. Add 6mg/L vancomycin. 6. Pour medium into sterile Petri plates.

	Ferric ammonium citrate Sodium citrate Sodium azide Agar **Vancomycin**	0.5 g/L 1.0 g/L 0.25 g/L 15.0 g/L **6 mg/L**	7. Dispense preparation into sterile Petri plates. 8. Final pH should be 7.1 ± 0.2 at 25 ℃.
Muller Hinton Media	Beef Extract Acid Hydrolysate of Casein Starch Muller Hinton Agar	2.0 g/L 17.5 g/L 1.5 g/L 17.0 g/L	1. Suspend 38.0 g of the Muller Hinton II Agar (liofilchem$_{srl}$, Italy) in 1L of distilled water. 2. Heat with frequent agitation and boil for one minute to completely dissolve the medium. 3. Autoclave at 121℃ for 15 min. Cool to room temperature. 4. Pour cooled Mueller Hinton Agar into sterile petri dishes. 5. Final pH 7.3 ± 0.2 at 25ºC. 6. Cool to room temperature. 7. Store the plates at 2-8 ºC.

Appendix 6: Disc diffusion test results of ESBL Enterobacterales isolated from Hooded Crows.

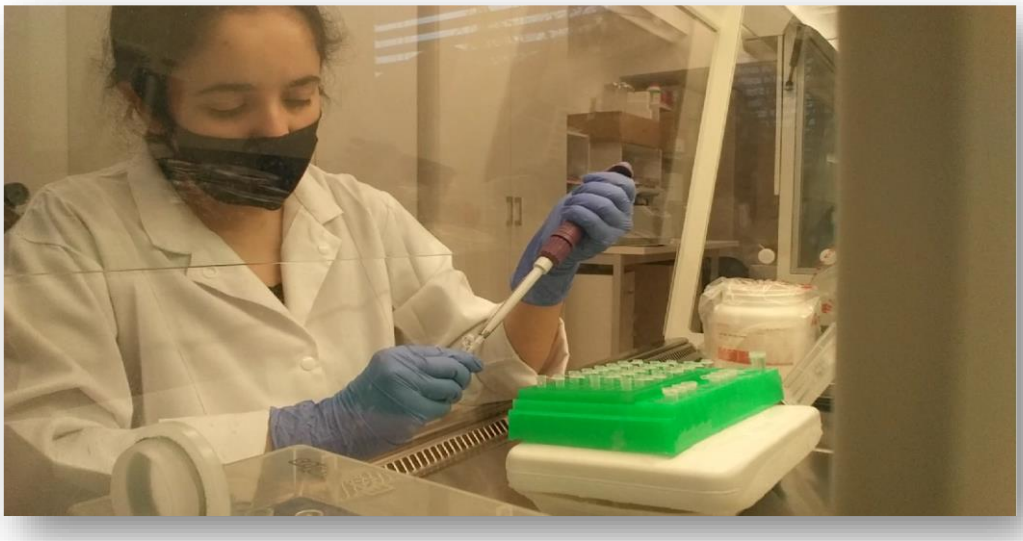
	Area					
	Urban areas				Rural area	
	Debrecen		Budapest		Balmazújváros	
Antimicrobials	Resistant	Susceptible	Resistant	Susceptible	Resistant	Susceptible
Ertapenem	3	206		6		4
Amoxicillin/ clavunic acid	15	194		6	1	3
Cefotaxim	209		6		4	
Ceftazidime	142	67	6		4	
Cefepime	205	4	6		4	
Polymyxin-B	54	155	4	2	2	2
Ciproflaxin	17	192		6	1	3
Sumetrolim	41	168		6	3	1
Amikan	2	207		6		4
Gentamicin	15	194		6	2	2
Tobramycin	13	196		6	2	2

Appendix 7. Field work



Dr. László Kövér and Isma Benmazouz during sample collection.

Appendix 8. Laboratory work



Isma Benmazouz during sample analysis.

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