Differences in the timing of reproduction between urban and forest European blackbirds (*Turdus merula*): result of phenotypic flexibility or genetic differences?

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Species which have settled in urban environments are exposed to different conditions from their wild conspecifics. A previous comparative study of an urban and a forest-living European blackbird population had revealed a three weeks earlier onset of gonadal growth in urban individuals. These physiological adjustments are either the result of genetic differences that have evolved during the urbanization process, or of phenotypic flexibility resulting from the bird's exposure to the different environmental conditions of town or forest. To identify which of these two mechanisms causes the differences in reproductive timing, hand-reared birds originating from the urban and the forest populations were kept in identical conditions. The substantial differences in the timing of reproduction between urban and forest birds known from the field did not persist under laboratory conditions, indicating that temporal differences in reproductive timing between these two populations are mainly a result of phenotypic flexibility. Nevertheless, urban males initiated plasma luteinizing hormone (LH) secretion and testicular development earlier than forest males in their first reproductive season. Moreover, plasma LH concentration and follicle size declined earlier in urban females than in forest females, suggesting that genetic differences are also involved and might contribute to the variations in the timing of reproduction in the wild.

Keywords: adaptation; gonadal cycles; life-history trait; luteinizing hormone; microevolution; urbanization

1. INTRODUCTION

In birds inhabiting mid and high latitudes, seasonal reproduction occurs at a time at which ultimate factors like food supply are most appropriate for survival of both parents and offspring (Thomson 1950; Lack 1968). Accurately timed reproduction is under strong natural selection because there is often only a short period of favourable conditions for breeding in the annual cycle (Murton & Westwood 1977). Long-term predictive cues, such as photoperiod, are often used for the proximate timing of the required physiological processes that must be initiated in advance of the breeding season (Farner & Lewis 1971; Dawson *et al.* 2001). In photoperiodic birds, increasing daylength in early spring initiates a cascade of neuroendocrine and endocrine events that lead to the development of gonads and the activation of reproductive behaviours, and eventually to the termination of these processes by photorefractoriness (Farner *et al.* 1983; Nicholls *et al.* 1988). The critical daylength (Hamner 1966) required for the initiation of reproductive development in spring can differ among species—as well as within species—depending on environmental conditions, like those associated with different geographical positions (Silverin *et al.* 1993; Lambrechts *et al.* 1996). In addition, supplementary cues such as food supply, weather conditions or behavioural stimuli modify the effects of photoperiod to fine-tune the timing of reproduction (Wingfield *et al.* 1992, 1993). Differences in

the adjustment of reproduction to different environmental conditions can either be the result of genetic differences or of phenotypic flexibility, i.e. an environment-induced change of the phenotype. Phenotypic flexibility is often based on reaction norms that are themselves the result of natural selection. Consequently, identifying the effects of genetic and environmental sources of phenotypic variation is important for the understanding of population differences in reproductive timing (Endler 1986; Falconer 1989).

A comparative study of annual reproductive cycles of two free-living European blackbird (*Turdus merula*) populations, one inhabiting an urban habitat, the other a forest habitat 40 km away, had revealed substantial differences in the temporal organization of reproduction (Partecke *et al.* 2005). Urban birds of both sexes develop their gonads approximately three weeks earlier in spring than their forest counterparts, whereas both populations regress their gonads at about the same time in mid summer. It has been proposed that since their colonization of central European cities in the early nineteenth century, urban populations of European blackbirds have adapted to this new environment, as evidenced for instance, by a higher breeding density compared with non-urban populations (Luniak *et al.* 1990). However, it has not been adequately tested whether urban populations are self-sustaining or whether there is a continual influx from the forest: while Erz (1964), based on estimated mortality and recruitment rates, suggested that the urban blackbird population of Cologne is only maintained by immigration from the

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surrounding regions, Batten (1973) found that the urban blackbird population in London is rather stable and even includes a surplus of juveniles.

The extended breeding season of urban birds may either be the result of micro-evolutionary changes that have evolved during the urbanization process (e.g. different photoperiod thresholds), or of phenotypic flexibility of individuals exposed to the different environmental conditions of town or forest. Urban populations live in a man-made habitat that differs environmentally from the original forest habitat in many ways. These include artificial light throughout the night, additional anthropogenic food, higher breeding density and a warmer microclimate (Luniak *et al.* 1990). The aim of the present study was to differentiate between these two alternatives (genetic versus environmental causes) and to elucidate how and to what degree they both contribute to the generation of the observed differences in the timing of reproduction.

2. MATERIAL AND METHODS

In 1998, we collected 30 urban (17 males, 13 females) and 30 forest (16 males, 14 females) European blackbird nestlings (average age of 8 days) from 10 nests in Munich (48°07' N, 11°34' E; 518 m asl) and from 10 nests in a forest (Raisting: 47°53' N, 11°04' E; 553 m asl) study site, where we had previously collected field data (see details in Partecke *et al.* 2005). Urban blackbirds hatched between 5 May and 28 May (median: 19 May), and forest birds between 17 April and 3 June (median: 11 May). In both sexes, hatching dates did not differ between the two groups (Mann–Whitney *U*-test; males: $Z = -1.51$, $p = 0.129$; females: $Z = -0.68$, $p = 0.496$). Nestlings were hand-reared in a room under natural daylight and an ambient temperature of $20 \pm 2.5^\circ\text{C}$. After having reached independence at an age of *ca.* 40 days, all birds were moved to individual cages in another room with natural photoperiod and an ambient temperature ranging from $+15^\circ\text{C}$ (minimum) in winter to $+30^\circ\text{C}$ (maximum) in summer. Birds could hear but not see each other. Food (Granvit; Hungenberg) and drinking water were available *ad libitum*. The experiment started on 1 August 1998 and was terminated on 20 October 2000. All the experimental procedures were carried out in accordance with the guidelines of the relevant German agencies.

(a) Sampling techniques

Every three to four weeks, blood samples for measurement of circulating plasma luteinizing hormone (LH) were collected between 09.00 and 12.00. Blood samples were obtained by puncturing a brachial wing vein with a 23 gauge needle. Blood (*ca.* 600 μl) was collected into heparinized microcapillaries. The plasma was separated from the red blood cells by centrifugation at 9000 r min^{-1} for 10 min (Force 7 Microcentrifuges, Denver Instrument Company Ltd) and afterwards stored at -70°C until hormone analysis.

One or two days after each blood sampling, birds were laparotomized under Isoflurane anaesthesia to assess gonadal size (Wingfield & Farner 1976). In males, the width of the left testis was measured to the nearest 0.1 mm. In females, the diameter of the largest follicle was measured to the nearest 0.1 mm up to a length of 7 mm. Measurements of follicles with a diameter of more than 7 mm were no longer accurate because, at this stage, follicles completely covered the inside of the incision; thus all values exceeding 7 mm were set to 7 mm. Incisions were treated with Actihaemyl gel (SolcoSwitzerland) and sealed with Histoacryl (Braun). Recovery from surgery was rapid and the incision healed quickly.

(b) Experimental protocol and definition

Birds of both populations underwent two complete annual reproductive cycles during the two years of the experiment. In the first experimental year (first year), blood samples were collected and laparotomies were started on 26 January 1999 (blood) or 28 January 1999 (laparotomy) until 20 August 1999 (blood), or 22 August 1999 (laparotomy). The sampling in the second experimental year (second year) started on 25 January 2000 (blood) or 26 January 2000 (laparotomy) until 24 August 2000 (blood) or 26 August 2000 (laparotomy).

To compare the timing of the reproductive season between urban and forest experimental birds, we used threshold values of gonads. In males, the onset and the end of 'functional' gonads were defined as the dates at which testicular width reached a value of 5 mm. This threshold value was selected on the assumption that testes start producing sperm at half-maximum volume (Gwinner 1986). In females, the onset and the end of mature gonads were defined as the dates at which the largest ovarian follicle reached a diameter of 2 mm. Egg-laying is possible within a few days after the follicle diameter reaches 2 mm (J. Partecke, personal observation). In both males and females, these dates were calculated by interpolation of individual fitted logistic growth curves obtained for each experimental bird. For a comparison of reproductive timing between free-living and experimental blackbirds, data for the timing of reproductive activity published in Partecke *et al.* (2005) were used.

During the first year, 16 urban and 15 forest males, and 13 urban and 14 forest females were investigated. Because of mortality, these numbers were reduced in the second year to 16 urban and 13 forest males, and 12 urban and 14 forest females.

(c) Hormone assay

Plasma LH levels were measured with a double-antibody radioimmunoassay (Follett *et al.* 1972, 1978). Chicken LH was iodinated following an established protocol. Primary antiserum against chicken LH (3–3) and standards (fraction PRC-AE1-s-1) were prepared and donated by P. J. Sharp. The secondary antiserum, goat anti-rabbit immunoglobulin 51–156, was purchased from Antibodies Incorporated, Davies, CA. Duplicate 40 μl aliquots of blood plasma were assayed and samples were analysed in three assays. Mean intra-assay variation was $14.5 \pm 4.8\%$ ($n = 3$) and inter-assay variation of the three assays was 8.5%. The mean detection limit for LH was 78 pg ml^{-1} .

A serial dilution of European blackbird plasma resulted in quantitative displacement of the antibody parallel to chicken standard LH. This assay has also been previously validated for use with European blackbirds (Schwabl *et al.* 1980).

(d) Statistical analyses

All statistical analyses were performed with SPSS, v. 10.0.7 (SPSS Inc., Chicago, IL), following Sokal & Rohlf (1998). Original datasets were tested for normality using the Kolmogorov–Smirnov–Lilliefors test (KSL-test) and for equality of group variances using the Levene test.

Changes in plasma LH levels over time were assessed using repeated-measures ANOVA routines with season as a within-subjects variable and origin (urban and forest population) as a between-subjects factor for each year (first year and second year). Plasma LH data were log-transformed to reduce heteroscedasticity. If the overall between-subject effect (origin) and/or the interaction of sampling date and origin were significant in repeated-measures ANOVA, univariate *F*-tests were used for *post hoc* comparisons between urban and forest birds (origin) at each

Figure 1. Seasonal changes in plasma concentration of LH (median; first and third quartile) and gonadal size (mean $\pm$ s.e.m.) of urban (triangles) and forest (circles) (a) male and (b) female European blackbirds in (i) the first and (ii) the second year. Asterisks denote a significant difference between the two groups ($p < 0.05$). Horizontal dashed lines indicate the minimum functional testis size and the follicle diameter above which egg-laying is possible within a few days. For sample sizes see § 2.

sampling date. One-way ANOVAs were performed to test for variations in the onset and end of functional gonads between urban and forest individuals within each sex using the sex-specific interpolated threshold values of the gonads. All tests were two-tailed and the significance level was set at $\alpha = 0.05$. Data of LH samples are presented as median $\pm$ first quartile and third quartile, respectively, and remaining data are presented as means $\pm$ 1 s.e.m.

3. RESULTS

(a) Seasonal cycles of plasma luteinizing hormone

In both experimental years, plasma LH concentrations changed seasonally in both sexes (males first year: $F_{8,232} = 18.851$, $p < 0.0001$; males second year: $F_{9,243} = 15.615$, $p < 0.0001$; females first year: $F_{8,200} = 28.632$, $p < 0.0001$; females second year: $F_{9,216} = 25.708$, $p < 0.0001$).

Plasma LH concentrations of males entering their first year showed an interaction of origin with season ($F_{8,232} = 2.370$, $p = 0.018$; figure 1a), but no significant

overall origin effect ($F_{1,29} = 1.581$, $p = 0.219$). Urban males started to secrete LH earlier in the season and secreted their highest plasma LH levels in March; at that time these levels differed significantly from those of forest males (figure 1a). Forest males displayed their highest plasma LH levels in May. For both groups, plasma LH levels declined simultaneously in May, reaching the lowest levels in July and August. In contrast to the first year, the pattern of plasma LH levels was similar in urban and forest males entering their second year, with no significant interaction of origin with season ($F_{8,232} = 0.859$, $p = 0.563$) and with no significant overall effects of origin ($F_{1,27} = 0.194$, $p = 0.663$; figure 1a).

In females, plasma LH concentrations showed an interaction of origin with season ($F_{8,200} = 2.518$, $p = 0.013$) and an overall between-subject effect ($F_{1,25} = 8.513$, $p = 0.007$) during the first year. Plasma LH levels of both groups increased at the same time, but maximum levels were lower and levels decreased earlier in urban females than in forest

Figure 2. Julian date (1 January = 1) of onset (left symbol) and end (right symbol) of (a) 'functional testes' and (b) 'functional follicles' in free-living (field) and experimental (laboratory first year and laboratory second year) urban (triangles) and forest (circles) European blackbirds. Data for free-living birds are only for adult birds and taken from Partecke *et al.* (2005). No error bars are presented for the field data because gonadal growth and regression were determined for the entire dataset within the urban and the forest population, resulting in one habitat-specific growth and regression curve. Data from experimental groups are presented as means $\pm$ s.e.m. Asterisks denote a significant difference between the two groups within each reproductive year ($p < 0.05$). For sample sizes see § 2.

females (figure 1b). The differences disappeared in the second year. There was only a tendency for a significant interaction of origin with season ($F_{9,216} = 1.790$, $p = 0.071$) but no overall significant between-subject effect ($F_{1,24} = 0.845$, $p = 0.367$; figure 1b).

(b) Gonadal development

Results similar to those of plasma LH were obtained for the timing of gonadal development (figure 1). In urban males, the onset of functional testes occurred 8.4 days earlier than in the forest males during the first year ($F_{1,30} = 10.007$, $p = 0.004$; figure 2). In the second year, both urban and forest males reached the state of reproductive activity simultaneously, with a non-significant mean difference of 3.6 days between urban and forest males ($F_{1,27} = 1.603$, $p = 0.216$). The timing of the end of active gonads did not differ between urban and forest males in both years (first year: $F_{1,30} = 1.997$, $p = 0.168$; second year: $F_{1,27} = 0.455$, $p = 0.506$).

Females of both populations achieved the state of reproductive activity at similar dates in both experimental years (first year: $F_{1,25} = 0.00$, $p = 0.989$; second year: $F_{1,24} = 0.256$, $p = 0.618$; figures 1 and 2). However, urban females regressed their gonads significantly earlier in both years than forest females (first year: $F_{1,25} = 8.458$, $p = 0.008$; second year: $F_{1,24} = 4.309$, $p = 0.049$; figures 1 and 2).

In a comparison between free-living and experimental blackbirds within each population, gonads started to grow earlier in the field than under laboratory conditions (figure 2). The difference between free-living and laboratory birds was greater in the urban group (Δ : males = 13.2–16.3 days; females = 35.3–38.8 days) than in the forest group (Δ : males = 0.1–1.6 days; females = 7.0–7.5 days). With respect to the end of functional gonads, testes of experimental and free-living birds regressed more or less simultaneously, whereas laboratory urban and forest females reduced their gonads later in the season compared with free-living conspecifics (figure 2).

4. DISCUSSION

The substantial differences in reproductive timing found between free-living urban and forest European blackbirds (Partecke *et al.* 2005) were reduced or even absent in captive blackbirds in the present study, which were exposed to identical environmental conditions. Experimental males from both populations displayed similar LH and gonadal profiles during their second year. Also, urban and forest females did not differ in the initiation of follicular growth in both years. These results suggest that the pronounced differences in the onset of reproduction between urban and forest-living European blackbirds are mainly the result of phenotypic flexibility to different environmental conditions.

This conclusion is not valid for the behaviour of urban males in the first year. They initiated testicular growth and increased plasma LH levels earlier than their forest conspecifics, leading to an advanced reproductive maturation compared with the forest males (a mean difference of 8.4 days). Our birds were collected very early in life and subsequently raised and kept under identical conditions during the entire experiment. Thus, we consider it likely that the observed differences in the timing of reproduction have a genetic basis. The lack of a difference in the subsequent year may either be the result of social synchronization or of changes in the temporal organization of consecutive annual events, such as migration and reproduction. In central Europe, the European blackbird is a partial migrant (Stephan 1999). It has been suggested that migratory disposition inhibits the activation of the hypothalamo-pituitary-gonadal axis, and, as a consequence, suppresses plasma LH secretion and testicular development in migratory individuals (Schwabl *et al.* 1984). We have indications that our experimental urban blackbirds had a reduced migratory disposition compared with our experimental forest individuals during the first year, whereas both groups exhibited similar migratory behaviour in the subsequent year (J. Partecke, unpublished data). Hence, the disappearance of a temporal difference in testicular growth during the second year may have an explanation in the change of

migratory behaviour from the first to the second spring migratory season preceding the reproductive phase.

An indication for a genetic difference in the timing of reproduction was also found in our hand-reared females. Although both groups initiated follicle growth synchronously, urban females did not achieve plasma LH levels as high or follicles as large as forest females in the first year, and their plasma LH concentration and follicle size declined earlier than in birds with a forest origin. These data suggest population differences in the degree of sexual maturation.

Although the present results argue in favour of a genetic basis for the differences found between urban and forest blackbirds, the present experiment has been performed on birds that had spent their first week of life in their natural habitats. Hence perinatal effects can not be completely excluded (Mousseau & Fox 1998). At this stage, we cannot rule out the possibility that maternal effects may have caused the observed differences in reproductive timing between experimental urban and forest blackbirds. However, if maternal effects were to be involved in the timing of reproduction, they would act differently on males and females because differences in reproductive timing between the populations were sex-specific.

Whereas temporal differences of gonadal growth between free-living and experimental forest birds were small, captive urban birds developed their reproductive system conspicuously later than their wild counterparts. This suggests that urban-specific environmental conditions advance the onset of reproduction in urban birds relative to that of forest individuals in their respective natural habitats.

Urban environments differ from non-urban habitats in several aspects that might influence the timing of reproduction. The urban microclimate is generally warmer than that of adjacent forest habitats (Klausnitzer 1989; Sukopp 1998; Partecke *et al.* 2005). Experimental studies in which photosensitive birds were exposed to the same long day lengths but different ambient temperatures provided some evidence for a direct advancing effect of higher temperatures on the reproductive system (Silverin & Viebke 1994; Wingfield *et al.* 1996, 1997, 2003). Furthermore, urban birds consume extra anthropogenic food offered at feeders during winter and early spring, and they also take advantage of all kinds of human waste. Moreover, the warmer microclimate in the urban habitat may induce an earlier increase of natural food abundance compared with the forest environment. There is evidence that food supplementation advances laying-date in many species (see reviews by Daan *et al.* 1989; Boutin 1990; Svensson 1995). However, most of these studies were conducted when the birds' gonads were already enlarged, and not during the early phase of gonadal growth. In studies that concentrated on the early phase of gonadal growth, food reduction resulted only in a slight delay of gonadal maturation in, for example, the European starling, *Sturnus vulgaris* (Dawson 1985; Meijer 1991) and the brambling, *Fringilla montifringilla* (Lofts *et al.* 1963).

All of these effects of temperature and nutritional conditions on reproductive development can nevertheless hardly explain the fact that our caged urban blackbirds developed their gonads two weeks later than their free-living counterparts. Particularly during winter and early spring, when gonads develop, ambient temperatures

were on average considerably higher in our birds' experimental housing than in the urban environment of Munich. Moreover, the quality of the food that we provided to our caged birds was presumably at least as high as that available for the birds in the city. Hence, if temperature and/or food are factors that affect the development of reproductive competence, an advance rather than the observed that delay of reproductive maturation would have been expected.

There are other factors that may induce an extension of the breeding season in urban blackbirds. A special feature of urban European blackbird populations is their higher breeding density, which in turn results in a smaller distances between neighbouring territories compared with blackbirds inhabiting non-urban areas (Stephan 1999). This is probably associated with a higher frequency of social stimulation, which may advance the activation of the reproductive system (Immelmann 1971; Wingfield & Farner 1993; Silverin & Westin 1995).

Furthermore, it has been repeatedly suggested that during the process of urbanization blackbirds have reduced their migratory behaviour (e.g. Luniak & Mulsow 1988). Hence the advanced onset of reproduction in urban birds might just be the result of the fact that most urban birds are sedentary and, therefore, start their reproduction earlier. However, other avian species show similar differences in the timing of reproduction between urban and non-urban populations, even though birds of both populations are sedentary (e.g. Tatner 1982; Dhondt *et al.* 1984; Bowman *et al.* 1998). Thus, the general phenomenon of an earlier breeding season in urban ecosystems can not be explained by a reduced migratory disposition. In our blackbirds it may, however, be an important factor causing intraspecific variation.

Finally, urban birds, in contrast to their forest conspecifics, are exposed throughout the year to a considerable amount of artificial light. The stimulatory role of the increasing vernal day length on the onset of the avian reproductive system is well known (e.g. Farner *et al.* 1983). In an urban environment, additional artificial night-lighting can be very intense. Evidence for a stimulatory effect of low-intensity artificial light on gonadal growth comes from experiments on house sparrows, *Passer domesticus* (Bartholomew 1949).

In conclusion, this study suggests that the profound differences in reproductive timing of European blackbirds in urban and forest environments can be mainly attributed to phenotypic flexibility. However, temporal differences in testicular development during the first year and follicular regression in both experimental years may well point to the possibility that genetic differences also play a role.

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