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ORIGINAL PAPER

A recent invasive population of the European starling sturnus vulgaris has lower genetic diversity and higher fluctuating asymmetry than primary invasive and native populations

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Abstract Fluctuating asymmetries (FA) are small stress-induced random deviations from perfect symmetry that arise during the development of bilaterally symmetrical traits. One of the factors that can reduce developmental stability of the individuals and cause FA at a population level is the loss of genetic variation. Populations of founding colonists frequently have lower genetic variation than their ancestral populations that could be reflected in a higher level of FA. The European starling (*Sturnus vulgaris*) is native to Eurasia and was introduced successfully in the USA in 1890 and Argentina in 1983. In this study, we documented the genetic diversity and FA of starlings from England (ancestral population), USA (primary introduction) and Argentina (secondary introduction). We predicted the Argentinean starlings would have the highest level of FA and lowest genetic diversity of the three populations. We captured wild adult European starlings in England, USA, and Argentina, measured their mtDNA diversity and allowed them to molt under standardized conditions to evaluate their FA of primary feathers. For genetic analyses, we extracted DNA from blood samples of individuals from Argentina and USA and from feather samples from individuals from England and sequenced the mitochondrial control region. Starlings in Argentina showed the highest composite FA and exhibited the lowest haplotype and nucleotide diversity. The USA population showed a level of FA and genetic diversity similar to the native population. Therefore, the level of asymmetry and genetic diversity found among these populations was consistent with our predictions based on their invasion history.

Keywords Exotic bird species · Fluctuating asymmetry · Genetic variability · *Sturnus vulgaris*

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Introduction

Fluctuating asymmetries are small stress-induced random deviations from perfect symmetry that arise during the development of bilaterally symmetrical

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traits (Ludwig 1932). Factors that cause fluctuating asymmetry (FA) can be either genetic or environmental in origin (Møller and Swaddle 1997). Developmental stability is the production of a phenotype, such as bilateral symmetry, under a given set of specified environmental and genetic conditions (Møller and Swaddle 1997). One of the factors that can reduce developmental stability and cause FA at a population level is the loss of genetic variation (Parsons 1990). Populations that have experienced a bottleneck or small populations of founding colonists frequently have lower genetic variation than their ancestral populations (Barret and Kohn 1991, Nei et al. 1975; Sakai et al. 2001). In part, this is because uncommon haplotypes are unlikely to be represented in founding, invasive populations (Futuyma 1997).

Reduced genetic variation appears to destabilize developmental processes and increases FA in a wide range of taxa (Møller and Swaddle 1997). For example, populations of cheetah (*Acynoxis jubatus*), a species that has experienced a considerable population bottleneck followed by intense levels of inbreeding, have elevated levels of FA in cranial morphology compared with a similar species of wild cat that have not experienced demographic declines (Wayne et al. 1986). Similarly, populations of British doer deer (*Capreolus capreolus*) established from a small number of introduced founder individuals have a lower genetic diversity and higher FA than relatively less disturbed populations (Baker and Hoelzel 2013).

Increased FA can also result from a large number of environmental factors, such as abnormal ambient temperatures, nutritional stress, parasitic infection, and habitat fragmentation (Møller and Swaddle 1997; Anciães and Marini 2000; Gebremichael et al. 2019). When an organism is exposed to a novel set of environmental conditions, or even to a novel element within the same environment, developmental processes may become disrupted resulting in increased individual expression of FA. For example, Møller (1992a) found that feather FA of male barn swallows (*Hirundo rustica*) increased with ectoparasitic load and Anciães and Marini (2000) recorded greater wing and tarsus FA within bird species that occupied fragmented forest habitats compared with those in contiguous forests.

The European starling, *Sturnus vulgaris* (hereafter starling) is native to Eurasia and the northern-most part of North Africa and has been introduced successfully in many countries, including the United States of America (USA), New Zealand, Australia, South Africa, and some Pacific and Caribbean islands (Blackburn et al. 2009; Craig 2020; Feare 1984; Rollins et al. 2009). The USA invasion started with the release of a small number of individuals from England in Central Park, New York (60 in 1890 and 40 in 1891, Feare 1984). The starling population in the USA has subsequently grown to at least 140 million individuals (Jernelov 2017). Starlings' invasion into Argentina came about through escapes from the pet trade near Buenos Aires in 1983, with birds imported from the USA (Navas 2002). As in the USA, the invasion of Argentina has been rapid and prolific, with starling populations booming in urban areas (Di Giácomo et al. 1993; Jensen 2008). Just 20 years after introduction, estimates of the relative density of starlings in urban and natural areas varied between 2.21 and 0.22 individuals ha^{-1}, respectively (Palacio et al. 2016, Rebolo and Fiorini 2010) and an estimate of the number of starlings in urban parks of Buenos Aires in 2010 suggests approximately 4,600 individuals occupy just those habitats (Rebolo and Fiorini 2010).

Consistent with the arguments discussed above, genetic diversity in sequences of mitochondrial DNA (mtDNA) within starling populations is lowest in the most recently colonized areas of Western Australia (Rollins et al. 2011). In this study, we documented the genetic diversity and FA of starlings from ancestral and introduced populations. We tested if starlings from a recent invasive population (Argentina) have higher FA and lower mtDNA diversity than those from an older invasive population (USA) and from the large original ancestral population (England). We captured wild adult starlings in Argentina, USA, and England and maintained them under standardized experimental conditions during the feather molt after which, we compared the FA of their primary feathers (left feather length minus right feather length). Feathers are a morphological trait that grows de novo each year and feather growth is affected by environmental and genetic factors (Gill 2001). Therefore, by controlling the environmental factors that individuals were exposed to during the molting, we evaluated how genetic variation was associated with feather growth and FA. We predict the populations established from a small number of founders (i.e. Argentina, where starlings were introduced in 1981 from the USA; and USA, where starlings were introduced in 1891 from

England; Fig. 1) will have higher levels of FA and lower genetic variability than a population from the ancestral range of the species (i.e. England). In addition, we also predict that there would be higher FA and lower mtDNA diversity in the starlings from the Argentinean population than in those from the USA population, as genetic diversity may have recovered somewhat in the USA population since their introduction in the nineteenth century.

Methods

Fluctuating asymmetry

We conducted experiments on wild-caught adult European starlings of undetermined sex (as FA is not expected to vary between sexes, J. P. Swaddle, unpublished data) to evaluate FA of primary feathers under standardized conditions. The number of experimental individuals was 20 from Bristol, England (51° 27′ 0″ N, 2° 35′ 0″ W), 20 from Williamsburg, Virginia, USA (37° 16′ 15″ N, 76° 42′ 25″ W), and 17 from Bernal, Buenos Aires, Argentina (34°42′0″ S, 58°17′0″ W). The individuals were

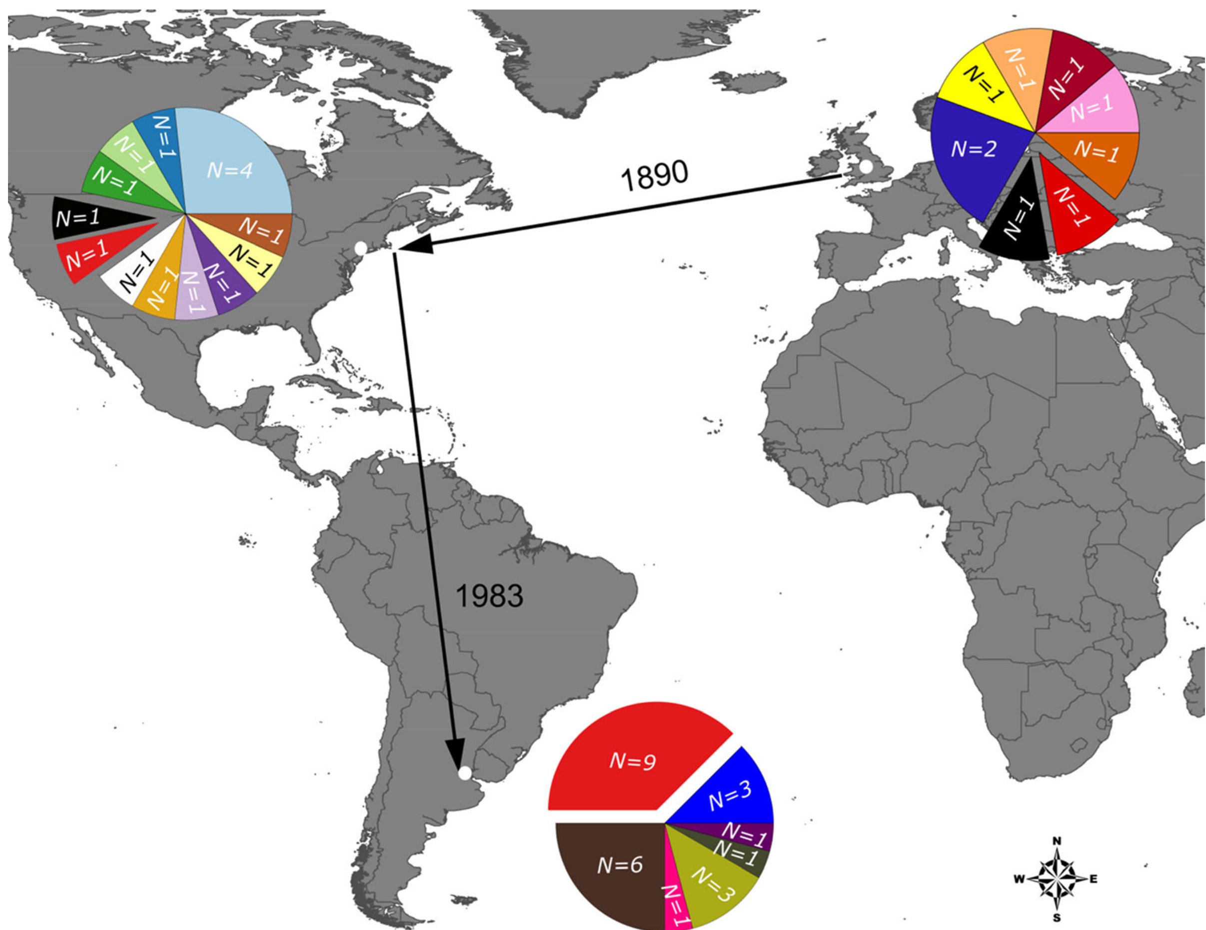

Fig. 1 Map showing the time and direction of European starlings (*Sturnus vulgaris*) introductions into America. White circles denote sampling localities. Pie charts show each population's haplotypes and their frequencies. Shared haplotypes (H2 and H12) are separated from main pie charts. For the Argentina chart: H1 blue, H2 red, H3 brown, H4 fucsia, H5 olive, H6 mosque, H7 purple. For USA chart: H8 light blue, H9 darker light blue, H10 soft green, H11 dark lime green, H12 black, H2 red, H13 white, H14 light orange, H15 light violet, H16 violet, H17 light yellow, H24 light brown. For the England chart: H12 black, H2 red, H23 orange, H22 pink, H18 carmine, H19 soft orange, H20 yellow, H21 dark blue

captured with mist nets during summer, at the end of their breeding season, and before they started molting. In England they were captured during June 1993, in the USA during June 2008, and in Argentina during November 2008. All the individuals from the same country were housed in one group in an indoor aviary of approximately 3.0 × 2.0 × 2.5 m (length × width × height). We decided to use one large free-flight room with lots of perches instead of individual small cages because in small cages the birds experience excessive feather wear and damage. They were housed at a constant temperature of approximately 20 °C, illuminated with a regular overhead fluorescent tube lighting, fed with ad libitum chick starter crumbs (with same nutritional characteristics in the three countries, Purina Start & Grow chick feed), and with drinking and bathing water available. The birds were maintained on a short day (8L:16D) photoperiod to induce feather molt (Witter and Swaddle 1994). They remained on this photoperiod throughout the experiment.

We examined birds and measured their feathers repeatedly until they were no longer growing indicating that the molt of the primary feathers was completed. When the feathers completed their growth (12 weeks on average), we measured the length of the primaries 3, 5 and 7 with Vernier calipers (± 0.1 mm) (Fig. 2). We avoided primaries 8 and 9 because they are much more likely to be damaged compared with the feathers we measured. We chose primaries 3, 5, and 7 because they represent several weeks of growth and they are less likely to become damaged. We measured feathers by sliding the Vernier caliper to the base of the feather where it emerges from the skin and

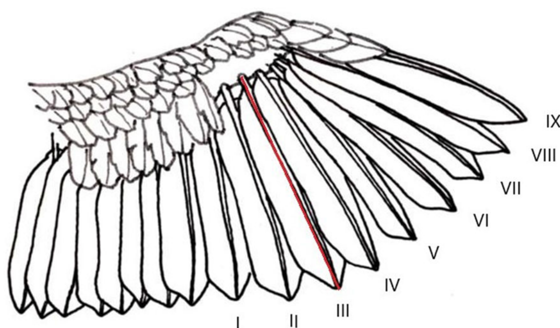

Fig. 2 Diagram of a wing where the numbers indicate the primary feathers classification. The red line shows the feather length that was measured three times for primary feathers 3, 5 and 7. Drawing by Ignacio Crudele

applied approximately equal pressure in all cases, and then measured the length to the tip of the feather (Fig. 2). To minimize measurement error, we measured each feather on both wings three times (Swaddle and Witter 1998). We always checked carefully the tips of the primaries and when any damage or wear was noted those values were excluded from the analyses (see Cuthill et al. 1993). Only one individual that experienced extensive feather damage and one other that did not complete molt (both from the Argentine dataset) were excluded (final sample size: N = 15 Argentina, N = 20 USA and N = 20 England).

As each feather was measured three times, we obtained three values of signed asymmetry (left feather minus right feather length) (formula 1). Then, we standardized for size differences among the feathers by dividing the signed asymmetry by feather length and averaged the values for each feather (relative signed asymmetry, formula 2). Then obtained the relative FA (absolute value of the relative signed asymmetry, formula 3). After that, we calculated for each individual, the composite asymmetry as the average of the relative signed asymmetries of the three feathers (formula 4) and the composite FA (as the average of the relative fluctuating asymmetries of the three feathers (formula 5).

$$\mathrm{F} = \mathrm{Feathers}(\mathrm{F} = 3, 5, 7)$$

$$i = \text{Repeat of the measure of the feather}(i : 1, 2, 3)$$

$$\mathrm{L} = \text{Measure of left feather}, \mathrm{R} = \text{Measure of right Feather}$$

$$\begin{aligned}\text{Signed asymmetry} &= \mathrm{SAi} \\ &= \mathrm{Li} \\ &- \mathrm{Ri}(\text{formula 1})\text{for each pair of measures}\end{aligned}$$

Relative Signed Asymmetry F = RSAF = χ(SAi / 0.5 (Li+Ri)) (formula 2) for each feather

$$\text{Relative Fluctuating Asymmetry F} = \mathrm{RFAF} = \mathrm{I\ RSAF\ I}(\text{formula 3})\text{for each feather.}$$

Composite Asymmetry = CA χ (RSAF) (formula 4) for each individual averaged across the three feathers.

Composite Fluctuating Asymmetry = CFA = χ (formula 5) for each individual averaged across the three feathers

As the distribution of composite asymmetry values closely approximated a normal distribution (visual

assessment of normal probability plot) and did not differ significantly from a mean of zero ($t_{54} = -0.97$, $p = 0.34$), the asymmetries measured were considered as fluctuating asymmetries (Swaddle et al. 1994). Previously, we have demonstrated that our measurements of signed asymmetry are highly repeatable for each of the three feathers within each country (F range: 4.5–51.4, $P < 0.0001$) (Swaddle and Witter 1994). We then used the composite FA of each individual to compare levels of plumage asymmetry among countries. Because of the particular "half normal" frequency distribution of the composite FA, we used a Box-Cox transformation (with $\lambda_1 = 0.3$ and to $\lambda_2 = 0.008$) to normalize the data (Swaddle et al. 1994), and performed a one-way ANOVA to explore if there were differences among countries and Tukey contrasts for pairwise comparisons. Statistical analyses were performed using R software, Version 3.4.0 (R Development Core Team 2013) and the R Studio, Version 3.6.2 (RStudio Team 2020). All tests were two tailed, values are reported as means ± SE and differences were considered significant at $P < 0.05$.

Genetic analyses

We analyzed blood samples from 15 of the 20 experimental individuals from Williamsburg, USA and 24 individuals from Bernal, Argentina (17 experimental and 7 non-experimental individuals captured in the same place). We did not have blood samples of the experimental individuals from Bristol but we obtained feathers (collected in 2009) from nine starlings captured in Oxford, England. Given there is constant gene flow among European starling populations in England (Neves et al. 2010) and that Oxford and Bristol are only 117 km apart, we assumed these starlings came from the same genetic pool. DNA was extracted from both blood and feather samples using an ethanol-salting out protocol. In order to sequence the mitochondrial control region we used the pair of primers svCRL2 and svPheH3 developed by Rollins et al. (2011). We performed polymerase chain reaction (PCR) amplifications in a total volume of 25 µl with 50–100 ng of total genomic DNA template, 0.2 µM of both forward and reverse primers, 0.2 mM of each dNTP, 1X PCR buffer (Invitrogen), 2.5 mM MgCl2, and 0.1 U Taq DNA Polymerase (Invitrogen). PCR conditions were 5 min of hot start at 94 °C; followed by 30 cycles of denaturation for 30 s at 94 °C, 15 s at 53 °C annealing temperature, and 30 s at 72 °C, and a final extension for 10 min at 72 °C. Five individuals from the USA could not be amplified. If "a" polymorphism in "the" primer sites is underlying our inability to amplify these samples, excluding them could introduce some bias to our diversity estimates. Amplification products were purified using the Exonuclease I and Shrimp Alkaline Phosphatase enzymes according to manufacturer's instructions (Thermo Scientific) and sequenced in an ABI 3130 XL (Applied Biosystems, Foster City, CA, USA) sequencer using ABI Big Dye^{TM} Terminator Chemistry.

Our mtDNA control region sequences were edited and aligned using BioEdit 7.2.5 (Hall 1999). Identification of the sequences as part of mtDNA noncoding control region D-loop highly variable domains I-III was confirmed by aligning our sequences with other populations of starlings obtained from the GenBank database. Population parameters of genetic diversity were defined as the total number of haplotypes, number of polymorphic sites, common and private haplotypes, haplotype diversity (probability that two randomly sampled haplotypes are different) and nucleotide diversity (π, average number of nucleotide differences per site in pairwise comparisons among sequences) and were all estimated in DNAsp 5.0 (Librado and Rozas 2009) for each population. Haplotype richness, after rarefaction to the minimum sample size of $N = 9$, was estimated using the *rarefy* function in the R package *vegan* (Oksanen et al. 2018). Population structure was assessed using exact tests (Raymond and Rousset 1995) implemented in Arlequin v3.5.1 (Excoffier et al. 2010).

Data availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Results

Fluctuating asymmetry

We observed significant differences in final asymmetry of the primary feathers among starlings from Argentina, USA, and England (ANOVA, $F_{2,52} = 30.09$, $P < 0.0001$, Fig. 3). Levels of

composite FA of primary feathers were higher in the Argentinean population compared with the ones from USA (Tukey HSD: mean difference 0.048 ± 0.009, $P < 0.001$) and England (Tukey HSD: mean difference 0.066 ± 0.009, $P < 0.001$). Starlings from the USA also exhibited higher levels of asymmetry than those from England, although the difference did not reach statistical significance (Tukey HSD: mean difference 0.018 ± 0.008, $P = 0.067$). The power for this test was approximately 60%, which means that is a moderately powerful experimental design and indicates that the difference in FA between birds of USA and England was not statistically supported.

Genetic analysis

Sequencing of the 942 bp of mtDNA control region in 48 starlings revealed 24 haplotypes consisting of 31 polymorphic sites among all samples (Fig. 1 and Table 1). All polymorphic sites have 2 variants, except from one site with 4 variants (polymorphic site 11, Table 1). The average nucleotide composition of these control region sequences was: 28.13% A, 26.65% T, 32.16% C, 13.05% G, with a bias against G. Among these haplotypes, six had already been described by Rollins et al. (2011) in Australian/UK populations: HapA, HapE, HapF, UKA, UKC, UKK (GenBank accession numbers FJ542126, FJ542133, FJ542128, HQ263631, HQ263633 and HQ263641, respectively). Additionally, four of the haplotypes

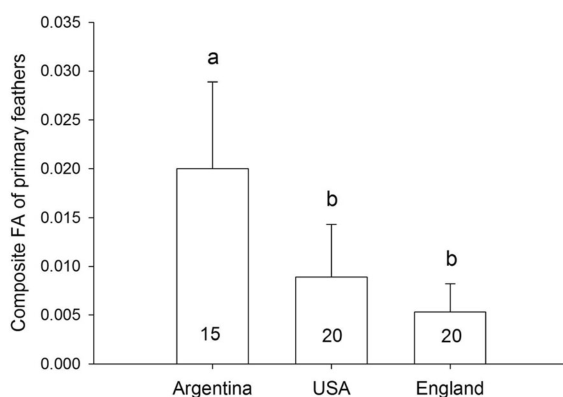

Fig. 3 Mean ± SE levels of Composite Fluctuating Asymmetry (FA) of the primary feathers 3, 5, and 7, for three populations of European starlings that molted in aviaries under similar conditions. Numbers inside bars show sample sizes. Different letters indicate significant differences ($P < 0.05$) in Tukey contrasts

found in our study were also screened in European Starlings in the USA: H_39 (NA005), H_5 (NA010), H_24 (NA002), H_27 (NA012) and one in the UK: H_32 (NA021, GenBank accession numbers MT795633-MT795650, Bodt et al. 2020, Table 1). No identical sequences were found when comparing overlapping regions of our sequences with the ones previously detected for starlings from South Africa (Berthouly-Salazar et al. 2013, GenBank accession numbers KF638591–617). New haplotypes have been deposited in GenBank under accession numbers MW513733-MW513740, MW513743, MW513744, MW513746-MW513748, MW513750.

Several haplotypes were unique to populations (6 in Argentina, 10 in USA and 6 in England (Fig. 1, Table 2). Two frequent haplotypes (H2, 38%; H3, 25%) were found in Argentina, one in the USA (H8, 27%), and one in England (H21, 22%). Interestingly, one of the six previously described haplotypes is the most frequent one in Argentina (called H2 in this study, HapF in Rollins et al. 2011, H_25 in Bodt et al. 2020). One haplotype that we discovered in an individual from England was previously detected in the USA (H22 in our study, H_32 in Bodt et al. 2020). Most haplotypes were found at low frequencies in only one population.

When comparing our findings to all previous mitochondrial studies (Rollins et al. 2011, Berthouly-Salazar et al. 2013, Bodt et al. 2020) in European starlings we discovered that four haplotypes that we encountered in the USA and one in the England starling's populations have actually also been detected in the publication of Bodt et al. 2020. For this reason, we included in Table 1 also the alternative haplotype's names in those studies and GenBank accession numbers to facilitate identification. We found that H2 (alternative names HapF and H_25), which is coded in red in Fig. 1 and Table 1, was previously detected in all studied regions (England, USA and Australia) and we also found it in all regions covered by our study (England, USA and Argentina). H12 (alternative names UKC and H_31), coded in black, was detected in England and North America in both our's and Bodt's study.

Diversity indices varied among geographic regions (Table 2). Birds sampled in Argentina showed the lowest haplotype and nucleotide diversity of all populations studied. While we found evidence of genetic differentiation between Argentina and the

Table 1 Variable sites in the mitochondrial DNA control region for 24 haplotypes found in samples from Sturnus vulgaris collected in Argentina, USA and England. Alternative haplotype names according to overlap with previously published data for starlings from Australian and UK (Rollins et al. 2011), South African (Berthouly-Salazar et al. 2013) and from USA (Bodt et al. 2020) are also included

Hap Name	Alt Hap Name	GenBank Acc.Nr.	5	19	23	24	37	38	47	69	88	93	125	139	142	190	215	216	274	325	374	519	623	647	663	667	679	850	881	898	902	926	941
H1		MW513733	T	T	C	C	A	T	A	A	G	C	A	A	A	G	T	A	C	C	A	T	A	A	T	C	C	C	T	C	A	G	C
H2	HapF H_25	FJ542128 MT795642	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	A	.
H3		MW513734	C	.	.	T	.	C	.	.	.	.	.	.	G	.	C	G	.	.	.	C	.	.	.	.	.	.	.	T	.	A	.
H4		MW513735	C	.	.	.	G	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	A	.
H5		MW513736	C	.	.	.	G	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	C	T	.	A	.
H6		MW513737	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	T	.	A	.
H7		MW513738	C	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	C	T	.	A	.
H8	H_39	MT795634	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	T	.	A	.
H9		MW513740	C	.	.	T	.	C	.	G	A	.	C	.	.	A	.	.	T	.	.	C	.	.	.	.	.	.	.	T	.	A	.
H10	H_5	MT795636	C	.	.	T	.	C	.	.	.	.	.	G	G	.	.	.	.	.	G	.	.	G	.	.	.	.	.	T	.	A	T
H11	H_24	MT795633	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	T	.	A	.
H12	UKC,H_31	HQ263633	C	.	.	T	.	C	.	.	.	.	.	.	G	.	.	.	.	.	.	C	.	.	.	.	.	.	.	T	.	A	.
H13	HapE, H_38	FJ542133	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	A	.
H14		MW513743	.	.	.	.	.	C	G	.	.	.	G	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	T	.	A	.
H15		MW513744	C	.	T	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	A	.
H16	UKA, H_7	HQ263631	C	.	.	T	.	C	.	.	.	.	.	G	G	.	.	.	.	.	G	C	.	G	.	.	.	.	.	T	.	A	T
H17	H_27	MT795637	C	C	.	T	.	C	.	.	.	.	.	.	.	.	.	.	.	T	.	C	.	.	.	T	.	.	.	T	.	A	.
H18	HapA, H_9	FJ542126	C	.	.	.	.	C	.	.	.	G	.	.	G	.	.	.	.	.	.	C	.	.	.	.	.	T	.	T	.	A	.
H19		MW513746	C	.	.	T	.	C	.	.	.	.	.	G	G	.	.	.	.	T	.	C	.	.	.	.	.	.	.	T	.	A	T
H20		MW513747	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	A	.
H21		MW513748	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	G	A	.
H22	H_32	MT795639	C	.	T	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	T	.	A	.
H23	UKK H_11	HQ263641 MT795638	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	T	.	A	.
H24		MW513750	.	.	.	T	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	A	.

Table 2 Mitochondrial DNA control region polymorphisms for each population of *Sturnus vulgaris*

	N[a]	Hap[b]	H_R[c]	Poly Sites[d]	Haplotype Diversity	π[e] (%)	Common haplotypes	Private Haplotypes
Argentina	24	7	4.6	13	0.793 ± 0.003	4.72	H2 (38%) H3 (25%)	6
USA	15	12	7.8	20	0.943 ± 0.003	5.65	H7 (27%)	10
England	9	8	8	15	0.972 ± 0.004	5.40	H20 (22%)	6

[a] Number of individuals,

[b] Number of haplotypes,

[c] Rarefied haplotype richness,

[d] Number of polymorphic sites,

[e] Nucleotide diversity

other populations (pairwise exact test p-value Argentina–USA = 0.00001; pairwise exact test p-value Argentina–England = 0.00064) no genetic separation was suggested for starlings from USA and England populations (pairwise exact test p-value England–USA = 0.18604). Even though the number of observed haplotypes in the USA population is higher in comparison with England's population (12 vs 8, respectively) this could be caused by a higher number of individuals sampled in the former population ($N = 15$ in USA vs $N = 9$ in England). This was corroborated by rarefying haplotype richness to the minimum population sample size (England). The estimated rarified haplotype richness (expected number of haplotypes if only 9 individuals were sampled from each population) ranged from 4.6 to 8 and was

similar for starlings from England and from the USA. The most recently introduced population (Argentina) had the lowest rarefaction haplotype richness (Table 2, Fig. 4). The rarefaction curves of haplotype richness do not reach an asymptote for randomly sampled starlings from USA and England populations. This suggests that our sampling might not fully describe local diversity for those populations and that we have only succeeded sampling part of the actual diversity.

Discussion

Levels of FA and genetic diversity differ among populations of European starlings from England, USA, and Argentina in ways that are largely predicted by their history of invasions into different continents through human interventions. Because the relevant conditions under which the starlings molted were controlled and similar in the three sites, these morphological differences cannot be attributed to differences in environmental conditions at the time of molting or extent of local adaptation to them during molt, but could be explained by the level of genetic diversity in the populations of starlings in the three areas or by environmental differences experienced during development. Several studies looked at how conditions during molt influence primary feather length asymmetry in European starlings. In particular Swaddle (1997) and Swaddle and Witter (1998) studied the change in length asymmetry between successive molts. Those studies show that the energetic and physiological environments of the birds influence within-individual change in feather length asymmetry. This is why we were careful to standardize the captive conditions of the birds across the three continents. The level of asymmetry and genetic diversity found among these populations was consistent with our predictions as starlings in Argentina, which derived from a primary invasive population (USA), have the highest FA and lowest genetic variation. To our knowledge, this is the first study in which the FA of two exotic and one native conspecific populations were studied under controlled ambient conditions minimizing the potential effects of environment or genotype-environment interactions (e.g. local adaptation) on FA (Kristoffersen and Magoulas 2009). Previous studies based on allozyme variation have shown that genetic variability of different populations of starlings widely distributed in the USA (Cabe 1998) is reduced compared to the population of origin in England (Ross 1983; but see Hofmeister et al. 2021a). Cabe (1998) found that the level of heterozygosity of the population in the USA is comparable to the one of England (Ross 1983), but the former lost 42% of the alleles at variable loci. This decrease in allelic diversity in the bottleneck population would be the remaining signature of the loss of rare alleles of the source population (Cabe 1998). Bodt et al. (2020) used the same markers of the current study to compare mitochondrial genetic diversity in European starlings from North America, Australia, and South Africa, and a portion of the native range in the United Kingdom. They found that the invasive populations had lower genetic diversity than the population in the native range and suggested this could be caused by genetic bottlenecks at introduction. Moreover, the North American population, which was intermediate in terms of propagule pressure, retained

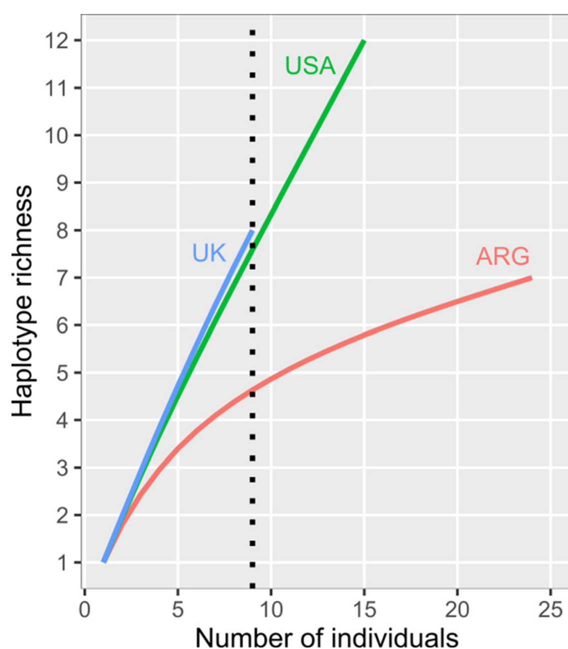

Fig. 4 Rarefaction curves of haplotype richness for three European starling's populations. The dashed line represents the smallest number of individuals per population (UK, $N = 9$) used to extrapolate the haplotype richness expected in the other populations (ARG and USA) if they have had that number of individuals sampled. USA = Common Starling's population from United States. UK = European starling's population from England. ARG = European starling's population from Argentina

the most genetic diversity. Due to the timescales, they stated this is unlikely to be caused by new mutations but could be explained by differences in genetic diversity of founders or by higher levels of differential survival between haplotypes in the USA and the other invasive populations. Unlike the findings of Cabe (1998) and Bodt et al. (2020), our work did not show significant differences in genetic variability between Starlings from England and USA. One possibility to explain this difference with respect to the Cabe´s results might be due to the use of different genetic markers that follow different evolutionary paths with different mutation rates. With respect to Bodt et al. (2020) results, surprisingly we found the same value of haplotype diversity for the UK population although they used 45 samples finding 30 haplotypes. Nevertheless, the value they obtained for the USA population was lower (0.876) than our estimation (0.943). One explanation for this might be that Bodt et al. (2020) used starlings from 14 localities whereas we captured starlings only in one locality (VA). Although starlings from North America have nearly continued gene flow (Cabe 1999; Hofmeister et al. 2021a), one possibility might be that starlings in the population we sampled have a higher genetic diversity as the result of local adaptation (Hofmeister et al. 2021a, 2021b).

Unlike Rollins et al. (2011), who found most haplotypes to be widespread suggesting no geographical association, most haplotypes identified in our study were at low frequencies in only one population. Shared haplotypes are only present in one individual from England and one from the USA but in 9 individuals from Argentina. Therefore common haplotypes only have similar frequencies when contrasting the ancestral population and the region of primary introduction. All haplotypes found in Argentina except one were not present in birds from the USA, the likely source of introduction. Although these haplotypes might exist in the USA at low frequency, they were probably not sampled in our study. An alternative explanation could be that these haplotypes have been lost in the USA after the introduction in Argentina.

Our results suggest that starlings from the population in Argentina display the lowest genetic diversity of the populations analysed in this study, based on a lower rarefied haplotype richness, inferior number of polymorphic sites, and minor nucleotide diversity. The genetic differentiation found could be due to geographic differences in evolutionary forces (selection, genetic drift, gene flow caused by spatial sorting) acting over time in different regions.

Although sample size was bigger in the population from Argentina ($N = 24$ individuals) the haplotype richness was the lowest, even after accounting for differences in sample size ($H_R = 4.6$). The low haplotype richness found in Argentina could be a result of inbreeding or genetic bottleneck of the introduced starlings. It is possible that our representation of haplotypes from randomly sampled starlings from England and USA is incomplete. Once the rarefaction curves flatten off, additional sampling is unlikely to add new haplotypes. However, rarefaction curves of haplotype richness suggest an incomplete sampling of haplotypes in USA and England (the shape of the curves indicates that almost every new sample might represent a new haplotype) and similar haplotype richness values after accounting for differences in sample sizes.

In recent years, a reference genome (Stuart et al. 2021) and a liver transcriptome for this species were sequenced and became available (Richardson et al. 2017). These resources could be used in the future to better analyze genetic variability allowing the development of new genetic markers that could be added in genetic studies.

Similarly to our results, Lovatt and Hoelzel (2011) found that FA and morphological variation were higher in bottlenecked populations of reindeers (*Rangifer tarandus*) compared to the source populations. Also Zachos et al. (2007) found a negative relationship between FA of non-metric skull and mandible traits and genetic variability in the roe deer (*Capreolus capreolus*). These results support the current findings, but what do our results mean for the starling invasion? If an increase in FA correlates with a reduction in fitness (e.g. Beardmore 1960), then it would be expected that the invasive population with a higher FA (Argentina) suffers from a worse performance. This could be a potential cause of the failure in the establishment and reproduction of individuals with low genetic variability in new areas. Nevertheless, the extent to which the FA of an individual reflects its developmental stability and predicts its fitness, depends on the character chosen for analysis (Clarke 1995). If bottleneck induced deleterious allele frequency shift, this may be reflected in a decrease of the biological function of individuals. However, the

distributional range expansion of 22 km per year of starlings in Argentina (Zufiaurre et al. 2016) suggests that their higher FA of primary feathers has not a remarkable effect on their fitness.

We expect the differences in primary feather FA among the starling populations we studied to be also present in other free-living starlings in these same locations. It is possible that local adaptation might result in lower expression of FA in England and the USA than in Argentina. As Argentinian starlings have been in that location for only a few decades, it is less likely that those birds have adapted to local conditions to the same degree as the birds in England and the USA. It could also be that environmental factors in Argentina, USA, and England vary to produce alternate patterns in the feather FA of free-living birds. Feather FA might be a sensitive indicator of many forms of environmental pollution and adverse environmental conditions during feather molt and regrowth (Møller and Swaddle 1997). Starlings have affinity for anthropogenic structures and their environment may be tied to the human environment (Zufiaurre et al. 2016). Nevertheless, they show a flexible use of space and can be also found in suburban and rural environments (Feare 1984; Hofmeister et al. 2021b). Therefore, it would be interesting to capture and measure free-living starlings just after they molt in their natural environments at the same sites our experimental birds were captured, to compare feather FA of free-living and experimental birds. A small difference between these values might give insight into how well adapted birds are to the particular stressors that caused FA.

As the aim of our study was to isolate genetic effects and minimize the influence of environmental factors on the production of FA, we placed our experimental birds in indoor aviaries in which variables were controlled throughout the study. Nevertheless, if molting is affected by the conditions to which starlings were exposed to before they were captured, it could be that we did not sufficiently control environmental variation in our experiment. However, because plumage molt is expensive in terms of molecular and energetic resources (Cornelius et al. 2011; Hoye and Buttemer 2011) and our captive birds were fed unrestricted amounts of high quality food, it would be expected that the environmental factors present during molting have a much higher effect on the feather structure than the ones the individuals experienced before this period. It would be also possible that the degree of suitability of the conditions used in the controlled aviaries varied across populations. For example, if these conditions were ideal for UK birds, it is possible that they were less suitable for birds in the US and Argentina that might have adapted to their new environments. In such a case, the degree of suitability might affect the estimates of FA but we cannot control this factor and assume the conditions we used are ideal for the three populations.

Within swallow (*Hirundo rustica*) populations, at an individual level, there is evidence of negative relationships between FA and reproductive rates (Møller 1992b). Additionally, the primary feather FA we reported here might have functional consequences for flight and therefore negatively impact daily energetic budgets (Swaddle 1997). Therefore, we could predict that populations with greater FA might have overall fitness deficits relative to populations with lower FA. However, the reality is that the measures reported here deliberately down-weight the influence of environmental factors on the expression of FA. Free-living populations of starlings in Argentina, USA, and England might not differ in FA as much as we report here, depending on their degree of local adaptation (as discussed above). It is more likely that any observations of population performance differences across these localities is driven by demographic effects of initially small populations sizes (i.e. more likely in Argentina), the extent of local adaptation, and direct effects of environmental factors (e.g., temperature, pollution, parasitic infections) on individual performance. It is possible that FA relates to these factors but it would be extremely surprising if FA were a driver of any performance-related differences among populations.

In conclusion, FA of primary feathers of starlings molted under controlled conditions was higher in Argentina than in USA and England. In line with the predicted effects of genetic variability on FA, the genetic diversity (mitochondrial marker) of starling populations was lower in Argentina, where the invasion was more recent, than in USA, where the invasion is older, and England, within the starling´s native range. These results contribute new evidence to support the relationship between genetic variability and the expression of symmetry in morphological characters.

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Availability of data and material The datasets of fluctuating asymmetries generated and analysed during the current study are available as Suplementary material and new haplotypes have been deposited in GenBank.

Declarations

Conflict of interest This manuscript is not being considered elsewhere and all co-authors have agreed to this submission. We have no conflicts of interest to disclose.

Ethical approval Experiment protocols have been established in compliance with the ethical standards, ensuring that all necessary precautions have been taken and the welfare of the birds has been respected. The capture and housing of starlings in England was permitted by the UK Home Office. The capture of starlings in the USA was permitted by the Virginia Department of Game and Inland Fisheries and all animal procedures were approved by the William & Mary Institutional Animal Care and Use Committee. In Argentina the work complied with the Argentinean Law for the Conservation of Wild Fauna (22421/81).

Consent to participate All coauthors gave their approval for the submission.

Consent for publication All coauthors gave their approval for publication.

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