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Morphological Clines and Weak Drift along an Urbanization Gradient in the Butterfly, *Pieris rapae*

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Abstract

Urban areas are increasing globally, providing opportunities for biodiversity researchers to study the process in which species become established in novel, highly disturbed habitats. This ecological process can be understood through analyses of morphological and genetic variation, which can shed light on patterns of neutral and adaptive evolution. Previous studies have shown that urban populations often diverge genetically from non-urban source populations. This could occur due to neutral genetic drift, but an alternative is that selection could lead to allele frequency changes in urban populations. The development of genome scan methods provides an opportunity to investigate these outcomes from samples of genetic variation taken along an urbanization gradient. Here we examine morphological variation in wing size and diversity at neutral amplified fragment length polymorphisms in the butterfly *Pieris rapae* L. (Lepidoptera, Pieridae) sampled from the center to the periphery of Marseille. We utilize established and novel environmental correlation approaches to scan genetic variation for evidence of selection. We find significant morphological differences in urban populations, as well as weak genetic structure and decreased genetic diversity in urban versus non-urban sites. However, environmental correlation tests provide little support for selection in our dataset. Our comparison of different methods and allele frequency clines suggests that loci identified as significant are false positives. Although there is some indication that selection may be acting on wing size in urban butterflies, genetic analyses suggest *P. rapae* are undergoing neutral drift.

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Introduction

Human populations have grown rapidly since 1950, with urban populations increasing nearly five-fold in size at an average rate of 2.6% per year [1]. This dramatic growth has led to massive cityscapes, which pose multifaceted challenges that threaten the survival of local species, largely mirroring trends of global change [2]. These challenges include the increasing fragmentation of natural habitats, novel climatic conditions and environmental hazards, and altered community structure and trophic interactions [3,4]. Studies of urban areas are thus emerging as important experimental systems in which to investigate how species respond to environmental changes on a recent timescale [5], which is a central goal of evolutionary ecological research.

Perhaps one of the main challenges facing urban species is to maintain viable, interconnected populations when suitable habitats are highly fragmented [6]. Species inhabiting patches of isolated habitat often experience fluctuation in population size, with the continual occupancy of each patch determined by the patch size, extinction probability, and colonization rate [7]. As patches are small in most urban landscapes, a common expectation is that non-urban populations will provide colonists to maintain occu-

pancy in small urban populations [8]. Small populations experience an increase in genetic drift [9,10], so we would expect to see a decline in genetic variation in urban populations and neutral genetic differentiation from non-urban populations. However, changes in genetic variation are likely to depend on the species mobility [11], as mobile species such as birds exhibit only subtle changes in genetic diversity related to urbanization.

While neutral divergence is one possible response to urbanization, a compelling alternative is that rapid evolutionary adaptation occurs, inducing directional changes in genetic and morphological variation [12]. Rapid evolution can be driven by strong selection on ecological traits, as Badyaev *et al.* [13] demonstrated in documenting the rapid evolution of bill size and courtship song in urban house finches. The relative importance of neutral and adaptive responses to urbanization, and the types of environmental factors that drive adaptive divergence when it occurs, are important questions for urban biodiversity studies. Understanding these factors could improve management strategies that seek to increase species diversity and persistence in urban landscapes [14]. The challenge lies in determining the role of specific environmental factors in generating observed patterns of morphological and genetic variation.

Genome scans are a promising approach to determine whether population genetic patterns result from adaptive genetic changes or genetic drift [15]. One class of genome scans, the *environmental correlation* approach, explicitly tests for correlations between environmental factors and allele frequency distributions [16]. One of the strengths of this approach lies in the individual-based design, which allows for a large number of independent data points to be sampled across a heterogeneous landscape, increasing sensitivity to subtle shifts in allele frequency that might occur as a result of adaptation from standing genetic variation [17]. This approach can be applied to species where population units are difficult to define, as often occurs when gradual changes in genetic variation develop under isolation by distance [17]. Joost et al. [16] proposed using logistic regression to test the effect of each environmental variable. Subsequent implementations of the *environmental correlation* approach have attempted to correct for correlations among individuals due to recent shared ancestry [18], for example by using generalized estimating equations (GEE) to account for relatedness of individuals at nearby sampling locations [19]. We introduce linear mixed-models (LMM) as an alternative to GEE, which allows for among-group variation to be modeled in a nested design and are widely used in genetic association studies [20].

Our study focuses on the butterfly *Pieris rapae* L. (Lepidoptera, Pieridae) sampled near Marseille, to test whether phenotypic changes in wing shape and differences in genetic diversity emerge in urban populations. Urban gradients are known to influence butterfly species diversity [14] and to favor species with certain ecological traits [21], and it has been argued that many urban butterfly populations are small population isolates [22]. However,

due to strong selection and because butterfly species often have multiple generations per year, genetic adaptation could occur rapidly. As a result, we test neutral and non-neutral hypotheses to understand the forces that have shaped genetic variability in urban populations of *P. rapae*.

Materials and Methods

Ethics Statement

No permits were required for the collection of butterflies in this study and the individuals sampled were killed instantly to minimize suffering.

Hypothesis Testing

We propose to test hypotheses of drift versus selection in *P. rapae* along an urbanization gradient in Marseille, focusing on two neutral models and one selection model. Under a neutral divergence with gene flow model, morphological differences should not be evident among urban and non-urban sites, genetic diversity should be reduced in urban sites, and changes in genetic structure among urban and non-urban sites should reflect weak genetic drift. Under a neutral geographical isolation model, morphological differences should not be evident among urban and non-urban sites and changes in genetic structure should reflect the isolation of urban populations (i.e. as an abrupt transition in multi-locus allele frequencies), while a decline in genetic diversity is not necessarily expected. Finally, under a model of local adaptation, morphological divergence should be evident in urban sites and a subset of loci should exhibit allele frequency changes that are significantly correlated with changes in environmental variation.

Sampling and Environmental Data

The collection of butterfly samples includes a total of 219 *P. rapae* individuals from 41 sites along a 100 km transect, extending from the center of Marseille northeast through fields and shrubland/forest (**Figure 1**, plotted using Google Earth software). Genetic data consists of 552 AFLP markers amplified from an EcoRI/MseI digest with three primer pairs (EACT-MCAG, EAAC-MCAC and EACT-MCTG). PCR products were labeled with FAM and electrophoresis was performed on a capillary sequencer with a ROX-500 size standard. A panel of 13 individuals was genotyped twice and loci were discarded if errors occurred in more than two individuals. The remaining 366 markers were used in downstream analyses.

We measured wing size variation among the sampled male butterflies, as females represented a small proportion of our total sample size (26%). Six wing size characters were recorded, including forewing length (FL), forewing width (FW), forewing area (FA), hindwing length (HL), hindwing width (HW), and hindwing area (HA; **Figure S1**). Digital images were scanned alongside a metric ruler and measurements were made in Adobe Photoshop CS3 software.

Data on road width (*var1*) were obtained from MapInfo (MapInfo Corporation); in addition, the density and number of buildings in a radius of 150 m were combined as a composite measurement of urbanization (*var2*). Land cover category (*var3*) and percent tree cover (*var4*) were obtained from GlobalMap (NASA Earth Observatory). The free database WorldClim was used to extract 19 climatic variables representing temperature and precipitation averages measured during 1950–2000. To reduce cross-correlations among climatic variables, we identified 16 highly correlated (Pearson's $r > 0.8$) WorldClim variables (**Table S1**), combined them in a principal component analysis after

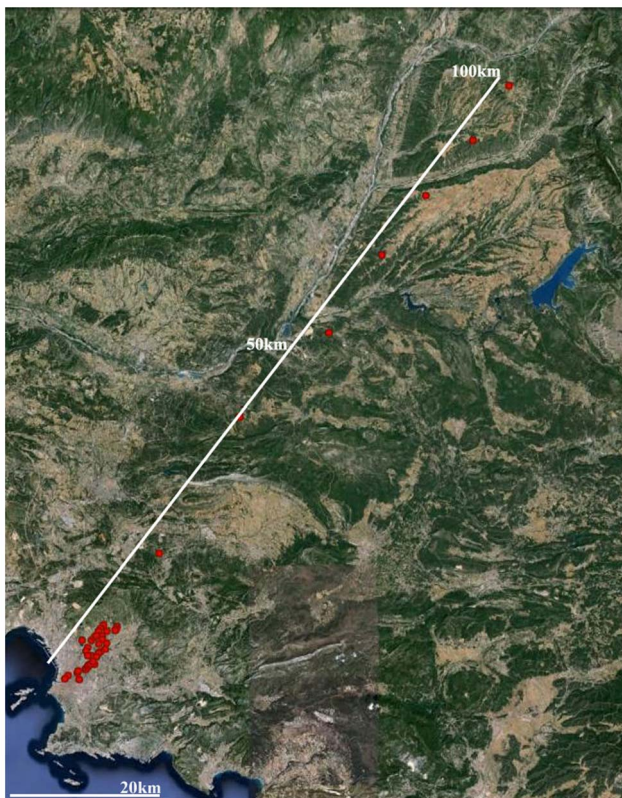


Figure 1. Sampling (red circles) of *Pieris rapae* along a 100 km urbanization gradient in Marseille.
doi:10.1371/journal.pone.0083095.g001

Table 1. Morphological differences in male wing size along the urbanization gradient, with significant values in bold.

		FL	FW	FA	HL	HW	HA
Correlation with distance from central Marseille	Pearson's <i>r</i>	−0.275822	−0.18196	−0.1701	−0.26493	−0.24648	−0.22381
	<i>p</i> -value	0.0005	0.0226	0.0332	0.0008	0.0019	0.0048
ANOVA-4 groups	Mean square	32.336	8.056	4552	28.5516	17.945	8760.8
	Residuals	2.335	1.286	834	2.049	1.508	1101.2
	<i>p</i> -value	0.0003	0.0133	0.0207	0.0003	0.0007	0.0054
ANOVA-3 groups (without 25–50 km)	Mean square	14.697	7.6739	4336.5	27.465	17.339	8230.7
	Residuals	2.117	1.243	801.8	1.862	1.437	1027.5
	<i>p</i> -value	0.0002	0.014	0.0214	0.0002	0.0007	0.0053

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standardization, and retained the first two eigenvectors (91.5% and 5.5% of the variation, **Figure S2**) as variables (*var5* & 6). The three remaining uncorrelated climatic variables, isothermality (*var7*), temperature seasonality (*var8*) and mean diurnal temperature range (*var9*) were analyzed individually.

Genetic, morphological and ecological data have been made publicly available on DRYAD (doi:10.5061/dryad.8837q).

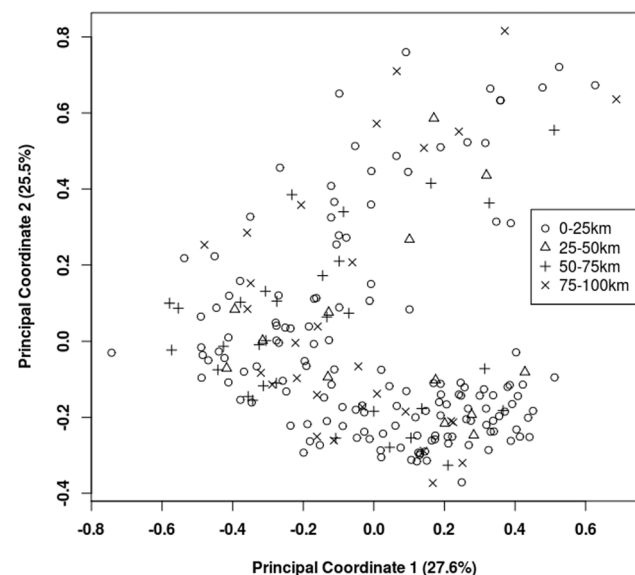
Analysis of Morphological Variation

We calculated the Pearson correlation coefficient between each morphological variable and the distance of the sampled butterfly from the center of Marseille, and used a *t*-test to assess statistical significance. We also grouped individuals into distance classes, 0–25 km, 25–50 km, 50–75 km, and 75–100 km from the center of Marseille to the periphery, and compared group means for each wing character in an analysis of variance (ANOVA) allowing for unequal variance among groups. We used an *F*-test to assess the significance of differences among group means. The ANOVA was repeated excluding the 25–50 km distance class, due to its smaller sample size. Finally, we calculated the Pearson correlation coefficient between each morphological character and genotypes at each AFLP locus. These correlation tests were corrected for multiple testing using the Dunn-Sidak correction ($1 - (1 - \alpha)/n$), where *n* is the number of variables multiplied by the number of loci (6×366), and the adjusted *p*-value is 2.34×10^{-5} . This correction assumes independence among tests and provides a similar, though slightly more conservative correction, than a Bonferroni adjustment.

Genetic Diversity and Structure

Genetic diversity was measured at each collecting site as the percentage of polymorphic loci and the expected heterozygosity or

Nei's gene diversity (*H_j*), using the software Genalex v.6.41 [23]. Using the groups based on distance classes, we measured pairwise *F_{ST}* and Nei's genetic distance. These analyses were conducted in the software AFLP-Surv [24], using a Bayesian method [25] developed for biallelic dominant markers [26]. Pairwise *F_{ST}* is typically robust to small deviations from demographic equilibrium and provides a measure of genetic differentiation among populations [27]. Finally, all non-urban and urban populations

**Figure 2.** Principal Coordinate Analysis of AFLP variation. doi:10.1371/journal.pone.0083095.g002**Table 2.** Genetic diversity, *F_{ST}* (in bold type) and Nei's genetic distance in pooled samples along the urbanization gradient (see Table S2 for non-pooled values).

Distance from central Marseille	Sample Size (N)	Expected heterozygosity (<i>H_j</i>)	% Loci Polymorphic	0–25 (km)	25–50 (km)	50–75 (km)	75–100 (km)
0–25km	146	0.105	31.5	-	0.0036	0.0035	0.0020
25–50km	10	0.125	52.2	0.0004	-	0.0000	0.0000
50–75km	20	0.126	49.5	0.0004	0.0000	-	0.0000
75–100km	43	0.116	36.1	0.0002	0.0000	0.0000	-

doi:10.1371/journal.pone.0083095.t002

were pooled into two groups to measure pairwise F_{ST} and Nei's gene diversity.

To examine population structure graphically, we used principal coordinate analysis (PCoA) on all individuals, calculating independent coordinates using a standardized covariance matrix of genetic distance, and plotted the first two coordinates to identify spatial clusters. We then used the Bayesian clustering program Structure v.2.3.3 [28] to assign individuals to populations based on inferred allele frequencies. Using collection sites as prior information, we estimated membership coefficients in one to four clusters (K) while allowing for admixture and correlated allele frequencies across populations. We examined 20 runs at each K to determine the maximum log-likelihood, setting the burnin to 100,000 and the MCMC sampling to 500,000 steps. Finally, we tested for isolation by distance (IBD) using a Mantel test of the correlation between genetic distance among individuals and Euclidean geographic distance in Genalex. Significance of IBD was tested using 999 permutations of the data.

Environmental Correlations and Genetic Clines

We used four approaches to test for environmental correlations, including logistic regression [16], generalized estimating equations [19], and two parameterizations of linear mixed models. All models were analyzed in R 2.14.0 (model formula and R code in **Methods S1**). The logistic regression model was implemented using a binomial error distribution and the logit link function, and significance was assessed using an analysis of variance F -test: $y_i = \text{logit-1}(\beta X_i)$. The GEE approach was implemented using autocorrelation correlation structure 1 ('ar1'), which corrects for relatedness among individuals sampled from neighboring localities. The GEE model also uses a binomial error distribution and a Wald test was used to assess significance: $y_i = \text{logit-1}(\beta X_i)$, $\text{Var}(y_i) = \phi \alpha_i$, where the scaling parameter (ϕ) and variance function (α_i) are used to model the covariance structure. For the two linear mixed models, the environmental variable was tested as a fixed effect, while the intercept was allowed to vary across groups. In one version of a LMM (our 'geographically stratified model'), individuals were grouped into four distance classes defined previously: $y_i = \beta_{0j}[i] + \beta_{1j}[i] X_i + \varepsilon_i$, where fixed effects and intercepts vary for j geographical groups. In the second version, the value of each environmental variable (e.g. urbanization, land cover, etc.) at the sample site was used to group individuals into classes (our 'environmentally stratified model'): $y_i = \beta_{0j}[i] + \beta_{1j}[i] X_i + \varepsilon_i$, where fixed effects and intercepts vary for j environmental classifications. Each LMM was implemented with autocorrelation correlation structure 1 ('ar1') and the binomial error distribution. The fixed effect of each variable was compared to a null model without the variable using a likelihood-ratio test to assess significance. All environmental correlation tests were corrected for multiple testing using a Dunn-Šidák correction ($n = 9$ variables $\times 366$ loci) with the adjusted p -value of 1.56×10^{-5} . For loci with significant correlations, we examined allele frequency clines along the Marseille transect using the *hzar* package in R. We plotted and estimated parameters for one-dimensional (1-D) clines, comparing this 1-D model to a null model of frequency changes independent of location using a likelihood ratio test.

Results

Morphological Variation

Measurements of the wing size of male butterflies were significantly negatively correlated with distance from the center of Marseille (**Table 1**). Mean values of each wing size character also differed significantly among distance classes in the ANOVA

(**Table 1**). These differences remained significant whether the small sample from the 25–50 km distance group was included or not (**Table 1**). On average, wing size characters were larger in male butterflies at urban sites than non-urban sites (**Figure S3**). Although two loci (14 and 339) were weakly correlated ($r > 0.25$) with morphological characters, neither was significant after correction for multiple testing.

Genetic Diversity and Structure

The percentage of polymorphic loci was high (mean value 31%), with polymorphism increasing from urban to non-urban sites ($r = 0.573$, p -value = 0.0003, **Table S2**). Expected heterozygosity decreased from urban to non-urban samples, but this was largely due to small sample sizes in urban sampling locations. When butterflies were grouped into distance classes, the expected heterozygosity increased from urban sites at 0–25 km (mean 0.10526 ± 0.00638) to non-urban sites at 25–50 km (mean 0.12518 ± 0.00747), 50–75 km (mean 0.12604 ± 0.00687), and 75–100 km (mean 0.11605 ± 0.00668), though this relationship is not significant. Non-urban populations (25–50 km, 50–75 km, and 75–100 km) were not differentiated based on F_{ST} or Nei's genetic distance (**Table 2**), but these groups were differentiated from urban samples. When all samples were pooled into two groups, F_{ST} (0.0038) of urban and non-urban samples was statistically significant (p -value = 0.004). Additionally, Nei's gene diversity was significantly higher (t -test p -value = 0.0001) in non-urban (0.1617 ± 0.0068) compared to urban populations (0.1457 ± 0.0066).

A plot of the first two principal coordinates (27.6% and 25.5% of the variation) provided no evidence of population structure (**Figure 2**). In the Structure analysis, log-likelihood values increased to a maximum at $K = 4$ ($\ln P(D) = -25839.7$). However, individuals were not assigned to discrete clusters when $K > 1$ (**Figure 3**), providing no evidence of structure. The IBD test was non-significant ($r = 0.062$, p -value = 0.063).

Environmental Correlations and Genetic Clines

We found significant correlations using three of the four statistical approaches (**Table 3**). GLM did not detect significant relationships after correction for multiple testing. In contrast, GEE detected 42 significant correlations for 29 loci at seven environmental variables (12 loci were significant at more than one variable). The geographically stratified LMM resulted in six significant tests across three variables, while the environmentally stratified LMM resulted in five significant tests across three variables. Only four loci were significant across more than one statistical model, specifically within the LMM approaches. However, none of the significant loci showed evidence of an allele frequency cline (**Figure S4**), nor were they correlated with morphological characters.

Discussion

Urban environments provide an important experimental system in which to investigate the role of ecological and evolutionary processes as species become established in highly disturbed habitats [12,29]. While urban environments often lead to relatively homogenous biotic communities [30], this can provide ecological opportunities for successful colonizing species. Urban habitats are particularly well suited to test the relative importance of landscape configuration in driving neutral genetic divergence [31] and ecological gradients in driving adaptive evolution [13]. With the goal of determining whether neutral or adaptive processes influence urban butterfly populations, we examined morphological

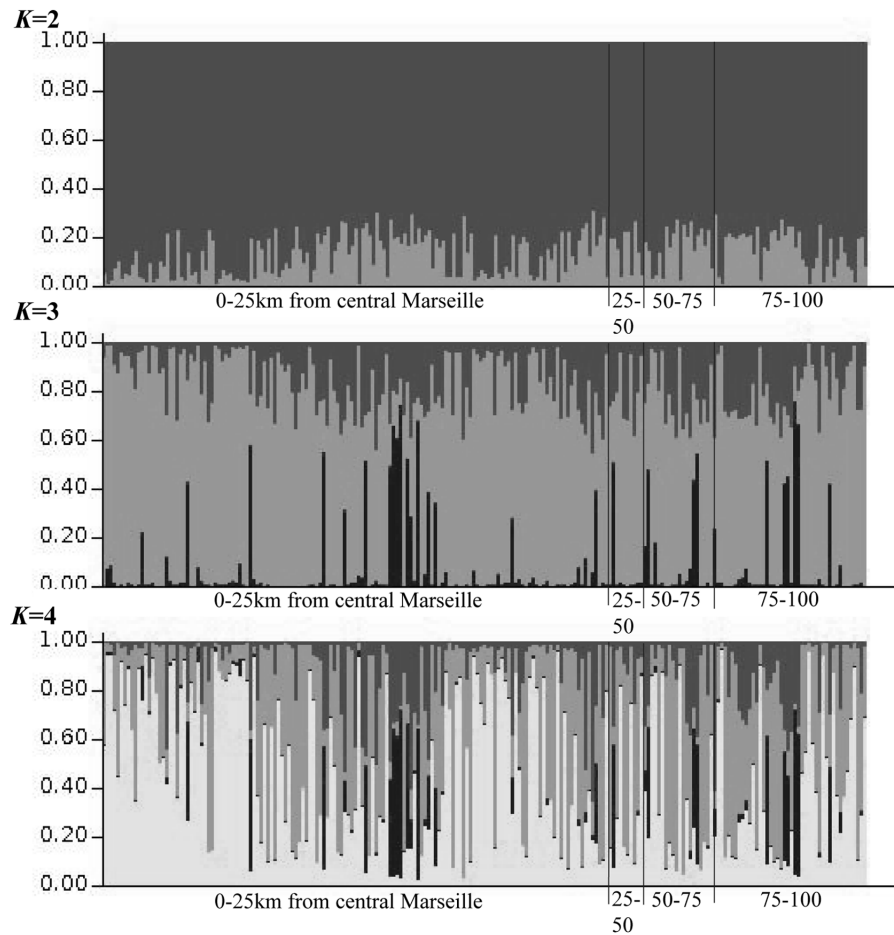


Figure 3. Proportional admixture coefficients of individuals at different levels of K clusters based on the Bayesian clustering algorithm Structure. Individuals are ordered left to right according to distance from the center of urban Marseille.
doi:10.1371/journal.pone.0083095.g003

Table 3. Loci with significant environmental correlations after correcting for multiple testing (p -value $< 1.56 \times 10^{-5}$).

	Urbanization	Road Width	% Tree Cover	Land Cover	Isothermality	Temperature Seasonality	Mean Diurnal Temp Range	Temp-Prec PC1	Temp-Prec PC2	Shared across Multiple Variables
Logistic Regression (GLM)	None	None	None	None	None	None	None	None	None	None
Generalized Estimating Equations (GEE)	88, 126, 130, 223, 334	1, 88, 126, 130, 223	7, 93, 120, 122, 145, 151, 176, 190, 210, 306, 362	7, 56, 93, 112, 120, 122, 128, 145, 156, 168, 176, 190, 208, 210, 239	113, 165, 196	16, 88	None	285	None	7, 88, 93, 120, 122, 126, 130, 145, 176, 190, 210, 223
Linear Mixed Model G (LMMg)	None	212	47, 225, 281, 288	None	None	None	325	None	None	None
Linear Mixed Model E (LMMe)	None	212	225, 281, 288	None	None	None	298	None	None	None
Shared across Multiple Tests	None	212	225, 281, 288	None	None	None	None	None	None	None

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and genetic changes along an urbanization gradient. The utilization of genome scan methods provides a direct test of whether genetic patterns result from adaptive processes [17].

The butterfly *Pieris rapae* exhibits changes in wing morphology across an urban-to-rural gradient in Marseille (**Table 1**). On average, males have larger wings in urban sites than in non-urban sites, across a variety of measurements including wing length, width and area. Selection could be favoring males that are better dispersers in urban sites, due to the distance among suitable nectar resources and the low population density of potential mates [32]. Large wing size is correlated with dispersal ability and thermal heating rate in butterflies [33] and has been found to be heritable in some *P. rapae* populations [34]. Furthermore, wing size is considered to be a polygenic trait with strong correlations to other fitness-related traits like fecundity and longevity [35]. It is plausible to suggest that urban populations of *P. rapae* are experiencing selection for increased wing size, though our data are not sufficient to exclude alternative neutral hypotheses.

While morphological trends are clear, we found only small differences in genetic variation in a large sample of AFLP markers. Notably, urban butterflies are weakly differentiated from non-urban populations and have a significant decline in diversity, while non-urban populations are not differentiated from one another. The observed genetic differences are not likely to be an artifact of sample size as the pooled urban population consists of a much larger sample of individuals than its non-urban counterpart (**Table 2**). Our observations are consistent with neutral divergence, where declines in variability result from the random effects of drift in a fragmented population [31,36]. Complete geographical isolation of urban populations is not well supported, as we would expect an abrupt shift in allele frequency across multiple loci, which we do not observe.

One additional possibility is that weak genetic divergence could be explained by selection, if certain alleles are favored in urban relative to non-urban environments. We tested this by examining the correlation between allele frequency changes and environmental variation in urban and non-urban habitats. Our environmental correlation tests included the GLM method [16], as well as the GEE method [19] and LMM methods to account for genetic correlations among sampled individuals. We detected 12 significant AFLP loci (3% of all loci) using the GEE method and none using GLM. Seven loci were detected in the LMM approaches. Only four loci were significant in multiple methods, all under the LMM models. However, these LMM models are quite similar in design, and their concordance is not entirely unexpected. The lack of shared significant loci across the other methods suggests that we might be detecting false positives. Furthermore, the analyses of allele frequency clines provide no evidence for geographical transitions in frequency along the urbanization gradient. Similarly, tests for associations of allele frequencies with wing size traits were not significant.

Recent simulation studies suggest that environmental correlation tests might suffer from a high rate of false positives [37], particularly when the underlying genetic structure is not modeled appropriately by the covariance matrix. While the intent of the GEE and LMM methods was to correct for underlying genetic correlations among sampled individuals, the subtle change in genetic structure might not be captured by these models. One solution to this problem is to evaluate the environmental correlation tests based on the output of multiple statistical models. In our dataset, we find little concordance among the different methods. We conclude that selection does not account for the observed genetic differences, but we must add the caveat that our sampling reflects only a small fraction of the *P. rapae* genome

(expected coverage <0.1% for a typical butterfly genome of 400 Mb). Given the observed shifts in morphological traits, it is possible that evidence of adaptation might be found with more comprehensive coverage. The recent developments in next generation sequencing in non-model organisms [38] should increase genome coverage and improve future efforts to detect genetic adaptation in *P. rapae*.

Numerous studies have focused on how biodiversity and ecological traits change along urban gradients [12,29], showing that urbanization favors highly dispersive generalists [39]. Comparatively little is known, however, about whether genetic changes occur when organisms are established in urban landscapes [31]. Here we show that butterfly populations in urban Marseille have diverged morphologically and genetically. Selection might favor larger sized male butterflies, whereas the observed genetic variation is consistent with neutral genetic drift. Future efforts will need to expand genetic sampling and incorporate quantitative genetic analyses of resident urban and non-urban populations to determine if morphological differences in urban *P. rapae* are heritable and under selection.

Supporting Information

Figure S1 Measurements of wing size taken on male butterflies. FL = forewing length, FW = forewing width, HL = hindwing length, HW = hindwing width.
(PDF)

Figure S2 Principal component analysis (PCA) of temperature and precipitation variables at the sample sites.
(PDF)

Figure S3 Boxplots of morphological variation in male butterflies taken at distances measured from the center of Marseille along a gradient of urbanization.
(PDF)

Figure S4 Plots of the allele frequency clines of significant loci as a function of distance (meters) from Marseille.
(PDF)

Table S1 Pearson correlations among variables, showing in bold climatic variables with significant correlations (r) greater than 0.80.
(PDF)

Table S2 Summary of AFLP genetic variation at each sampling site.
(PDF)

Methods S1 Statistical model formulas and details of implementation in the computing environment R.
(PDF)

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Author Contributions

Conceived and designed the experiments: IW MDC SM. Performed the experiments: IW SDS. Analyzed the data: SDS IW. Contributed reagents/materials/analysis tools: SDS MDC SM. Wrote the paper: SDS IW MDC SM.

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