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Song and the city : a comparison between urban and forest blackbirds

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Morphological and genetic differentiation between urban and forest blackbirds.

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Habitat-dependent divergence in both sexual and ecological traits can lead to population differentiation. We have previously found divergence in song and response to song between urban and forest blackbirds in the Netherlands. In this chapter, we test whether there is also habitat-dependent differentiation in morphology and neutral genetic markers in these locations. We find several morphological differences between urban and nearby forest populations. In combination with results from other studies conducted across Europe, a general pattern emerges for some traits like bill and wing length. This suggests that habitat-dependent selection is playing a role in morphological differentiation of some traits. We also find significant genetic differentiation at neutral markers between urban and nearby forest populations indicating that population divergence can happen at a very small geographic scale. Furthermore, within our study the populations with a similar habitat were genetically more similar to each other than populations of a different habitat. We interpret this as support for the idea that at a small geographic scale colonization of cities might have occurred from nearby urban areas and that there might be current exchange between populations with the same habitat.

Introduction

Ecology and sexual selection often play an important role in speciation (Podos 2001; Slabbekoorn & Smith 2002a). Divergent ecological selection can lead to trait differentiation between populations and sexual selection may contribute to prezygotic mating barriers. A powerful force behind population divergence is when ecological and sexual selection act in concert. This is the case when ecology has an influence on sexual signals leading to habitat-dependent signal divergence (Podos 2001; Slabbekoorn & Smith 2002b).

In this thesis, we have shown habitat-related divergence in song, a sexual signal in birds (Catchpole & Slater 2008; Collins 2004), and habitat-dependent variation in response to the divergent song trait in the European blackbird (*Turdus merula*) in three cities and three nearby forests in the Netherlands. Urban males sing with higher motif and twitter frequencies, larger twitter proportions and longer pauses than forest birds (chapter 2). Divergence in temporal song features are due to habitat-related differences in territory density and seasonality whereas the divergence in spectral song features is probably caused by habitat-dependent variation in background noise levels (chapter 2, 3 and 4). The habitat-dependent divergence in motif frequency influences male-male communication with urban and forest males responding differentially to motifs with either a low or high frequency, which could potentially promote population divergence (chapter 2).

There are several studies showing ecological differentiation between urban and forest blackbirds in a variety of traits such as migratory tendency (Partecke & Gwinner 2007), physiological response to stress (Partecke *et al.* 2006a) and morphology (e.g. Evans *et al.* 2009a). Furthermore, genetic differences at neutral markers between urban and nearby rural blackbird populations have also been found (Evans *et al.* 2009b; Gregoire 2003). Hence, we have shown urban divergence in song (i.e. a sexual trait) and there are other studies revealing urban divergence in various ecological traits and neutral genetic markers. However, there are no studies showing divergence in song, ecological traits and genetics for the same study populations. Furthermore, the various studies concern rather different geographic scales. In this chapter, we investigate whether the very same populations that were shown to have diverged acoustically, also diverged morphologically and genetically. Our study populations concern two city-forest pairs that are not far away from each other and for which the city and forest within a pair are only a few kilometres apart, which means that we focus on differentiation at a rather small geographic scale.

Morphology

Various studies have revealed morphological differentiation between populations of birds from the same species occupying different habitat types (Smith *et al.*, 1997; Schlotfeldt & Kleindorfer, 2006). Ecological circumstances in cities and natural habitats are distinct in various factors, because cities contrast with most other habitats in having little vegetation, many solid surfaces, different food sources, different predators and a higher temperature (e.g. Landsberg 1981; Woods *et al.* 2003). Despite the relative novelty of urban habitat, a few

studies show that there is already differentiation in morphological traits between urban and non-urban conspecifics in some bird species (dark-eyed juncos: Rasner *et al.*, 2004, house finches: Badyaev *et al.* 2008) including blackbirds (Evans *et al.* 2009a).

Lippens and Van Hengel already suggested in 1962 that urban and forest blackbirds may differ morphologically in The Netherlands (Lippens & van Hengel 1962). Results from two PhD-dissertations conducted in one city-forest pair in Germany and three city-forest pairs in France also suggested differences between urban and forest birds (Gregoire 2003; Partecke 2003). These findings led to a large-scale study including 11 city-rural habitat pairs covering a large part of the distribution of blackbirds showing an overall habitat effect with urban birds having longer wings and stubbier bills than rural ones (Evans *et al.* 2009a). Furthermore, pairwise comparisons between urban and adjacent rural populations often revealed significant differences in various traits (e.g. body mass and tarsus length), although these were not always consistent or occasionally even in the opposite direction between urban-rural pairs.

Genetics

Local differentiation of genes coding for traits under divergent selection can happen in the presence of gene flow (e.g. Senar *et al.* 2006). Habitat-related variation of functional genes can therefore indicate the existence of adaptations to divergent selection pressures, but neutral genetic markers are more suitable to address questions regarding reproductive isolation by investigating patterns of neutral gene flow. Evidence on functional and neutral genetic differentiation related to anthropogenic selection pressures is limited. Urban habitat-related differences in morphology of dark-eyed juncos and house finches seem to have at least some genetic basis and urban and non-urban populations are distinct from each other at neutral markers in both species (Evans *et al.* 2009b; Rasner *et al.* 2004; Yeh 2004). The situation in blackbirds is similar to these two species, because a common garden experiment with hand-raised birds from one city and one forest location in Germany revealed that behavioural and morphological differences between populations reflect functional genetic differentiation. A lack of genetic differentiation at neutral markers (AFLP) in a study by (Partecke *et al.* 2006b) was probably due to a limited resolution of the analysis, because an opposite pattern emerged when the same samples were used in a larger study with microsatellites (Evans *et al.* 2009b). The latter study included 12 city-rural pairs located between Spain, Tunisia, England and Estonia for which rural locations may have included forests, but possibly also other rural habitat types. The results show that genetic differentiation is largest among urban populations, intermediate among urban and rural populations and smallest among rural populations. This is in line with findings from a study in France on two cities and three forests showing two significant genetic distances between locations: one between a forest and nearby city (~ 40 km) and one between two nearby cities (~ 40 km). Hence, the urban populations do not form one large monophyletic group and the forest populations are relatively similar to each other. Evans *et al.* (2009b) interpret this pattern as evidence for the hypothesis that establishment of urban populations happened through independent colonization events rather than in a leapfrog manner with urban individuals repeatedly dispersing to uncolonized cities.

Aim of this study

We compare morphology and neutral genomic DNA of blackbirds in two city-forest pairs in the Netherlands. It is interesting to know whether there are morphological and genetic differences related to habitat in these sampling sites in order to deduce the possible consequences of the song divergence observed at these locations (chapter 2). Furthermore, results from our sampling sites in combination with results from previous studies can be used to verify how consistently morphological traits differ between urban and forest populations. This might shed light on the causes underlying morphological differences. Importantly, the centres of a city and forest within a pair in our study were just 5 to 6 kilometres apart with mostly habitable areas in between, whereas the distances within city-rural pairs in other blackbird studies (Evans *et al.* 2009a; Gregoire 2003; Partecke 2003) was always larger (23 to 40 km). In our small-scale study, the existence of morphological or genetic differentiation would therefore be strong support for the idea that urbanization has a major influence on divergence between blackbird populations. Furthermore, the distance between our two city pairs was 90 kilometres, which is similar to Gregoire 2003: distances between four locations within two city-forest pairs was 40 kilometres and an additionally forest was situated 200 kilometres away. The geographic scale of Evans *et al.* 2009b was much larger with distances between pairs of usually several hundred kilometres (minimal and maximal distances were respectively 130 and 2900 kilometres). Between our study locations there are also various other cities and forests making it more probable that the pairs are to some extent into contact with each other. We therefore expect that there might be a higher level of habitat-dependent gene flow between the two city-forest pairs than observed in the previous studies.

Material & Methods

Population- and individual sampling

We conducted our study in four locations in the Netherlands, which formed two pairs of a city and a nearby forest. The centre of a city and a forest within a pair were only five to six kilometres apart and the distances between the two pairs was 90 kilometres. The cities Arnhem (51°58N, 5°54E) and Breda (51°35N, 4°46E) are relatively old and medium-sized Dutch cities with 144,000 and 171,000 inhabitants, respectively (source: Central Bureau of Statistics of the Netherlands). We mainly sampled in small parks in both the city centre and adjacent residential areas. The forest locations associated with these urban areas are named The Veluwe and The Liesbos. The Veluwe (52°01N, 5°57E) is a relatively large area of about 100,000 hectares near Arnhem mainly covered with mixed forest and moorland, which we sampled between Het Rozendaalse Veld, Beekhuizen and De Koningsheide. The Liesbos is a relatively small, old-growth forest of 200 hectares nearby Breda with mainly deciduous trees and some coniferous trees at the edges.

The city-forest pair consisting of Arnhem and The Veluwe will from here on be referred to as “pair I” and the pair with Breda and The Liesbos will be referred to as “pair II”. Individuals were captured with mist nets in combination with playback of blackbird song. This was done in the years 2005, 2006 and 2007 between early April and the end of July. This period is during the

breeding season of blackbirds (Clement & Hathway 2000) ensuring that local individuals rather than migratory ones were sampled. Birds were ringed to prevent resampling of individuals. Sex and age were determined with the plumage and beak colour (Conings *et al.* 1999). Males are black with a yellow to orange bill and eye ring, females are dark-brown with no conspicuous eye ring and a brown beak with a variable amount of dull yellow. Juveniles are similar to females, but they have an entirely dark bill and their plumage is lighter brown and more speckled.

Morphology

Birds were weighed to the nearest 1.0 g by placing them in a cotton bag attached to a pesola. It is known that body mass of birds can vary considerable during the time of day and during the season. To avoid a bias in our data, we caught urban and forest blackbirds at similar times during the day and balanced sampling across the breeding season. Tarsus length was measured with a sliding callipers to the nearest 0.1 mm by gently bending at the intertarsal joint and the toes, and measuring the distance between the bending points (Svensson 1992). Although this measurement is slightly larger than the actual tarsus length, it has a smaller measurement error (Alatalo & Lundberg 1986). Bill and skull length were also measured with callipers to the nearest 0.1 mm. The bill length was the distance between the tip of the bill and the base of the skull. To determine the skull size, we subtracted the bill length from the distance between the tip of the skull and the furthest point at the back of the skull. Wing length was measured to the nearest 1.0 mm by flattening the primaries and extending them to their maximum length using a ruler with a vertical stop. All morphological measurements were taken by the same person (E.A.P. Ripmeester) to avoid observer-specific variation. Furthermore, all length measurements were taken twice and their averages were used in the statistical analyses.

Genetics

Blood was taken from a brachial wing vein (~50 µl). Samples were stored in 500 µl of buffer (0.15 M NaCl, 0.05 M Tris-HCl, 0.001 M EDTA, pH = 7.5) at 4°C. DNA was extracted from blood samples with DNeasy columns (Qiagen, Westburg) applying the protocol of the manufacturer. Individuals were genotyped at eight microsatellite loci (Table 5.1). PCR reactions were performed in a T3 Thermocycler (Biometra) in volumes of 10 µl with a MgCl₂ concentration of 1.5 mM and contained 10-15 ng of total genomic DNA, 0.2 mM of each dNTP, 0.5 µM of each primer, 0.25 units of *Taq* DNA polymerase (Qiagen, Westburg), and 1 µl Qiagen PCR buffer. PCR programmes consisted of 3 min of denaturation at 94°C followed by 35 cycles of denaturation at 94°C for 30 s, annealing at temperatures between 53 to 62°C for 30 s (Table 5.1), extension at 72°C for 45 s and finally 72°C for 7 min. Amplification products were resolved on an MegaBACE and scored with MegaBACE Fragment Profiler (GE Healthcare Europe GmbH). Exact tests for deviations from Hardy-Weinberg equilibrium were performed in Genepop version 4.0.7 for each population and locus separately (Guo & Thompson 1992). One locus significantly deviated from Hardy-Weinberg in all four study sites and was therefore excluded from further analysis (Table 5.1). Tests in Genepop version 4.0.7 for genotypic linkage equilibrium showed no sign of linkage between any of the remaining seven loci.

Table 5.1. Information regarding the 8 microsatellites used in this study. The original reference, EMBL accession number, applied annealing temperature and information regarding Hardy-Weinberg deviations is given for each locus. Microsatellite Ase50 was discarded from further analyses as it was not in Hardy-Weinberg equilibrium in all four sampling locations ($p < 0.01$).

Locus	Original reference	EMBL accession number	Ta (°C)	Hardy-Weinberg equilibrium
Ase40	Richardson <i>et al.</i> 2000	AJ276642	62	yes
Ase50	Richardson <i>et al.</i> 2000	AJ276779	60	no
Cuμ28	Gibbs <i>et al.</i> 1999	AF122894	60	yes
Cuμ32	Gibbs <i>et al.</i> 1999	AF122895	53	yes
PAT MP2-43	Otter <i>et al.</i> 1998	AM056063	60	yes
TguGga13-017	Simeoni <i>et al.</i> in press	CK313422	62	yes
TguEST06	Slate <i>et al.</i> 2007	CK307697	62	yes
TguEST11	Slate <i>et al.</i> 2007	AC159936	62	yes

Statistics

Statistical analyses on the morphological data of adult birds were conducted in R version 2.6.2 (Ihaka & Gentleman 1996). Assumptions of statistical tests regarding normal distribution and heteroscedasticity were visually verified. We started by using linear models including the factors “habitat” (city vs. forest), “city-forest pair” (pair I vs. pair II) and “gender” (male vs. female), as well as their interactions. However, these linear models gave results that contradicted graphical inspection of the raw data. This was due to the relatively low sample sizes of females in the forest locations, which harmed the statistical power to identify interaction effects and consequently weakened the statistical analyses. We therefore analysed the data for males and females separately per city-forest pair using Welch Two Sample t-tests. In addition, visual inspection of the data suggested that there was geographic variation between the two city-forest pairs in both genders in one of the morphological characteristics, the wing length. To verify this, we made linear models on wing length including the factors “habitat” and “city-forest pair” in combination with the interaction between these two factors for both genders separately.

Genetic analyses were conducted on data from all adults as well as a selection of juveniles. Juveniles were only included when they were sampled at least 200 metres away from any adult sample to ensure that these juveniles were unrelated to the adults. Fstat version 2.9.3 was used to calculate F_{st} values (i.e. genetic distances) and corresponding significance levels between all pairs of locations (Weir & Cockerham 1984). Furthermore, in an attempt to deduce possible gene flow patterns between the study locations, we performed individual assignment tests based on allelic frequencies with GeneClass version 1.0 (Cornuet *et al.* 1999). The principle behind this analysis is that individuals are expected to have particularly high probabilities to be assigned to their own sampling location as well as to locations with relatively closely related individuals. Hence, comparing relative assignment probabilities of the four study locations consisting of two city-forest pairs may provide information on the importance of habitat and

geographic distance on reproductive isolation. We used Bayesian assignments tests with 10,000 simulated individuals per population and the “leave one out” option to calculate individual assignment scores (Rannala & Mountain 1997). This resulted for each individual in four categories of probability scores (“own location”, “same habitat & different pair”, “different habitat & same pair” and “different habitat & different pair”). We used Wilcoxon signed-rank tests in SPSS version 16.0 for pairwise comparisons of probabilities between these four main categories. Furthermore, we subdivided the category “same habitat & different pair” into two sub groups: one for individuals originating from the forest and one for individuals from the city. We compared the probabilities of these two sub groups with a Mann-Whitney U test in SPSS to see if there would be a difference in genetic similarity between the two cities versus the two forests. For graphical representation of the data, we calculated relative probability scores such that within each of the individuals the four probability scores added up to 1.0.

Permits

Individuals were caught and sampled in the field with permission of the following organizations and authorities responsible for the study areas: Staatsbosbeheer Regio Oost, Staatsbosbeheer Regio Zuid, Gemeente Arnhem and Gemeente Breda. Ringing licenses were given to us by Vogeltrekstation Arnhem (licence numbers: 951 and 831). The Leiden University committee for animal experiments approved our study (licence number: DEC 05085).

Results

Morphology

We collected morphological data from 48 city males, 38 forest males, 31 city females and 13 forest females. There were some missing data points for tarsus length ($n=16$) and bill length ($n=2$), because these characteristics were not measured from the start of the data collection. Descriptive data and the number of individuals measured per location per sex for each morphological characteristic are shown in Figure 5.1. Birds from cities and adjacent forests significantly differed in several morphological traits. Significant differences were mainly found in males even though the morphological patterns for females and males look similar (Figure 5.1). This is possibly because the female sample size was relatively small and females vary considerable in body weight depending on whether they are carrying eggs or not. The female data thus support the patterns observed in males, although they often have too little statistical power.

Urban males were significantly heavier than forest males in both city-forest pairs (Welch Two Sample t-test: pair I; $t = 2.7$, $df = 35.4$, $p < 0.01$, pair II; $t = 2.1$, $df = 43.9$, $p = 0.04$). A similar difference in body weight was found in females in pair I ($t = 2.3$, $df = 14.1$, $p = 0.03$), but not in pair II ($t = 0.3$, $df = 12.4$, $p = 0.78$). No differences in male tarsus and bill length were found in pair I (tarsus length; $t = 0.2$, $df = 29.9$, $p = 0.85$, bill length; $t = 1.1$, $df = 36.5$, $p = 0.28$) nor in the females of both pairs (tarsus length pair I; $t = 0.3$, $df = 11.6$, $p = 0.78$, tarsus length pair II; $t = 0.8$, $df = 8.5$, $p = 0.43$, bill length pair I; $t = 0.9$, $df = 9.8$, $p = 0.39$, bill length pair II; $t = 1.0$, $df = 10.8$, $p = 0.34$). Urban males in pair II had a significantly

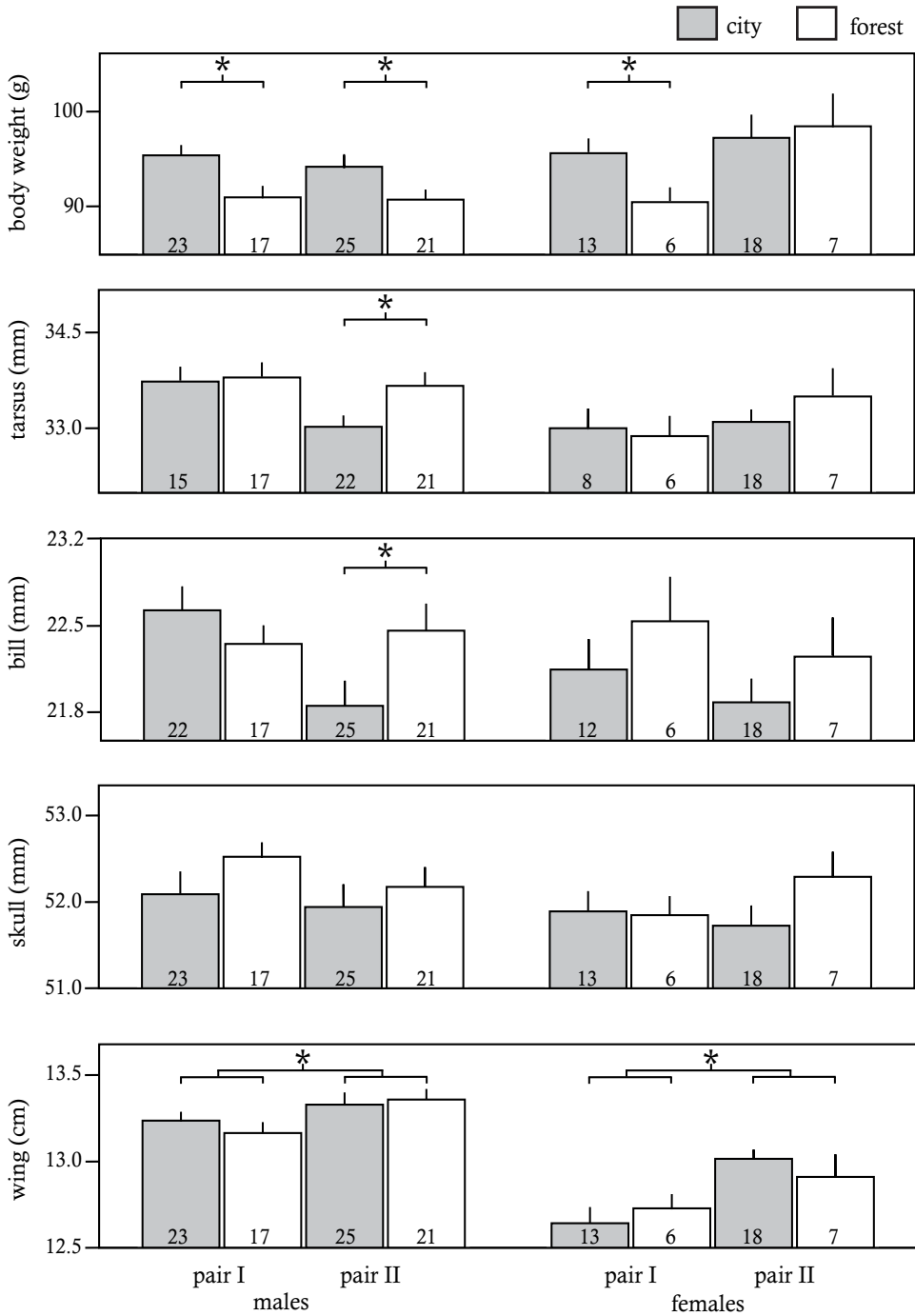


Figure 5.1. Means and standard errors of body weight, tarsus length, bill length, skull length and wing length of urban (grey bars) and forest (white bars) blackbirds separated by city-forest pair and gender. Number in bar indicates the group size. * means $p < 0.05$.

shorter tarsus and bill length than forest males (tarsus length; $t = 2.3$, $df = 39.4$, $p = 0.03$, bill length; $t = 2.0$, $df = 43.3$, $p = 0.048$). No differences in skull size were found between urban and forest birds in neither of the pairs or sexes (male pair I; $t = 1.4$, $df = 35.1$, $p = 0.17$, male pair II; $t = 0.7$, $df = 43.9$, $p = 0.51$, female pair I; $t = 0.1$, $df = 14.8$, $p = 0.89$, female pair II; $t = 1.5$, $df = 14.2$, $p = 0.15$). Similarly, urban and forest birds did not differ in wing length in both pairs and both sexes (male pair I; $t = 0.9$, $df = 33.4$, $p = 0.38$, male pair II; $t = 0.3$, $df = 43.9$, $p = 0.75$, female pair I; $t = 0.7$, $df = 15.5$, $p = 0.50$, female pair II; $t = 0.7$, $df = 8.1$, $p = 0.48$). An additional analysis on wing length showed that wings were significantly shorter in pair I than pair II in both males and females (linear model for males: effect city-forest pair; $F_{1,85} = 4.8$, $p = 0.03$, effect habitat; $F_{1,84} = 0.1$, $p = 0.8$, interaction city-forest pair x habitat; $F_{1,83} = 0.6$, $p = 0.4$, linear model for females: effect city-forest pair; $F_{1,43} = 14.0$, $p < 0.01$, effect habitat; $F_{1,42} = 0.04$, $p = 0.8$, interaction city-forest pair x habitat; $F_{1,41} = 1.0$, $p = 0.3$).

Genetics

The number of genotyped individuals was 39 in Arnhem, 26 in The Veluwe, 45 in Breda and 29 in The Liesbos. Individuals were successfully genotyped at 6.9 ± 0.04 unlinked loci. We found significant F_{ST} values for the genetic distances between all study locations: every location was genetically different at the tested microsatellite loci from the location within the same city-forest pair having another habitat and from the city and forest location of the other pair (Table 5.2).

The assignment test on 139 individuals confirmed the insight from F_{ST} values reflecting divergence among all four populations, but it also revealed additional information regarding genetic similarities (Figure 5.2). Individuals had a much higher probability to be assigned to their own location than to any of the other three locations (Wilcoxon signed-rank test: $z > 5.1$, $p < 0.001$). Moreover, probability scores were significantly higher for the “same habitat & different pair-category” than for the “different habitat & same pair-category” ($z = 2.2$, $p = 0.03$). A similar tendency was found for probability scores to be higher for the “same habitat & different pair-category” than for the “different habitat & different pair-category” ($z = 1.8$, $p = 0.08$). Individual assignment probabilities did not differ between the two categories representing the locations having a different habitat type than an individuals’ sampling location ($z = 0.5$, $p = 0.6$). Furthermore, the probability scores were not different for the urban and forest subgroups of the category “same habitat & different pair” (Mann-Whitney U test: $z = 0.6$, $p = 0.5$).

Table 5.2. F_{ST} values between all study locations. * means p-values < 0.01 .

		pair I		pair II	
		city	forest	city	forest
pair I	city	-	0.032 *	0.015 *	0.023 *
	forest	0.032 *	-	0.023 *	0.014 *
pair II	city	0.015 *	0.023 *		0.022 *
	forest	0.023 *	0.014 *	0.022 *	-

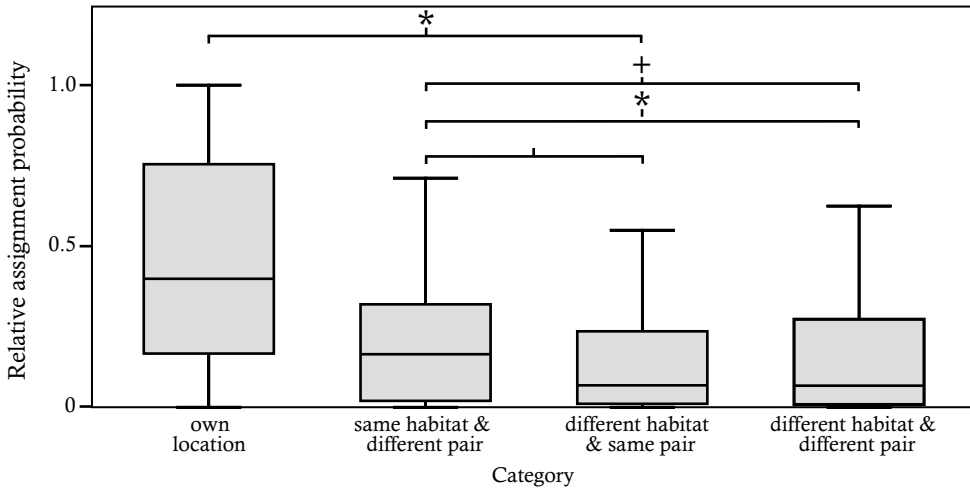


Figure 5.2. Relative assignment probabilities of individuals for the four different location categories. Box plots show the median (line), interquartile range (box) and 95% range (whiskers). * indicates p-values < 0.05 and + indicates $0.05 < p < 0.1$.

Discussion

Habitat-dependent morphological differentiation

Bills were shorter in urban birds than forest conspecifics in one of the city-forest pairs and not in the other pair. Evans *et al.* (2009a) found an overall effect for urban birds to have stubbier bills (i.e. lower ratio between bill length and bill height) than rural ones, which was also reported by Gregoire 2003. Results of these studies are thus relatively consistent and suggest a general pattern in which city birds often have shorter bills than forest or rural birds (Table 5.3). Short bills facilitate picking up items up food from hard surfaces and shallow soils whereas pointier bills are better for probing in deep soils (Cuthill *et al.* 1992). Hence, short bills might be an adaption to the many hard surfaces and shallow soils in cities, although they might also be the result of wear caused by feeding on hard feeding substrates (c.f. Hulscher 1985).

In our study, both urban and forest birds from pair I had significantly shorter wings than birds in pair II. Hence, the geographic location rather than habitat influenced the wing length in our study. The large-scale study across Europe found an overall effect for urban birds to have longer wings than conspecifics in adjacent rural areas (Evans *et al.* 2009a) in line with findings from a study in Germany (Partecke 2003), but contradicting a study in French (Gregoire 2003) (Table 5.3). Urban birds thus often have longer wings than non-urban conspecifics although the opposite pattern occurs in France. In general, wing length increases with the tendency to migrate (Fitzpatrick 1998; Seebohm 1901). Urban birds migrate less often in winter than forest conspecifics (Partecke & Gwinner 2007). Habitat-dependent variation in migratory tendency can thus not account for the general pattern of divergence in wing morphology between urban and non-urban areas. Instead, the divergence in wing morphology might be related to the relative openness of cities, because dense vegetation is known to select

Table 5.3. Overview of the results of various studies on the morphology of wild caught adult blackbirds. We show only the results of the males for our current study, because although morphological patterns of males and females were similar, the female comparisons were often not significant presumably due to a low sample size. Male and female data is considered for the other three studies.

Study	Morphological trait	Number of urban-nonurban pairs	Number of pairs with significant differences and their direction	Significant overall habitat effect
current study	Bill ²	2	1 x urban < forest	-
Evans <i>et al.</i> 2009a		11	5 x urban < rural	urban < rural
Gregoire, 2003		3	-	urban < forest
Partecke, 2003		-	-	-
current study		2	0	-
Evans <i>et al.</i> 2009a	Wing	11	1 x rural < urban	rural < urban
Gregoire, 2003		3	-	urban < forest ¹
Partecke, 2003		1	1 x forest < urban ²	-
current study		2	1 x urban < forest	-
Evans <i>et al.</i> 2009a		11	2 x urban < rural 1 x rural < urban	no
Gregoire, 2003	Tarsus	3	-	urban < forest
Partecke, 2003		1	1 x urban < forest	-
current study		2	2 x	-
Evans <i>et al.</i> 2009a		11	1 x urban < rural	no
Gregoire, 2003		-	-	-
Partecke, 2003	Body weight	1	0	-
current study		2	2 x	-
Evans <i>et al.</i> 2009a		11	1 x urban < rural	no
Gregoire, 2003		-	-	-
Partecke, 2003		1	0	-
current study	Skull	2	0	-
Evans <i>et al.</i> 2009a		-	-	-
Gregoire, 2003		-	-	-
Partecke, 2003		-	-	-

“-” indicates that a study did not measure a morphological trait or did not test for either differences within urban-nonurban pairs or an overall habitat effect for all pairs combined.

¹ There was a significant interaction effect between gender and habitat indicating that there was an effect of habitat in males, but not in females.

² This significant difference between urban and forest birds was significant in males and not in females.

for shorter wings to improve manoeuvrability (Rayner 1988; Winkler & Leisler 1985).

We found that urban birds are heavier than conspecifics in adjacent forests, which might be related to food availability. Blackbirds do not rely much on anthropogenic food sources, in contrast to for instance house sparrows (*Passer domesticus*) which consequently have elevated cholesterol level in cities (Gavett & Wakeley 1986a; Gavett & Wakeley 1986b). Urban blackbirds do eat a lot of earthworms in the Netherlands, because they can find them relatively easily on fields of grass commonly present in Dutch cities. A longitudinal study showed a positive relationship between annual rainfall and blackbird body mass, which they suggested to be related to earthworm availability (Yom-Tov *et al.* 2006). Habitat-dependent variation in diet may therefore explain why urban birds are heavier than forests ones in our study. However,

only one significant difference in body weight was found in all the pairs of the other studies and this was for urban birds in Spain to be lighter than nearby forest ones (Table 5.3). The Dutch situation in which habitat-dependent food availability appears to influence body weight is thus an exception to the general pattern that urban and nearby forest birds have a similar weight.

Urban birds had shorter tarsi than forest birds in one of our two city-forest pairs. A similar pattern was reported in the studies of both Partecke (2003) and Gregoire (2003). Furthermore, the same significant difference between urban and non-urban birds was found in two of the eleven urban-rural pairs of Evans *et al.* 2009a and in just one pair of that study the non-urban birds had the shortest tarsi. Urban birds thus regularly have shorter tarsi than conspecifics in adjacent non-urban areas although there are just as often no differences with occasionally even the opposite pattern (Table 5.3). Variation in tarsus length might be related to differences in vegetation such as typical leaf litter depth.

To summarize, there are many morphological differences between urban and non-urban birds in adjacent areas. In general, populations of urban birds have almost always shorter bills, often longer wings and sometimes smaller tarsi than populations in nearby non-urban areas. This pattern does not apply to all urban-rural pairs, because there are various cases in which in one or more traits no difference or even the opposite pattern is observed. The locality-specific differences in the general pattern might be attributable to two factors. Firstly, it could be the result of founder effects when a few individuals with a nonstandard morphology colonize new areas (Grant & Grant 1995; Saccheri *et al.* 2006). Secondly, locality-specific differences might arise as a consequence of habitat differences such as microclimatic circumstances, food availability and predator abundance. To conclude, urban and nearby forest populations are often morphologically divergent, even at a very small geographic scale, with some of the traits showing moderately consistent divergence pointing at possible habitat-dependent selection pressures as a driving force.

Habitat-dependent genetic differentiation

We found significant genetic structure in our study locations: each of the four populations was genetically different from the other three populations. Hence, also the urban and forest population within city-forest pairs were significantly differentiated from each other suggesting a non-panmictic situation. Although blackbirds disperse on average only a distance of 200 metres, they can disperse over long distances of several hundreds of kilometres (Paradis *et al.* 1998). Hence, given that the travelling distance between urban and nearby forest populations is very short, the significant genetic divergence between adjacent populations is remarkable. We believe that some behavioural mechanism is likely responsible for a reduction in dispersal across habitats. Our playback results in chapter 2 are in line with such an impact.

In birds there is in general little genetic differentiation between adjacent locations, presumably due to the ability of birds to disperse over long distances. A study on orange-tufted sunbirds (*Nectarinia osea*) within an urban population showed the existence of two distinct song dialects with sharp boundaries on a microgeographic scale (< 1.5 km²) (Leader *et al.* 2000) with males responding strongest to their own dialect (Leader *et al.* 2002). Nevertheless,

in this urban population the two dialect groups were not associated with neutral genetic differences (Leader *et al.* 2008). A cross-fostering experiment with great tits (*Parus major*) from two qualitatively different parts within one forest separated by ~3 km showed significant differences in nestling condition and shape between birds originating from either low- or high density areas within the same forest (Shapiro *et al.* 2006). These were interpreted as genetic adaptations to local conditions related to competition for food and nesting availability at a very small geographic scale. Furthermore, a study on urban and rural populations of house finches (*Coproductus mexicans*) located 6-10 kilometres apart showed genetic divergence in bill shape as well as genetic differentiation at neutral markers probably related to habitat differences (Badyaev *et al.* 2008). Hence, genetic differentiation in birds is possible at very small geographic scales without important geographic boundaries, but presumably only when nearby locations are ecologically sufficiently distinct.

We did not only show that urban and nearby forest populations are genetically differentiated, but also that individuals are more likely to be assigned to populations with the same habitat as an individuals' home habitat than to populations of another habitat. Evans *et al.* 2009b performed similar assignment tests in their blackbird study and found that rural populations are less differentiated from each other than urban populations, which suggests that urban populations often had a rural location as their source population. Results from blackbirds in France support this idea, because especially the two urban populations were genetically differentiated from each other (Gregoire 2003). A slightly different pattern emerges from our study, because our assignment tests suggest that there is no difference between urban or forest individuals in how likely they are to be assigned to the other study location with the same habitat type as their own (i.e. urban populations were genetically just as similar to each other as the forest populations were to each other).

This apparent discrepancy in results might be explained by regarding the geographic situation. Evans *et al.* 2009b suggested that colonization of cities occurred via independent events across Europe. However, colonization events might not be independent from each other at smaller geographic areas like our study. At shorter distances it is more likely that urban birds have colonized new nearby cities in a leapfrog manner (proposed by Luniak *et al.* 1990). The relative genetic similarity of urban birds in our study could thus be the result of colonization via dispersion from nearby colonized cities in the Netherlands. In this case, the observed genetic pattern might be due to habitat-dependent founder effects whereby gene flow between recently colonized cities and nearby forests have not erased genetic relations between source and current populations. Habitat probably also played an important role after colonization in this scenario, because otherwise it seems likely that nearby urban and forest populations would have mixed again causing an erosion of the genetic pattern. This is supported by the finding that genetic distances between urban and nearby rural populations were not negatively correlated with the year of urban colonization (Evans *et al.* 2009b). With our data it is impossible to distinguish between founder effects and the influence of gene flow after colonization. Nevertheless, it is likely that habitat played an important role in genetic population divergence either via habitat-dependent colonization or habitat-dependent gene flow between existing populations.

Conclusion

We have demonstrated the presence of divergence in song, ecological traits and genetics within the same set of study populations. Our findings add to the growing body of evidence that urban birds are morphologically differentiated from nearby non-urban conspecifics. Some of the morphological traits show a moderately consistent pattern of divergence suggesting habitat-dependent selection pressures. The genetic differentiation between cities and adjacent forests indicates that population divergence can occur at very small geographic scales. Furthermore, we found that within our study the populations with a similar habitat were genetically more similar to each other than populations of a different habitat. At a small geographic scale, it therefore seems plausible that colonization of cities has historically occurred from nearby urban areas and that there might be current exchange between populations with the same habitat. Habitat-dependent divergence in song and responses to song could contribute to this, which is in line with our findings that urban and forest male blackbirds respond differentially to a song trait showing habitat-dependent divergence (chapter 2). Future research on the influence of song divergence on male dispersal and female mating decisions could provide insight in the role of sexual signals in population differentiation.

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