**Discussion**

In this paper, we build off of recent empirical work identifying the presence of parallel urban-rural clines in the frequency of hydrogen cyanide (HCN) across eastern North American cities (Thompson et al. 2016; Johnson et al. 2018, *this issue*). We used spatially-explicit, agent-based simulations to examine the formation of parallel clines in non-additive traits—of which HCN is an example—under varying levels of genetic drift, gene flow and selection. We found that independent of how drift is manipulated (e.g. through serial founder events or a gradient in maximum population size), drift lead overwhelmingly to the formation of positive clines and steeper drift gradients generated more strongly positive clines, although these effects decreased with increasing migration. However, clines formed by drift alone were substantially weaker than those generated by selection, suggesting an upper limit to the strength of phenotypic clines in non-additive traits due to drift. Finally, when selection operated counter to the prevailing drift gradient, stronger selection was required to generate clines as strong as those observed in the absence of drift. Below, we begin by discussing the relevance of our results for the evolution of cyanogenesis clines in white clover. We then suggest that urban environments—in combination with knowledge of the genetic architecture of focal traits—can provide the replication necessary for understanding the contributions of drift, selection, and gene flow to the evolution of clines in non-additive traits. We end by more broadly discussing the role of drift and other evolutionary mechanisms in the formation of phenotypic clines.

*Evolution of cyanogenesis clines in Trifolium*

Understanding geographical variation in allele and phenotype frequencies often provides insight into the evolutionary mechanisms structuring patterns of genetic variation in natural populations. In white clover, pioneering work by Hunor Daday identified broad-scale latitudinal clines in the frequency of HCN across multiple continents (Daday 1954a, 1958) and altitudinal clines across the central European Alps (Daday 1954b). This work has since been extended and clines in the frequency of HCN appear quite common, with higher frequencies occurring in warmer and drier habitats (de Araújo 1976; Ganders 1990; Kooyers and Olsen 2012, 2013; Kooyers et al. 2014; Thompson et al. 2016). These patterns are thought to reflect the benefits of producing HCN in warmer environments where herbivores are more common, the cost of producing HCN in frost-prone habitats, and the benefits of producing cyanogenic glycosides in regions subject to moderate drought stress (Daday 1965; Kakes 1989; Kooyers and Olsen 2013; Kooyers et al. 2014). Thus, investigations into the mechanisms structuring spatial variation in HCN frequencies have focused largely on selection (but see Kooyers and Olsen 2012; Johnson et al. 2018, *this issue*), despite the genetic architecture of HCN making populations particularly susceptible to loss of HCN via drift.

The presence of repeated correlations between environmental variables and phenotype frequencies is often considered strong evidence for the role of natural selection in generating adaptation. However, clines may also form via neutral processes (Vasemägi 2006; Colautti et al. 2010) and this may be exacerbated in traits with a non-additive genetic basis (e.g. epistasis). For example, we found that gradients in the strength of drift from rural (weak drift) to urban (strong drift) populations preferentially led to urban populations having lower HCN frequencies, consistent in direction with phenotypic clines in HCN observed across cities (Thompson et al. 2016; Johnson et al. 2018, *this issue*). Thus, when a trait’s genetic architecture predisposes populations to evolve in a particularly direction under the effects of drift alone, drift must be rejected as a null hypothesis prior to invoking the role of selection in generating adaptive differentiation. Observing more clines in natural populations than would be expected under drift alone is one way to address; null distributions for the proportion of clines expected under drift can be generated through simulations and compared to the actual proportion observed in nature. Ideally, simulations would be parameterized with empirical estimates of migration rates and effective population size to provide system-specific estimates of expected proportions. Unfortunately, we lack these data for white clover populations making it difficult to compare the clines observed across cities to the proportions estimated in these simulations.

While drift preferentially generated positive clines in HCN, the mean strength of clines formed by drift was substantially lower than those involving selection. This occurred because although strong drift in urban populations drives HCN frequencies downward, the weak drift in rural populations and the absence of selection results in rural HCN frequencies fluctuating around their initial frequency. In contrast, simulations involving selection favoured HCN+ genotypes in rural populations and HCN– genotypes in urban populations, resulting in a steeper gradient in phenotype frequencies between urban and rural populations and thus stronger clines. To understand whether drift is likely to generate clines in HCN as strong as those observed across cities, we compared the magnitude of slopes from our simulations to those observed across cities for which there are data (Thompson et al. 2016; Johnson et al. 2018, *this issue*). Since cities varied in transect lengths, it was necessary to standardize distance values by placing them all on the same scale, resulting in distance values that vary between 0 (urban) and 1 (rural) for all regressions (see supplementary material: “Comparison of standardized slopes from simulated and empirical data”). Even under a weak gradient in drift and high migration (minimum *urban N* = 100, *m* = 0.05), the distribution of observed slopes overlapped completely with the distribution of simulated slopes (–0.30 < βsimulated < 0.27; –0.08 < βobserved < 0.30), suggesting drift can generate clines as strong as those observed across cities. However, the absence of decreases in neutral genetic diversity in urban populations suggests that genetic drift is not an important mechanism structuring urban-rural phenotypic clines in HCN (Johnson et al. 2018, *this issue*). Similarly, the absence of clines at neutral microsatellite markers in North American clover populations suggests that latitudinal clines in HCN are adaptive (Kooyers and Olsen 2012), at least in the invaded range. Thus, while there is overlap in the strength of observed clines and those simulated by drift, additional evidence from neutral markers strongly suggests that many clines in cyanogenesis are adaptive.

*Urban environments as replicated systems to study non-adaptive evolution*

Clines have traditionally been studied along latitudinal (e.g. Huey et al. 2000; Caicedo et al. 2004; Campitelli and Stinchcombe 2013), altitudinal (Clausen et al. 1948; Luo et al. 2015; Zhao and Wang 2015) or longitudinal (Samis et al. 2008, 2012; Kooyers et al. 2014) transects, since such transects are often associated with gradients in putative selective agents (e.g. temperature, precipitation, biotic interaction strength). Importantly, geographical transects can also be associated with variation in demographic factors important for determining the extent to which drift will act in populations. This is especially true during invasions or rapid range expansions when founder events result in smaller marginal populations exchanging fewer migrants, leading to reduced genetic diversity and increased differentiation relative to more central populations (Alleaume-Benharira et al. 2006; Eckert et al. 2008; Excoffier and Ray 2008). Such spatial variation in demography may provide the foundation upon which parallel clines in non-additive traits can form. Studies of non-additive trait differentiation across replicate geographical gradients in selection and demography, coupled with knowledge of the focal trait’s genetic architecture, would provide a strong test of the relative contribution of drift, selection, and gene flow in the formation of parallel clines. However, logistical challenges associated with replicating continental-scale latitudinal, altitudinal or longitudinal transects has precluded such replication for many systems.

Urban environments represent globally replicated disturbances to the landscape and are frequently associated with convergent evolutionary changes within populations. For example, changes in biotic and abiotic factors associated with urbanization has led to parallel evolutionary responses in taxa as diverse as plants (Yakub and Tiffin 2016), fish (Reid et al. 2016), lizards (Winchell et al. 2016) birds (Mueller et al. 2013) and insects (Diamond et al. 2018, *this issue*). Similarly, fragmentation associated urbanization has increased the strength of drift in many urban populations, leading to reduced neutral genetic diversity and increased differentiation (Noël et al. 2007; Munshi-South et al. 2016; Lourenço et al. 2017). The frequent observation of increased drift in urban populations, together with our results showing parallel changes in HCN frequencies under gradients of drift, suggests that urban populations may be prone to directional phenotypic change due solely to stochastic forces. However, the importance of drift in structuring urban phenotype frequencies will depend on the genetic architecture underlying traits and the natural history of focal organisms that vary in their tolerance to environmental pressures imposed by urbanization.

The natural history and biology of focal taxa is likely to affect their susceptibility to stochastic changes in allele and phenotype frequencies. Owing to their tolerance of disturbed habitats, non-native species often increase in frequency in urban environments at the expense of native species, which are more susceptible to environmental changes associated with the development of cities (McKinney 2006). Native species (i.e. those that pre-date urbanization) may therefore be more vulnerable to stochastic changes in demography with increasing urbanization leading to increased genetic drift in urban populations of native, but not exotic, species. In support of this view, red-backed salamanders (Noël et al. 2007), white-footed mice (Munshi-South et al. 2016) and fire salamanders (Lourenço et al. 2017)—three species native to the regions in which they were studied—all show reductions in effective population size or reduced neutral genetic diversity in urban populations. In contrast, white clover—originally native to Eurasia—shows inconsistent effects of urbanization on neutral diversity across 8 cities in Ontario, Canada (Johnson et al. 2018, *this issue*) suggesting no increased effects of drift in urban populations. While data is still limited, urban populations of native species appear more susceptible to the effects of genetic drift than non-native species. Predictions on the effects of urbanization on the strength of genetic drift should therefore be based at least in part of the natural history of species being studied.

*Genetic drift and the formation of clines*

The propensity for gradients in the strength of genetic drift to generate clines in non-additive traits depends largely on the genetic architecture of the trait being examined. For example, drift operating alone should generate clines in additive quantitative traits or at individual loci in proportion to the initial allele frequencies resulting in no mean change in allele frequencies when averaged across all simulated clines (Colautti and Lau 2015). Our results support this as in the absence of migration and selection, clines at individual loci underlying HCN (i.e. *CYP79D15* and *Li*) occurred with equal frequency resulting in a mean slope of zero across 1000 simulations (Figure 4B). In contrast, clines in HCN were overwhelmingly positive, suggesting that the epistatic interaction among underlying loci makes natural populations particularly susceptible to the formation of clines via drift. This prediction can provide a way of assessing whether observed clines are due to drift or other evolutionary mechanisms; repeated clines in the same direction at individual loci underlying non-additive traits excludes drift as a possible mechanism producing clines. However, isolation-by-distance can lead to repeated clines at single-loci (Vasemägi 2006) and would have to be ruled out prior to invoking selection.

While genetic drift is sufficient to generate clines in non-additive traits, it is insufficient on its own to maintain them. We have simulated cases where genetic drift leads to the formation of clines on contemporary time scales under non-equilibrium conditions as these are the conditions most likely to occur in urbanizing areas. Nonetheless, in the absence of migration or selection, local genetic drift acting within independent populations is expected to eliminate clines as different alleles become fixed or lost across populations. However, small amounts of migration, which averages allele frequencies among neighboring populations and slows the loss or fixation of alleles, may reduce the extent to which drift eliminates clines and allow for more long-term maintenance of clines. Selection, on the other hand, can maintain clines indefinitely, although clines become increasing non-linear as time proceeds and populations become fixed for alleles on opposing sides of the population where alleles have equal fitness. Again, migration may slow the fixation of alleles resulting in more long-term maintenance of linear clines in phenotype frequencies.

The presence of gradients in the strength of drift has important consequences for the ability of selection to generate clines in non-additive traits. We found that opposing drift gradients constrained the formation of parallel clines due to selection; selection had to be five times stronger to generate predominantly positive clines in the presence of opposing drift gradients relative to simulations that lacked drift (Figure 7). The presence and strength of clines in such cases thus reflects a balance between drift and selection: drift may lead to directional changes in the frequency of non-additive traits but this may be countered by selection if the fitness differential between genotypes is sufficiently strong. An interesting scenario not modeled here concerns the ability of selection to maintain clines formed via drift. Using HCN as an example, if selection is constant and favours HCN+ genotypes across the entire transect, the increased fitness of HCN+ genotypes may counter their stochastic loss in urban populations via drift. Equilibrium could be reached when the rate at which the frequency of a non-additive trait decreases via drift equals the rate at which its frequency increases due to selection. The maintenance of clines in non-additive traits under drift-selection equilibrium represents an interesting avenue for future research.

The formation of phenotypic clines in non-additive traits via neutral processes is likely to be a common phenomenon. Neutral processes are known generate clines at single loci (Vasemägi 2006) and in additive quantitative traits (Colautti and Lau 2015). Here we have shown that gradients in the strength of drift lead to deterministic phenotypic clines in HCN. This, together with predictable changes in other epistatically-determined phenotypes due to stochastic processes (e.g. *Eichhornia paniculata*, Barrett et al. 2009), suggests that non-additive traits are especially susceptible to deterministic changes in frequency via stochastic forces. This pattern arises because random changes in the frequency of alleles at individual loci underlying non-adaptive phenotypes disproportionately affects phenotype frequencies since phenotypes depend also on alleles present at other loci. Thus, phenotypes that result from interactions among multiple genes or metabolic pathways (e.g. HCN) should be most vulnerable to deterministic changes via drift. We further predict that the extent to which non-additive traits are subject to directional changes via drift will increase with the number of interacting loci due to a greater number of possible allelic combinations that can influence resulting phenotypes. Additional theoretical and empirical work exploring changes in the frequency of non-additive phenotypes—whether due to epistasis or dominance at individual loci—is required to generate predictions and assess the generality of this phenomenon in natural populations.

*Conclusion*

We have shown that gradients in the strength drift can lead to repeated spatial clines in the frequency of a non-additive phenotype, despite equal frequencies of positive and negative clines at underlying loci. Drift should thus be considered a null model to be rejected prior to invoking the selection in the formation of clines, especially when phenotypes result from interactions among multiple genes or metabolic pathways. Rejecting drift as a possible mechanism structuring clines in non-additive traits can come in a number of ways. First, showing the absence of clines at neutral loci across the genome despite the presence of clines in the focal trait is strong evidence that adaptive processes are at work as drift is expected to affect all loci. Controlling for clines at neutral loci has been used to reject neutral processes in formation of cyanogenesis clines (Kooyers and Olsen 2012; Johnson et al. 2018, *this issue*) and is a common approach used in other systems as well (e.g. Keller et al. 2009; Campitelli and Stinchcombe 2013). Second, showing that more clines are observed in nature than would be expected under drift suggests that other mechanisms are responsible for generating clines. Expectations could be derived from simulations parameterized with estimates of gene flow and effective population size from natural populations to provide system-specific null models. A disadvantage of this approach is that it does not inform the mechanism structuring any *one* cline but rather rejects drift as a mechanism producing *all* clines. Finally, showing that individual loci underlying focal traits consistently cline in the same direction strongly suggests that other mechanisms are generating clines since drift should not display directionality at individual loci. The second and third points above require large-scale replication and we suggest that urban environments can provide the replication necessary to understand the relative contributions of adaptive and non-adaptive processes in the formation of parallel clines. Finally, we suggest that observations of parallel clines take explicit consideration of the genetic architecture underlying focal traits to generate suitable null models, as traits with a non-additive genetic basis are likely to show deterministic changes in frequency due solely to stochastic forces.