Urbanization drives adaptive evolution in a Neotropical bird

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Abstract
Urbanization has dramatic impacts on natural habitats and such changes may potentially drive local adaptation of urban populations. Behavioral change has been specifically shown to facilitate fast adaptation of birds to changing environments, but few studies have investigated the genetic mechanisms of this process. Such investigations could provide insights into questions about both evolutionary theory and management of urban populations. In this study, we investigated whether local adaptation has occurred in urban populations of a Neotropical bird species, *Coereba flaveola*, specifically addressing whether observed behavioral adaptations are correlated to genetic signatures of natural selection. To answer this question, we sampled 24 individuals in urban and rural environments, and searched for selected loci through a genome-scan approach based on RADseq genomic data, generated and assembled using a reference genome for the species. We recovered 46 loci as putative selection outliers, and 30 of them were identified as associated to biological processes possibly related to urban adaptation, such as the regulation of energetic metabolism, regulation of genetic expression and changes in the immunological system. Moreover, genes involved in the development of the nervous system showed signatures of selection, suggesting a link between behavioral and genetic adaptations. Our findings, in conjunction with similar results in previous studies, support the idea that cities provide a similar selective pressure on urban populations and that behavioral plasticity may be enhanced through genetic changes in urban populations.

Key words: selection, behavioral adaptation, phenotypic plasticity, selective sweep, South America
The establishment and spread of urban centers is a major modification of natural landscapes caused by recent anthropogenic activity (McKinney 2006; Grimm et al. 2008) and it is known to have severe impacts on the structure of natural biological systems (Alberti 2005, 2015). Urbanization primarily modifies the landscape by suppressing natural habitat and altering characteristics of the environment in the area. As cities comprise new environments, species living there have to deal with many previously inexperienced selective pressures such as elevated levels of air, acoustic and visual pollution (Isaksson 2015; Mohan and Kandya 2015), reduced and fragmented habitat size (Harms 1999; Gibb and Hochuli 2002; Cane et al. 2006; Delaney et al. 2010) and the overall different substrates in cities, which affect reproduction, locomotion and communication (Warren et al. 2006; Kight et al. 2012; Winchell et al. 2016). Additionally, the establishment of cities usually decreases overall biological diversity (a phenomenon known as homogenization; McKinney and Lockwood 1999) and organisms that can persist in the area or colonize it posteriorly have to deal with this altered biotic environment. Therefore, understanding the adaptation of natural populations to new urban environments has important practical implications, such as assisting in selecting reintroduction source populations (Corlett 2016; He et al. 2016) and achieving urban ecological resilience (Johnson and Munshi-South 2017).

Such questions have already been addressed in some studies across different taxa and have shown that adaptation to urban environments can happen in a relatively short period of time (reviewed in Johnson and Munshi-South, 2017). Urban populations exhibit, for example, different morphological characteristics for locomotion in urban substrates (Winchell et al. 2016) and for low dispersal of seeds (which increases offspring survival in patchy environments; Cheptou et al. 2008; Dubois and Cheptou 2017). Specifically, behavioral modifications in bird species have been documented as a way to facilitate fast adaptation, with urban populations showing less aversion to the human presence (Mueller et al. 2013) and modified behavioral patterns in order to cope with anthropogenic noise and artificial light (Kempenaers et al. 2010; Mendes et al. 2011; Slabbe koorn 2013; Cartwright et al. 2014; Brischoux et al. 2017). Behavioral phenotypes are one of the most plastic phenotypic characters (Snell-Rood 2013), and some variation in this phenotype can arise, be maintained and inherited by the offspring almost independently from the underlying genetic variation (Jablonka and Lamb 2005; Pigliucci et al. 2006). Therefore, investigating such fast behavioral adaptations are good opportunities to address fundamental evolutionary questions such as the interplay between phenotypic plasticity and genotypic evolution in the rapid adaptation of bird populations to new environments (Hohenlohe et al. 2010a; Nadeau et al. 2014; Harris and Munshi-South 2017). Since cities across the globe can be seen as an artificial experiment with an unprecedented number of replicates (Johnson and Munshi-South 2017), they provide a suitable model to investigate such theoretical questions about rapid adaptation.

Recent advances in the access to genomic information from non-model organisms (Stapley et al. 2010; Ekblo m and Galindo, 2011) also provide the methodological tools to address this question. One approach that has become popular to detect signatures of selection is the genome scan approach, which consists in seeking the genome for patterns of genetic variation that could be indicative of recent selective sweeps (e.g., low diversity levels and/or high linkage disequilibrium; Smith and Haigh 1974; Kaplan et al. 1989; Stinchcombe and Hoekstra 2008; Oleksyk and Smith 2010). The search for selective sweeps have been used in a myriad of organisms and exhibited success in identifying putative loci under selection underlying phenotypic modifications in natural populations (e.g., Hohenlohe et al. 2010a; Bruneaux et al. 2013; Andrew et al. 2013; Bernatchez et al. 2016; Schwensow et al. 2017; Bosse et al. 2017; Harris and Munshi-South 2017). Our
theoretical understanding about the dynamics of rapid adaptation on the molecular level is, however, still limited (Orr 2005a, b), and there is evidence suggesting genetic signatures left by rapid local adaptation may differ from the classic model of a selective sweep (Hermisson and Pennings 2005). As the type and extent of these signatures might differ, depending on how much time has passed since the beginning of selection (Oleksyk and Smith 2010; Hohenlohe et al. 2010b), an interesting approach would be integrating analyses that search for different types of signatures (e.g., combining approaches that search for high levels of linkage disequilibrium (LD) with those looking for high correlations of allele frequency and environmental variation; Pavlidis et al. 2010; Hohenlohe et al. 2010b; Schoville et al. 2012).

Therefore, in this study, we aim to combine approaches that search for different types of genomics signatures of natural selection to investigate the genetic base of behavioral adaptation in urban Neotropical passerine. Birds are particularly useful to answer questions about behavioral adaptations in cities, since they are conspicuous in cities and allow for easy behavioral and genetic sampling. Additionally, it is known that anthropogenic noise is a strong selective pressure on their behavioral phenotypes, specifically through masking of acoustic signals used for communication (Slabbekoorn 2013). As acoustic communication is crucial for several activities related to the reproductive success of birds (e.g., courtship and territory defense), it is expected that urban individuals exhibit modifications in song patterns to maximize efficiency of communication, which is indeed observed in several studies (Bermúdez-Cuamatzin et al. 2011; Francis et al. 2011; Luther et al. 2015). However, the underlying mechanisms, specifically regarding whether these modifications result from phenotypic plasticity or selection on genetic variation, are still unclear. Here, we perform a first investigation of the effect of urbanization in the genetic variation of the bird species *Coereba flaveola*, a Neotropical oscine passerine conspicuous in urban areas. We sampled the genetic variation of this species in different levels of urbanization and searched for loci under selection. We chose this species because 1) previous studies have shown that urban individuals change their singing patterns in response to anthropogenic noise (Winandy et al. 2021a, 2021b), providing a chance to investigate the role of genetic variation on behavioral adaptation; and 2) a recent reference genome was assembled for the species, which allowed us to increase the accuracy and reliability of the detection of selection signature from reduced genomic representation sequencing approaches (Stapley et al. 2010). Our specific goals were to: 1) investigate whether there are signatures of local adaptation in urban populations; 2) if so, identify which genomic loci are under local adaptation and 3) annotate when possible the function of selected loci, investigating their relationship with biological adaptations to urbanization.

**Materials and Methods**

**Sampling**

Our sampling comprised 24 individuals of *Coereba flaveola* from urban and rural sites around the city of Salvador, Brazil (Figure 1; Table S1). Twelve individuals were sampled in urban green areas across an urbanization gradient inside the city representing different levels of urban noise (ranging from 47 dB to 66 dB of environmental noise intensity, Table S1). Twelve individuals were also sampled in non-urban areas, outside the city of Salvador; samples were taken from four independent sites (three samples per site, Figure 1), in order to yield independent information about the genetic variation that presumably is not under selection from
urbanization (environmental noise intensity in non-urban areas were around 38-40 dB, Table S1). Individuals were collected with airguns as well as mist nets set alongside a sound speaker playing recorded *C. flaveola* songs in order to attract individuals into the net.

### DNA Extraction, library preparation and data assembly

Total DNA was extracted from muscle tissue using the DNeasy Blood & Tissue (Qiagen ©) kit, following manufacturer instructions, and stored at -20 °C. We implemented the reduced-representation sequencing approach known as Restriction-site Associated DNA Sequencing (i.e., RADSeq; Andrews et al. 2016), which uses restriction enzymes to randomly cut genomic DNA and sequence the resulting fragments. This technique has been widely used in population genomics to infer both neutral and selective processes (Davey and Blaxter 2010). In this study, we implemented the ezRAD protocol (Toonen et al. 2013; Knapp et al. 2016), which consists of an initial DNA digestion using the enzyme DpnII (New England Biolabs, Inc) and posterior library preparation using Illumina kit TruSeq Nano DNA HT (Illumina, Inc.). Specifically, we did a post-extraction DNA concentration using Agencourt AMPure XP magnetic beads (Beckman Coulter) in a proportion of DNA 1 : 0.6 Beads. Concentrated DNA was eluted in 44 μl HPLC grade H2O and added to a mix with DpnII enzyme and enzyme buffer, following concentrations recommended in the protocol. DNA quantification was performed with Qubit (Thermo Fisher Scientific, Inc.) after post-digestion clean-up, to assess the quantity of DNA to be used in library preparation, and after library was finished, to assess the final concentration to be pooled into the sequencing machine.

Library preparation was done using Illumina kit TruSeq Nano DNA HT (Illumina, Inc.) and following manufacturer instructions. Briefly, the workflow consisted of: 1) end-repair of enzyme cut sites overhangs; 2) size selection using magnetic beads provided by the kit manufacturer; 3) adenylation of 3' Ends (A-tailing) in order to ligate adapters; 4) ligation of adapters containing barcode sequences for multiplexing; 5) post-ligation cleanup with magnetic beads; 6) DNA enrichment through PCR amplification; 7) post-amplification cleanup with magnetic beads. Modifications to the original protocol were done only in step 2 to select fragment sizes suitable to 75 cycles paired-end sequencing we implemented. Therefore, we modified DNA:Bead proportion, using 1:0.7 to eliminate fragments longer than 350 bp and, then, 1:1.2 to eliminate fragments shorter than 150 bp.

Final libraries were sequenced in four lanes of the NextSeq 500/550 v2 kit (mid-output of 19.5 Gb and 150-cycle; Illumina, Inc.). Sequence fragments were assembled using the reference genome available for *C. flaveola*; we utilized only the scaffolds used for annotation, which consisted of 3848 scaffolds with a N50 of 487,855 comprising 85% of core eukaryotic genes (Antonides et al. 2017). We assembled our fragments to the reference genome utilizing the iPyrad pipeline (Eaton and Overcast, 2020), which implements a set of developed software for genome analysis in seven steps. This pipeline performs the initial filtering of low-quality sequences, mapping of reads to reference genome, within-individual assembly of sequences reads, heterozygosity and error rate estimation, and finally the search for homologous regions across all individuals to identify single nucleotide polymorphisms. In iPyrad, we set an initial filter to remove adapters and primers sequences in our dataset and performed trimming of reads (up to 15 pb) to reduce spurious SNP calling due to low quality sequencing towards the end of reads. The minimum coverage for SNP identification was set to 6, and the clustering
threshold (to align reads both within and among individuals) was set to 0.9 (i.e., 90% similarity). To generate the final file used for posterior analysis, we allowed a maximum of 50% of missing data per locus, following (Huang and Knowles, 2016). The final file contained all sequenced fragments that were successfully mapped to a reference scaffold, as well as their respective polymorphic sites within our sample.

**Intrapopulation analyses of positive selection**

We combined different approaches that look for different types of genetic signature that would suggest positive selection. These approaches can be classified as intrapopulation analysis (using only the dataset from urban areas) and interpopulation analyses (using the total dataset, consisting of differentiation tests and genotype-environment association analyses). Intrapopulation approaches were utilized to look for deviations from linkage disequilibrium as well as an excess of low and high-frequency alleles in loci from urban areas; both these signatures suggest a recent selective sweep (Sabeti et al. 2002; Voight et al. 2006; Hohenlohe et al. 2010b). Deviations from linkage disequilibrium were estimated using the algorithm OmegaPlus v. 3.0 (Alachiotis et al. 2012). An excess of low and high frequency alleles was estimated from the shape of the site-frequency spectrum (SFS). To infer selective sweeps from the SFS shape, we calculated the composite likelihood ratio (CLR) as described by (Nielsen et al. 2005) using the algorithm SweeD v 3.0 (Pavlidis et al. 2013) The input file for both analyses was the vcf file containing the SNPs calculated from urban samples only, with the information of where each SNP mapped on the scaffolds of the reference genome. Therefore, we provided to OmegaPlus and SweeD algorithms the information of the length of each scaffold as well as the distance between SNPs. Both algorithms require us to choose a grid size as well as window size (for OmegaPlus only) by which to separate local regions within the scaffold, in order to calculate LD and SFS across the genome. Since these parameters may vary depending on the dataset and the biological group being analyzed, we chose to empirically summarize *a priori* the number of polymorphic sites and the distance between them from our sample. Most scaffolds (around 500 out of the total of 611 reference scaffolds that were present in the assembly of urban individuals only) showed few SNPs (between 1 and 3), suggesting a small grid number. We performed the analyses varying the grid number from 3 to 5, which incorporated around 70% of all available scaffolds (the remaining not included 30% consisted of those scaffolds showing 2 or less polymorphic sites). When analyzing the distribution of distance between SNPs across all scaffolds, we noticed that most SNPs were highly clustered in the contig, with few SNPs being further than 10 kb apart. Therefore, we chose this value to assure linkage disequilibrium would be calculated between SNPs close together (incorporating then most of the available polymorphic sites). This rationale assumes that the few SNPs farther than 10kb apart are not significantly linked.

The levels of LD and the shape of SFS (summarizing the number of low and high frequency alleles and described by the CLR) was calculated for all windows across all scaffolds in the urban individuals. In order to identify loci under the selection, we used neutral simulations to create a distribution of values of LD and CLR that would be expected in the absence of selection (referred hereafter as the neutral distribution). We looked for values of observed LD and CLR that deviated from this neutral distribution in order to identify outlier SNPs. Since both non-selective (i.e., mutation, migration and genetic drift) and selective microevolutionary processes are expected to shape observed patterns of genetic diversity; Luikart et al. 2003; Stapley et al. 2010; Hohenlohe et al. 2010b), we incorporated the effect of non-selective processes in the estimation of the neutral distribution,
as done by some authors (Harris and Munshi-South, 2017). Initially, we assessed genetic structure among populations and then performed neutral simulations under different demographic scenarios to estimate the most likely demographic history of the urban population. The best demographic scenario was then used to simulate how the statistics of selection used in OmegaPlus and SweeD (i.e., LD and CLR) would behave in the absence of selection. To estimate genetic structure, we used a principal component analysis (PCA) through the package pcdadapt (Luu et al. 2017) in the R software (R Core Team, 2021). We additionally used the algorithm implemented in fineRADstructure (Malinsky et al. 2018), a haplotype-based approach that performs well in scenarios of recent divergence (as is expected in divergence due to urbanization). The high resolution of fineRADstructure derives from using haplotype linkage information to estimate a co-ancestry matrix, i.e., a summary of neared neighbor haplotype relationships (Lawson et al. 2012). We used the script provided by Edgardo M. Ortiz to convert iPyrad output to fineRADstructure format (available in https://github.com/edgardomortiz/fineRADstructure-tools). Parameters were kept default and we used the R script provided with the package to plot results.

Based on the structure found, we then inferred the demographic history of urban and rural populations using the software fastsimcoal v. 2.6 (Excoffier et al. 2021), coupled with a model-selection approach. FastSimcoal implements a composite multinomial likelihood approach to estimate parameters from the joint site-frequency spectrum (jointSFS) of populations. The joint site frequency spectrum of urban and rural populations was calculated using the script easySFS.py, by Isaac Overcast available in https://github.com/isaacover/astcast/easySFS. This script uses the software ∂a∂i (Gutenkunst et al. 2009) to convert VCF files outputted by iPyrad in SFS files to be used in fastsimcoal for parameter estimation. We considered one population and projected it down to 10 individuals in the SFS, maximizing the number of segregating values for the project value used, as recommended by Gutenkunst et al. (2009).

We implemented parameter estimation under three possible demographic models (Figure 2) and retained the estimated parameters of the best model chosen through Akaike Information Criterion (Akaike 1998). Since no relevant genetic structure was recovered in different tests (see Results), three scenarios with a single population were tested: 1) a scenario of demographic stability; 2) a scenario of population bottleneck; 3) a scenario of population expansion. The parameters estimated were as follows: effective population size of present population (Ncoe), time of urbanization affecting population demography (Turbo) and effective population size of ancestral population, i.e. before urbanization (Nanc). Generation time was assumed as one year. The files used to describe the scenarios in fastsimcoal are given in the Supplementary Material.

We implemented 1,000 simulation replicates based on the best fitted demographic scenario. The simulated datasets were similar to the empirical dataset in number of independent sequenced fragments present in the assembly as well as sequence length (see Supplementary Methods). We then calculated LD and CLR from these simulated datasets using the same parameters explained above. Resulting values were used to create the neutral distribution and outlier SNPs were identified by assuming a threshold of values that fell outside the 95% percentile of the neutral distribution.
Interpopulation analysis of positive selection

We also implemented interpopulation approaches to detect signatures of selection, comparing patterns of genetic variation across both urban and rural areas. We specifically calculated 1) the genetic differentiation across urban and rural sites (differentiation analysis) and 2) the correlation of SNPs frequencies across the whole sample (urban and rural) with environmental variables (genotype-environment association approaches). Differentiation analyses look for SNPs showing greater levels of genetic differentiation (between different selective pressures) than the background levels averaged across the genome (Hoban et al. 2016), whereas genotype-environment association (GEA) approaches look for SNPs whose frequency is highly correlated with environmental values after accounting for underlying population structure (Manel et al. 2010).

For differentiation analyses, we used the $F_{ST}$ based approach implemented in BayeScan v.2.1 (Foll and Gaggiotti, 2008), which uses a Bayesian approach based on a multinomial-Dirichlet model to assess the probability of a SNP being under positive selection. We implemented an urban-rural comparison in Bayescan on unlinked SNPs (i.e., randomly selecting one SNP for each sequenced fragment assembled in iPyrad), setting prior odds to 1000. We also performed the approach implemented in the R package pcadapt, which uses principal component analysis to detect SNP that are outliers regarding how they are related to population structure (Luu et al. 2017). This approach has been shown to perform well with different levels of missing data and admixture between populations. In order to detect selection outliers, BayeScan and pcadapt implement a False Discovery Rate (FDR) approach, which corrects for errors resulting from multiple statistical tests on the same dataset (Thornton and Jensen, 2007). FDR can be interpreted as the probability that a SNP is erroneously identified as a selection outlier; we set this value to 0.05 (i.e., there was a 5% chance that an outlier SNP was erroneously identified in these analyses).

A GEA analysis was implemented in the LFMM software (Frichot et al. 2013). This approach screens the genome seeking for SNPs showing high correlation between allele frequency and environmental variation, while simultaneously accounting for underlying genetic structure using “latent factors”. As the predictor variable, we used the variation in environmental noise, measured at sampling sites. Measurements followed the same procedures cited above to identify a noise gradient across sampling sites (see Supplementary Methods for more information). To detect correlation outliers, we implemented the FDR approach using the script available in the software, setting the value to 0.05.

Functional annotation

In order to investigate whether outlier SNPs were related to specific biological functions, we retrieved the fasta sequence of reference scaffolds to which the outlier SNPs were mapped and used them in the software Blast2GO (Conesa et al. 2005). This softwares performs functional annotation based on a reference database and summarizes biological functions using Gene Ontology (GO) terms, a standardized categorization of biological processes in which different genomic regions may be involved. Using GO terms allows a general association of genomic sequences from different individuals to putative biological functions (Ashburner et al. 2000). We chose to use the entire scaffold to which an outlier SNP was mapped, instead of just the sequenced fragments in which the SNP was found, because 1) a query performed with a longer sequence would allow more confidence in the search results; 2) we wanted to account for the possibility that outlier SNP reflected linked selection on nearby...
genes (which would not be detected if only the sequenced fragment was used). We used the NCBI online dataset as reference, restricting our search to the taxon Aves. Query settings were kept in the default options: the algorithm was blastx-fast, E-value of $10^{-3}$, word size of 6, HSP Length cutoff of 33, Annotation Cutoff of 55 and GO weight of 5. Scaffolds that successfully blasted in the database were mapped and annotated, which returned a list of biological functions and processes to which those sequences were possibly related. We summarized this list and highlights biological processes that were previously associated to urban adaptation (e.g., physiological and immune system adaptation for metabolism of xenobiotics; Harris et al. 2013; Harris and Munshi-South, 2017). Additionally, we looked for possible biological processes associated with behavioral adaptation.

### Results

**Genomic dataset**

Illumina sequencing generated on average 3,050,307 reads per sample, which were assembled into 5,329 variable loci, matched to 2,368 scaffolds in the reference genome and yielded a total of 7,787 SNPs. Sequenced fragments had on average 118.41 bp, ranging from 101 to 185 bp. The dataset used for intrapopulation analyses (containing urban individuals only, $N = 12$) consisted of 783 variable fragments (on average 117.34 bp, ranging from 111 to 163 bp) and a total of 1,148 SNPs. For interpopulation analyses, we used both urban and rural samples ($N = 24$), and randomly selected one single SNP from each sequenced fragment, yielding a dataset with 5,329 unlinked SNPs.

**Neutral simulations for within-population analyses**

All population structure tests recovered $K = 1$ as the best clustering of populations in our dataset (see Figure S1 for scatterplot of PCA scores based on pcadapt results). Therefore, we implemented a single population model in fastsimcoal coalescent simulations. AIC-based model selection indicated a scenario of population bottleneck as the best model that fitted the empirical data (Table 1). This bottleneck was estimated by fastsimcoal to have occurred around 150 generations ago, which is assumed as the time of intensification of urbanization. Comparing the nucleotide diversity between urban and rural sites corroborates the bottleneck scenario: average diversity in urban areas was significantly lower (mean: $0.35 \pm 0.14$; median 0.33) than in rural areas (mean: $0.46 \pm 0.18$; median: 0.5; Wilcoxon test’s $W = 6502148$, $P < 0.01$; Figure S2).

**Scans for positive selection**

We recovered a total of 31 putative outlier SNPs (Figure 3; Table S2). These were detected from pcadapt ($N = 12$) and LFMM ($N = 13$; both tests used an FDR of 0.05), as well as SweeD ($N = 6$; CLR higher than the 95 percentile of the neutral distribution). One SNP was found in both pcadapt and SweeD tests (Table S2). Bayescan and OmegaPlus tests did not recover any outliers. Putative outlier SNPs were located in a total of 51 scaffolds in the reference genome, among which 30 were successfully blasted in the NCBI database. A final number of 14 scaffolds were fully annotated and returned GO terms describing biological processes (Table S2).
We distributed the scaffolds (hereafter referred as outlier regions) in five groups based on their overall biological function (Tables 2 S2): 1) regulation of cell growth, division, movement and organization (N = 5); 2) immune system (N = 1); 3) regulation of energetic metabolism (N = 4); 4) regulatory genes controlling location and rate of DNA expression (N = 10); and 5) genes related to the neural system (N = 4). One scaffold was annotated as a transmembrane protein, with no further information on the literature that could allow us to assign it into any of the categories above. Four scaffolds were blasted to hypothetical proteins (with no known function) and one scaffold was blasted solely to the published genome of *C. flaveola*, with no information on function (Table S2). A reduced list of the outlier regions is presented on Table 2.

Figure 1 highlights a few regions worth mentioning due to their putative relevance in urban adaptation (see next session): jockey/pol (two different reference scaffolds coding for this gene were found by LFMM and *pcadapt*, and possibly related to regulatory processes); ERVK113 (found by *pcadapt* and related to the immune system); FIGNL1 (the same reference scaffold was signaled as an outlier in SweeD and *pcadapt*; it is involved in DNA break repair, regulating cell growth and division). Genotype-environment association analyses recovered two regions involved in the regulation of energetic metabolism (*ARID5B* and *INPPL1*) and one region involved in the control of cell proliferation (*GALNT7*). Finally, *pcadapt* recovered a few genes (e.g., *OTUD7A* and *SLIT3*) involved in neural development and synapse signaling.

**Discussion**

We investigated the population genomics of the Neotropical bird *C. flaveola* along urban and rural areas and uncovered putative SNPs under selection in urban environments (Tables 2 and S2). Our results agree with recent studies addressing similar questions, which have found genomic regions under selection related to metabolism, immunity and oxidative stress in urban populations of the rodent *Peromyscus leucopus* (Harris and Munshi-South 2017), the insect *Bombus lapidarius* (Theodorou et al. 2018) and the red fox *Vulpes vulpes* (DeCandia et al. 2019). Examples of annotated genomic regions related to metabolism in our study include *ARID5B*, which increases lipid storage (Claussnitzer et al. 2015), and *INPPL1*, which regulates the action of insulin-stimulated kinases and might confer resistance to dietary obesity (Habib et al. 1998). *INNPL1* also reduces protein kinase B (*AKT*) activation, a gene involved in the regulation of the insulin signaling pathway. Interestingly, *AKT* was previously found as a putative selection outlier locus (Harris and Munshi-South 2017). Congruences between our results and those by previous authors suggest a common adaptation to urban environments related to changes in the type of dietary resources available. As examples of annotated regions in *C. flaveola* related to immunity and oxidative stress, we highlight the gene ERVK113 (found by *pcadapt*) which belongs to a class of transposable elements (the endogenous retrovirus) that has been shown to shape the evolution of transcriptional networks underlying the infectious interferon response in mammalian lineages (Chuong et al. 2016). Additionally, two genes found in our analyses, *GALNT7* and *TRABD2B*, may suggest an adaptive response to high levels of physiological stress and consequent increase of cancerogenous cells in urban populations (Nourazarian et al. 2014; Momenyan et al. 2016). *GALNT7* is a glycopeptide transferase essential for the regulation of cell proliferation and has been implicated in tumorigenesis (Bennett et al. 1999; Nie et al. 2016), whereas *TRABD2B* is a metalloprotease that acts as a negative regulator of the Wnt signaling pathway (a family of genes implicated in oncogenesis; (Zhang et al. 2012). The recovering of immune system related genes in
different taxa (i.e., birds and mammals), different areas in the globe (i.e., tropical and temperate cities), and by using different genomic sequencing approaches (i.e., RADSeq in this study, and RNASeq in, for example, Harris and Munshi-South, 2017) strengthens the idea that changes in the immune system and regulation of cell proliferation might result from an universal selective pressure in urban environments around the world, and might be important in underlying adaptation in several other taxa.

In addition to the results listed above, which are in agreement with previous genomic studies investigating urban adaptation, our findings suggest two additional processes of rapid local adaptation that are less evidenced in the literature: 1) changes in regions responsible for regulating DNA expression (i.e., regulatory regions), and 2) changes in the development, structure and functioning of the nervous system. The process of adaptation through changes in regulatory pathways has been suggested in several studies addressing deep evolutionary scales (Carroll, 2008; Feinberg and Irizarry, 2010; Bräutigam et al. 2013; Johnson, 2017), but fewer studies have demonstrated the role of transcription regulation in rapid local adaptation (Gilad et al. 2006; López-Maury et al. 2008). Among the list of putative selection outliers in our analysis, the gene jockey/pol was identified in two different reference scaffolds by LFMM and pcadapt results. This gene codifies an RNA-dependent DNA polymerase, which synthetizes cDNA from RNA-strands and is known to be involved in the activity of retrotransposons (mobile genomic segments which can be copied and inserted in different regions; (Finnegan, 2012). Studies have suggested that transposon activity might be involved with periods of great and rapid evolutionary change, stimulated by the appearance of different selective pressures, specially abiotic stress (Oliver and Greene 2009; Zeh et al. 2009; Belyayev 2014). In addition to jockey/pol, other genes responsible for regulating chromatin structure and gene expression were retrieved in our analysis (e.g., AFF4, ARID2 and ZNF438X1, see Table S2). This dataset of loci involved in regulating genomic expression is in agreement with recent studies on urban adaptation by birds (McNew et al. 2017; Caizergues et al. 2022), and supports the idea that the activity of transcription regulators might have differentiated between urban and rural populations, promoting evolutionary changes in the regulation of gene expression, rather than changes on the gene itself, to deal with novel environmental pressures.

We also recovered outlier regions inferred to be related to the development and structure of the nervous system, suggesting behavioral adaptation in urban populations (if we assume the nervous system as a genetic basis for behavioral phenotype; Chiel and Beer 1997). Behavioral adaptation is expected to be important in the prompt response of animals to changing or new environments (Snell-Rood 2013) and most studies addressing behavioral adaptation relate it to behavioral phenotypic plasticity (e.g., Sih et al. 2011). Indeed, adaptive phenotypic plasticity is probably a better solution for short-term problems (Yeh and Price 2004; Ghalambor et al. 2007; Mathot et al. 2012), since this process is independent of microevolutionary changes in the genetic pool of adapting populations, which usually may take a longer time to occur. The outlier regions recovered here suggest that behavioral adaptation might have occurred in our focal species through selection on genes influencing levels of attention and excitation and higher behavioral plasticity, aspects thought to be important in urban environments (Sol et al. 2013; Blumstein 2014). These genes include the CHRM5 gene, which codes for a receptor protein influencing many effects of acetylcholine in the central and peripheral nervous system as well as modulating the activity of midbrain dopaminergic neurons (Eglen and Nahorski, 2000; Fink-Jensen et al. 2003). Acetylcholine is known to be related to increased attention levels (Himmelheber et al. 2000; Jones, 2005), whereas dopamine plays important roles in executive functions, motor control, motivation, arousal,
reinforcement and reward (Björklund and Dunnett 2007). Selection on this gene could therefore be related to regulation of response to acetylcholine and dopamine neurotransmitters, affecting levels of attention and activity in urban individuals.

Higher behavioral plasticity may arise from selection on genes related to neural development and plasticity (Hofmann 2003; Bottjer and Arnold 2003; Chen and Tonegawa 2003). Examples of such genes in our outlier dataset are SLIT3 and EPHA7. SLIT3 belongs to a family of secreted extracellular matrix proteins and plays an important signaling role in the neural development of most bilaterian animals (Itoh et al. 1998; Piper et al. 2000; Dallol et al. 2003). EPHA7 is involved in brain development by modulating cell-cell adhesion and repulsion, showing a repellent activity on axons (Depaepe et al. 2005; Egea and Klein 2007). EPHA7 might also be related to vision acuity, since it is involved in the guidance of corticothalamic axons and in the proper topographic mapping of retinal axons (Rashid et al. 2005). Selective pressures on visual acuity might be important on urban bird populations since birds tend to be closely associated with human activity (Gil and Brumm, 2014), rely more on visual cues (e.g., Matyjasik, 2005; Spottiswoode and Stevens, 2010) and are exposed to artificial light during the night in urban settings, which may drive behavioral and physiological modifications (Kempenaers et al. 2010; Dominoni et al. 2013). Finally, the outlier gene OTUD7A is mostly expressed in neuronal soma and dendrites, and also showed localization to the postsynaptic region of excitatory synapses, suggesting a role in synapse development and maturation, as well as brain morphological development (Uddin et al. 2018; Yin et al. 2018). Therefore, we suggest that genetic modifications underlying rapid behavioral adaptation of C. flaveola to cities might have involved fine adjustments to a well-established neural architecture, increasing the plasticity and/or modifying the structure of this architecture. Interestingly, selection on genes related to neural functions has been suggested in a recent study investigating Great Tits (Parus major) across several cities in Europe (Salmón et al. 2021). Although the genomic regions under selection are not the same found in this study, this agreement suggests a common selective pressure on behavioral phenotypes, coupled with adaptation through genetic changes in neuronal genes.

In conclusion, our study suggests that urban individuals of C. flaveola suffer selective pressures in urban environments and therefore show signatures of genetic adaptation in regions related to the regulation of gene expression, regulation of energetic metabolism, the immune system and nervous system development. Additionally, since the neural regions found here are not related to specific song genes affecting singing patterns (Catchpole and Slater 2008), we also suggest that the different song patterns observed in urban individuals of C. flaveola as a response to increasing noise levels (Winandy et al. 2021a, 2021b) are a consequence of an overall increase in behavioral plasticity in those individuals that helps them cope with urban selective pressures (higher background noise being only one of them). We highlight that our approach is a post-hoc investigation of a list of genomic scaffolds that were mapped to SNPs estimated to be under selection. We aimed to test the hypothesis that urban individuals show genetic signatures of selection in regions associated with behavior and we do so by looking for those signatures and correlating them to known genomic regions when possible. We highlight that the list retrieved here is meant to be an initial investigation of the genomic architecture underlying behavioral adaptation in urban birds, especially because it is restricted to one urban replicate with a modest sample size. The processes of behavioral adaptation may undergo different mechanisms in different regions, and new patterns can be uncovered in further studies with larger sample sizes. Nevertheless, despite these limitations, we found a consistent set of putative adaptive genomic regions controlling neural development in urban individuals.
Genetic variants in these genes might play a role in neuronal embryogenesis and ontogenetic plasticity in urban birds, promoting higher behavioral plasticity in such a novel environment. Future investigation should address this hypothesis by focusing on whole genome and transcriptomic approaches, as well as targeted-sequencing of regions of interest, with increased sample size, in order to enhance our understanding of the link between genotype and phenotype in natural populations.
Acknowledgement
We would like to thank the following organizations for providing funds for this study: FAPESB (RED0045/2014; JCB0026/2016), CNPq (443249/2014-8, 465767/2014-1) and CAPES (23038.000776/2017-54). R.M. thanks CAPES for the master scholarship. H.B.-F. acknowledges the CNPq Research Productivity Fellowships (307037/2018-5). We would also like to thank the staff of the Genome Investigation and Analysis Laboratory (GENIAL) in CEFAP-USP, and the Laboratório de Genética e Evolução de Aves - LGEMA, USP, for their support during molecular procedures. Additionally, we thank the helpdesk staff in LNCC (Laboratório Nacional de Computação Científica), for the bioinformatics support. Finally, we thank Gabrielle Winandy and Rafael Felix for help with the collection of specimens in the field.

Authors' contributions
RM and HBF designed the study. RM performed sample collection and sequencing. RM, PMM and HBF analyzed, wrote and reviewed manuscript.

Conflict of interest statement
The authors declare no conflict of interests.

Data archiving statement
The files used for the analyses implemented in this study will be available on Github. The final genomic data generated will be deposited in NCBI.

Ethics statement
All specimens for the study were collected through the sampling permit number 42077-1, provided by government agency ICMBio - Instituto Chico Mendes de Conservação da Biodiversidade, Brazil, which regulates the methods used in this work. All specimens and tissues were deposited in the Museu de História Natural da Universidade Federal da Bahia (Natural History Museum of the Federal University of Bahia).
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Figure 1. Map of the study area with sites where *Coereba flaveola* individuals were collected (numbers follow Table S1). Six sites were sampled in the city of Salvador (1-6; bottom map) and four sites in non-urban areas (7-10, upper right map). Colors indicate levels of urbanization: red areas indicate urbanized surfaces, while green areas indicate forested surfaces. Yellow and gray areas in the city represent dunes and roads, respectively, whereas gray areas in the upper right map represent farmlands. Non-colored (white) areas in both maps represent water bodies (including the surrounding ocean). Data for this map was downloaded from http://mapbiomas.org. Upper left map shows South America and the dashed red square indicates the location of the study area. Dashed black square in the upper right map represents the location of urban areas in relation to non-urban sites.
Figure 2. Demographic scenarios simulated in fastsimcoal. Bars represent the single population encompassed urban and rural individuals, as uncovered by structure analyses (see Results). Changes in bar width represent changes in population size from past (top) to present (bottom). The dashed line represents the time of urbanization ($T_{urb}$), which was estimated by fastsimcoal (see Supplementary methods for details).
Figure 3. Manhattan plots for two interpopulation analysis (pcadapt and LFMM) and one intrapopulation analysis (SweeD) for detecting positive selection. Horizontal axis depicts SNP number, whereas vertical axis depicts the p-value of each SNP for the statistic used in each analysis (except on SweeD plot, in which the vertical axis depicts the CLR statistic, see Methods). Grey dots represent neutral loci, whereas black dots represent putative selected loci. Outlier SNPs that were recovered as related to urban adaptation are highlighted as red dots with their respective acronyms (see Table 2 for full description of the genes).
Table 1. Results of model selection using AIC for the demographic scenarios simulated in fastSimCoal. K represents number of parameters in each model. ln(L) represents the estimated likelihood for each model. Delta AIC (ΔAIC) represents the difference between AIC value from each model and the value from the model with lowest AIC. Ncoe, T urb and Nanc are population parameters estimated in fastSimCoal, corresponding, respectively, to present population size, time of urbanization event and ancestral population size (see Supplementary Methods for more information).

<table>
<thead>
<tr>
<th>Scenario</th>
<th>K</th>
<th>ln(L)</th>
<th>AIC</th>
<th>Delta AIC</th>
<th>Ncoe</th>
<th>T urb</th>
<th>Nanc</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stable</td>
<td>1</td>
<td>-3028.681</td>
<td>6059.362</td>
<td>2040.194</td>
<td>1044855</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Bneck</td>
<td>3</td>
<td>-2006.584</td>
<td>4019.168</td>
<td>0</td>
<td>1044219</td>
<td>158</td>
<td>6153180.86</td>
</tr>
<tr>
<td>Growth</td>
<td>3</td>
<td>-3211.471</td>
<td>6428.942</td>
<td>2409.774</td>
<td>1044498</td>
<td>412</td>
<td>820615.598</td>
</tr>
</tbody>
</table>

Table 2. A reduced list of the genes identified in Blast2GO. "Biological function" is related to the major biological process the gene is associated: CGM = Cell growth and movement; IM = Immune system; MR = Metabolism regulation; NS = Nervous system; RGE = Regulation of Gene Expression. Description of the biological function of the sequence was shortened and only major references were kept. Check Table S2 in supplementary results to see all putative outlier regions, with complete descriptions and references.

<table>
<thead>
<tr>
<th>Biological function</th>
<th>Analysis</th>
<th>Acronym</th>
<th>Name</th>
<th>Description</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>CGM</td>
<td>SweeD</td>
<td>FIGNL1</td>
<td>Fidgetin-like protein 1</td>
<td>Involved in DNA double-strand break (DBS) repair via homologous recombination (HR).</td>
<td>Yuan and Chen (2013)</td>
</tr>
<tr>
<td>CGM</td>
<td>LFMM</td>
<td>GALNT7</td>
<td>N-acetylgalactosaminyltransferase 7 isoform X1</td>
<td>Essential for the regulation of cell proliferation and has been implicated in tumorigenesis.</td>
<td>Bennett et al. (1999); Nie et al. (2016)</td>
</tr>
<tr>
<td>CGM</td>
<td>LFMM</td>
<td>CSPG4</td>
<td>chondroitin sulfate proteoglycan 4-like</td>
<td>CSPG4 has been reported to exhibit a role in the growth and survival as well as in the spreading and metastasis of tumor cells.</td>
<td>Ilieva et al. (2018); Rivera et al. (2012)</td>
</tr>
<tr>
<td>IS</td>
<td>pcadapt</td>
<td>ERVK113</td>
<td>endogenous retrovirus group K member 113 Pol protein-like</td>
<td>Participates in initial response to retrovirus infection.</td>
<td>Chuong et al. (2016)</td>
</tr>
<tr>
<td>MR</td>
<td>LFMM</td>
<td>ARID5B</td>
<td>AT-rich interactive domain-containing protein 5B</td>
<td>Transcription coactivator that plays a key role in adipogenesis and liver development (overall, it reduces mitochondrial thermogenesis and increases lipid storage).</td>
<td>Baba et al., (2011); Claussnitzer et al. (2015)</td>
</tr>
<tr>
<td>MR</td>
<td>pcadapt</td>
<td>PASK</td>
<td>PAS domain-containing serine/threonine-protein kinase</td>
<td>Serine/threonine-protein kinase involved in energy homeostasis and protein translation. Probably plays a role under changing environmental conditions (oxygen, glucose, nutrition), rather than under standard conditions</td>
<td>Rutter et al., (2001); Wilson et al. (2005)</td>
</tr>
<tr>
<td>NS</td>
<td>pcanpact</td>
<td>EPHA7</td>
<td>ephrin type-A receptor 7-like</td>
<td>Depaepe et al. (2005); Rashid et al. (2005); Egea and Klein (2007)</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>NS</td>
<td>pcanpact</td>
<td>SLIT3</td>
<td>slit homolog 3 protein-like</td>
<td>Itoh et al. (1998)</td>
<td></td>
</tr>
<tr>
<td>NS</td>
<td>pcanpact</td>
<td>OTUD7A</td>
<td>OTU domain-containing protein 7A isoform X1</td>
<td>Uddin et al. (2018); Yin et al. (2018)</td>
<td></td>
</tr>
<tr>
<td>RGE</td>
<td>pcanpact</td>
<td>AFF4</td>
<td>AF4/FMR2 family member 2-like</td>
<td>Lin et al., (2010)</td>
<td></td>
</tr>
<tr>
<td>RGE</td>
<td>LFMM, pcanpact</td>
<td>jockey/pol</td>
<td>RNA-directed DNA polymerase from mobile element jockey-like</td>
<td>Ivanov et al. (1991)</td>
<td></td>
</tr>
<tr>
<td>RGE</td>
<td>pcanpact</td>
<td>ARID2</td>
<td>AT-rich interactive domain-containing protein 2</td>
<td>Euskirchen et al. (2012)</td>
<td></td>
</tr>
</tbody>
</table>

Eph receptors are a group of receptors that are activated in response to binding with Eph receptor-interacting proteins (Ephrins). Has a repellent activity on axons and is for instance involved in the guidance of corticothalamic axons and in the proper topographic mapping of retinal axons to the colliculus.

Slit is a family of secreted extracellular matrix proteins which play an important signaling role in the neural development of most bilaterian animals.

The OTUD7A gene encodes a member of a family of deubiquitinating enzymes. Also localizes to the postsynaptic density in the brain, suggesting a role in synapse development and maturation.

Key component of the super elongation complex (SEC), a complex required to increase the catalytic rate of RNA polymerase II transcription.

RNA-dependent DNA polymerase, indicating the activity of mobile elements such as retrotransposons.

Involved in transcriptional activation and repression of select genes by chromatin remodeling (alteration of DNA-nucleosome topology).