ARTICLE

Natural selection and parallel clinal and seasonal changes in *Drosophila melanogaster*

Running title: Parallel clinal and seasonal changes

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M.F.R. and R.C. designed the research. M.F.R. performed the research and analyzed the data. M.F.R., M.D.V. and R.C. discussed the results and conclusions. M.F.R. wrote the manuscript with input from M.D.V and R.C.

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Data accessibility

All the data used in this project are available on the NCBI Short Read Archive (BioProject accession numbers PRJNA256231 and PRJNA308584, and NCBI Sequence Read Archive SRA012285.16).

Abstract

Spatial and seasonal variation in the environment are ubiquitous. Environmental heterogeneity can affect natural populations and lead to covariation between environment and allele frequencies. *Drosophila melanogaster* is known to harbor polymorphisms that change both with latitude and seasons. Identifying the role of selection in driving these changes is not trivial, because non-adaptive processes can cause similar patterns. Given the environment changes in similar ways across seasons and along the latitudinal gradient, one promising approach may be to look for parallelism between clinal and seasonal change. Here, we test whether there is a genome-wide relationship between clinal and seasonal variation, and whether the pattern is consistent with selection. We investigate the role of natural selection in driving these allele frequency changes. Allele frequency estimates were obtained from pooled samples from seven different locations along the east coast of the US, and across seasons within Pennsylvania. We show that there is a genome-wide pattern of clinal variation mirroring seasonal variation, which cannot be explained by linked selection alone. This pattern is stronger for coding than intergenic regions, consistent with natural selection. We find that the genome-wide relationship between clinal and seasonal variation could be explained by about 4% of the common autosomal variants being under selection. Our results highlight the contribution of natural selection in driving fluctuations in allele frequencies in *D. melanogaster*.

Introduction

Species occur in environments that vary both in space and time (Ewing 1979; Cardini et al. 2007; Dionne et al. 2007; Hancock et al. 2008; Zuther et al. 2012; Campitelli and Stinchcombe 2013; Kooyers et al. 2015). Traits that covary with the environment in space, also called clinal, are usually thought to be the result of spatially varying selection (Endler 1977). However, non-adaptive processes such as isolation by distance, range expansion and

63 admixture can produce similar patterns of spatial change (Wright 1943; Vasemägi 2006;
64 Excoffier et al. 2009; Duchen et al. 2013; Bergland et al. 2016). Therefore, disentangling the
65 relative contributions of natural selection and demographic processes in producing clinal
66 variation is challenging.

67 *Drosophila melanogaster* has been used as a model to study clinal variation because,
68 in addition to being a model species, it is a sub-Saharan fly species that has recently invaded
69 most of the world (David and Capy 1988). These flies migrated into Eurasia approximately
70 15,000 years ago (Li and Stephan 2006), but the colonization of the Americas and Australia
71 likely happened in a single event within the last several hundred years (Bock and Parsons
72 1981; Keller 2007). The establishment of populations in dramatically different environments
73 (*e.g.*, temperate regions) was followed by the emergence of adaptations to these new
74 environments (David and Capy 1988; Yukilevich et al. 2010).

75 Clinal variation has been documented for *D. melanogaster* in many characters both at
76 the phenotypic and genetic level. For instance, at the phenotypic level, flies from higher
77 latitudes are darker (David et al. 1985), bigger (Arthur et al. 2008) and show higher incidence
78 of reproductive diapause than flies from lower latitudes (Schmidt et al. 2005). At the
79 genotypic level, latitudinal clines have been identified for allozymes, chromosome
80 inversions, copy-number variation and single nucleotide polymorphisms (Mettler et al. 1977;
81 Knibb 1982; Oakeshott et al. 1982; Schmidt et al. 2000; de Jong and Bochdanovits 2003;
82 Sezgin et al. 2004; Fabian et al. 2012; Kapun et al. 2016; Schrider et al. 2016). Surprisingly,
83 not much is known about the actual selective pressures in natural fly populations, but
84 temperature is directly or indirectly underlying much of clinal adaptation (Knibb 1982;
85 Krimbas and Powell 1992; Božičević et al. 2016).

86 A classic approach to discern between adaptive and neutral differentiation has been to
87 look at parallel latitudinal variation between continents (Hoffmann et al. 2002; Hoffmann and

88 Weeks 2007; Turner et al. 2008; Paaby et al. 2010; Reinhardt et al. 2014; Schrider et al.
89 2016). If a trait varies clinally in two or more continents, a plausible scenario is latitudinally
90 varying selection acting on ancestral variation (Endler 1977; Barton 1983; Barton 1999).
91 However, some of the clinal patterns observed in North America may be attributed to
92 migration of African flies to low latitude regions and European flies to high latitude regions
93 (Caracristi and Schlotterer 2003; Yukilevich and True 2008; Duchen et al. 2013; Kao et al.
94 2015; Bergland et al. 2016). The main evidence in favor of the secondary contact hypothesis
95 is that there is a cline in the proportion of African ancestry in North America (Kao et al.2015;
96 Bergland et al. 2016), and a weak cline in African ancestry in Australia (only marginally
97 significant; see Bergland et al. 2016).
98 Separating the role of selection and demography in maintaining clinal polymorphisms
99 is not trivial, given that the axis of demography and environmental change are confounded in
100 this system (i.e., migration and the environment are structured along the south-north axis). A
101 simpler, more plausible model would be that admixture introduced "pre-adapted" alleles, that
102 then contributed to clinal adaptation (Flatt 2016). One approach that can help disentangle the
103 role of adaptation is to explore signatures of parallel latitudinal and seasonal changes (Cogni
104 et al. 2014; Cogni et al. 2015).
105 *Drosophila* has been widely used in the study of seasonal adaptation. One of the
106 earliest examples of seasonal genetic variation was observed in chromosomal inversions in *D.*
107 *pseudoobscura* (Dobzhansky 1943). In *D. melanogaster*, it has been shown that flies
108 collected in the spring are more tolerant to stress (Behrman et al. 2015), show higher
109 diapause inducibility (Schmidt and Conde 2006), have increased immune function (Behrman
110 et al. 2018) and have different cuticular hydrocarbon profiles than those collected in the fall
111 (Rajpurohit et al. 2017). Furthermore, genome-wide analyses have identified polymorphisms
112 and inversions that oscillate in seasonal timescales in several localities in the United States

113 and Europe, but the mechanism behind it has not been fully resolved (Bergland et al. 2014;
114 Kapun et al. 2016; Machado et al. 2018; Wittman et al. 2017). Identifying polymorphisms
115 that fluctuate seasonally is challenging, because effect sizes are small, and populations are
116 subject to stochastic environmental events (Machado et al. 2018). A recent analysis suggested
117 seasonal fluctuations in allele frequencies is probably small, and temporal structure
118 independent of seasons may be more important in this system (Buffalo and Coop 2019).
119 Although it is hard to characterize the role of selection in producing latitudinal or
120 seasonal change in allele frequencies, we could gain power by analyzing parallelism between
121 latitudinal and seasonal changes. Two adaptive mechanisms are expected to induce
122 correlations between clinal and seasonal fluctuations in allele frequencies. First, the
123 environment changes similarly with latitude and through seasons (at least with respect to
124 temperature). Second, the onset of spring changes with latitude, and so seasonal changes in
125 polymorphisms alone could produce clinal variation, a mechanism termed seasonal phase
126 clines (Roff 1980; Rhomberg and Singh 1986). Demographic processes that can generate
127 clinal patterns like isolation by distance and secondary contact from diverged populations are
128 independent to variation across seasons, given the short time scale at which the seasonal
129 processes happen (Bergland et al. 2014; Cogni et al. 2015). Therefore, parallel latitudinal and
130 seasonal variation in a trait is strong evidence in favor of natural selection. Indeed, it has been
131 observed in *D. melanogaster* that both the prevalence of reproductive diapause and the
132 frequency of a variant in the couch potato gene associated with diapause inducibility vary
133 latitudinally and seasonally (Schmidt et al. 2005; Cogni et al. 2014). The frequency of this
134 diapause-inducing variant drops in the summer and is positively correlated with latitude, as
135 we would expect if it were being driven by natural selection.
136 Besides this classic example, a few studies have looked for signatures of parallel
137 clinal and seasonal variation in *D. melanogaster* (Bergland et al. 2014; Cogni et al. 2015;

138 Kapun et al. 2016; Behrman et al. 2018; Machado et al. 2018). Cogni et al. (2015) found an
139 association between clinal and seasonal change in central metabolic genes, that are likely
140 important drivers of climatic adaptation. Kapun et al. (2016) dissected the role of selection in
141 shaping clinal variation in *D. melanogaster* cosmopolitan inversions. By integrating clinal
142 and seasonal variation, information on the stability of inversion clines, and analysis of the
143 effect of climatic variables on inversion frequencies, they concluded that several inversion
144 clines are consistent with natural selection. A recent study also investigated the association
145 between clinal and seasonal variation and showed the direction of allele frequency change
146 with latitude is concordant with the change with seasons in many polymorphisms across the
147 *D. melanogaster* genome (Machado et al. 2018). Nevertheless, parallel clinal and seasonal
148 variation has not been used to characterize the role of selection in driving genome-wide
149 fluctuations in allele frequency.

150 Here, we aim to answer whether the clinal and seasonal patterns observed in *D.*
151 *melanogaster* are consistent with natural selection. If natural selection is a key process
152 underlying clinal and seasonal change, flies collected in the north should be more similar to
153 flies collected in early spring, whereas southern flies should be more like flies collected in the
154 fall (Cogni et al. 2014; Bergland et al. 2014; Cogni et al. 2015; Kapun et al. 2016; Behrman
155 et al. 2018; Machado et al. 2018). We quantified the relationship between clinal and seasonal
156 change along the *D. melanogaster* genome. Given the localized effects of selection, and the
157 expectation that constraint depends to some degree on genic annotations, we also expect the
158 parallelism to vary across genomic annotations. We also assessed if the coupling between
159 clinal and seasonal change stems from just a few selected polymorphisms, or if spatial and
160 temporal selection acts pervasively across the genome. Finally, we estimated how much of
161 the common polymorphisms should be under selection to explain the observed degree of
162 coupling between clinal and seasonal change. By integrating these two independent sources

163 of evidence, we further disentangle the contributions of selection in maintaining the patterns
164 of clinal and seasonal variation in *D. melanogaster* populations.

165 Material and Methods

166 Population samples

167 We analyzed 20 samples from seven locations along the United States east coast, collected by
168 (Bergland et al. 2014) (10 samples), and (Machado et al. 2018) (10 samples) (see Table S1).
169 The samples were based on pools of wild-caught individuals. We decided to not include
170 previously collected samples from Maine because they were collected in the fall, whereas all
171 of our other samples were collected in the spring, and we also did not include the DGRP
172 sample from North Carolina, as it is hard to ascertain when they were obtained (Fabian et al.
173 2012; Mackay et al. 2012; Bergland et al. 2014). The Linvilla (Pennsylvania) population was
174 sampled extensively from 2009 to 2015 (six spring, seven fall samples), and was therefore
175 used in our analysis of seasonal variation. We also replicated our results using data from four
176 Australian samples (Anderson et al. 2005; Kolaczkowski et al. 2011). All the data used in this
177 project are available on the NCBI Short Read Archive (BioProject accession numbers
178 PRJNA256231, PRJNA308584 and NCBI Sequence Read Archive SRA012285.16).

179 Mapping and processing of sequencing data

180 Raw, paired-end reads were mapped against the FlyBase *D. melanogaster* (r6.15) and *D.*
181 *simulans* (r2.02) reference genomes (Gramates et al. 2017) using BBSplit from the BBMap
182 suite (https://sourceforge.net/projects/bbmap/; version from February 11, 2019). We removed
183 any reads that preferentially mapped to *D. simulans* to mitigate effects of contamination (the
184 proportion of reads preferentially mapping to *D. simulans* was minimal, never exceeding
185 3%). Then, reads were remapped to *D. melanogaster* reference genome using bwa (MEM
186 algorithm) version 0.7.15 (Li and Durbin 2010). Files were converted from SAM to BAM

187 format using Picard Tools (http://broadinstitute.github.io/picard). PCR duplicates were
188 marked and removed using Picard Tools and local realignment around indels was performed
189 using GATK version 3.7 (McKenna et al. 2010). Single nucleotide polymorphisms (SNPs)
190 and indels were called using CRISP with default parameters (Bansal et al. 2016). We applied
191 several filters to ensure that the identified SNPs were not artifacts. SNPs in repetitive regions,
192 identified using the RepeatMasker library for *D. melanogaster* (obtained from
193 http://www.repeatmasker.org), and SNPs within 5bp of polymorphic indels were removed
194 from our analyses, because these are more likely to be sequencing errors. SNPs with average
195 minor allele frequency in the clinal and seasonal samples less than 5%, with minimum per-
196 population coverage less than 10x (or 4x for the Australian samples) or maximum per-
197 population coverage greater than the 99th quantile were excluded from our analyses. Also,
198 SNPs with multi-allelic states were removed from the analysis. We did not include SNPs in
199 any of the sex chromosomes. SNPs were annotated with their genic classes using SNPeff
200 version 4.3o (Cingolani et al. 2012).

201 **Clinal and seasonal changes in allele frequency**

202 Pool-seq data contain an additional component of error due to sampling (Kofler et al. 2011;
203 Lynch et al. 2014). We model the additional error by computing the effective number of
204 chromosomes (N_E) at each site:

205 $$N_E = \left(\frac{1}{D} + \frac{1}{N_C}\right)^{-1}$$

206 where N_C is the number of chromosomes in the pool and D is the read depth at that site
207 (Kolaczkowski et al. 2011; Feder et al. 2012; Bergland et al. 2014).

208 To assess latitudinal variation, we used generalized linear models (GLM) to regress
209 allele frequency at each site against latitude according to the form,

210 $$y_i = \alpha + \beta^{\text{clinal}}\text{Latitude} + \epsilon_i$$

211 Where y_i is the allele frequency in the ith population, α is the intercept, β^{clinal} is the
212 regression coefficient for latitude, and ϵ_i is the binomial error given the N_E at that
213 polymorphism. This kind of regression is suitable for the analysis of clinal variation of allele
214 frequencies estimated from Pool-seq, because it accounts for differences in read depth and
215 number of chromosomes, and the non-normality of allele frequencies (Bergland et al. 2014;
216 Machado et al. 2016).

217 To assess seasonal variation, we followed a similar procedure. We regressed allele
218 frequency at each site against a binary variable corresponding to spring or fall according to
219 the form,

220 $$y_i = \alpha + \beta^{\text{seasonal}}\text{Season} + \gamma\text{Year} + \epsilon_i$$

221 where y_i is the allele frequency in the ith sample, α is the intercept, $\beta^{seasonal}$ is the seasonal
222 regression coefficient (Season is a dummy variable: June and July were encoded as Spring,
223 and September, October and November as Fall), γ is the regression coefficient for year, and
224 ϵ_i is the binomial error given the N_E at that SNP.

225 We defined clinal and seasonal SNPs using an outlier approach, because we do not
226 have an adequate genome-wide null distribution to compare our estimates. We considered
227 that SNPs were outliers if their regression P-value fell in the bottom 1% (or 5%) of the
228 distribution. All statistical analyses were performed in R 3.5.0 (R Core Team 2018).

229 **Relationship between clinal and seasonal variation**

230 We examined the relationship between clinal and seasonal variation by regressing the clinal
231 against seasonal slopes for all SNPs:

232 $$\beta_i^{\text{clinal}} = \alpha + \beta\beta_i^{\text{seasonal}} + \epsilon_i$$

233 where β_i^{clinal} is the estimated clinal slope for the ith SNP, α is the intercept, β is the slope,
234 $\beta_i^{seasonal}$ is the estimated seasonal slope for the i^{ith} SNP, and ϵ_i is the gaussian error at that

SNP. The regression line was fit using Huber's M estimator to improve robustness to outliers. The clinal and seasonal slopes were z-normalized to facilitate interpretation of the magnitude; this way, the regression coefficient can be interpreted as a correlation coefficient. To confirm our results are robust to potential model misspecifications, we implemented a permutation test in which we rerun the generalized linear regressions for each SNP using shuffled season and latitude labels 2,000 times. The same procedure was implemented for most of the statistical tests, except where indicated otherwise.

To assess differences in the relationship between clinal and seasonal variation across different genic classes (exon, intron, 5' UTR, 3' UTR, upstream, downstream intergenic, splice), we added the following interaction term:

$$\beta_i^{clinal} = \alpha + \gamma_i \beta_i^{\text{seasonal}} \text{Class}_j + \epsilon_i$$

where γ_i is the regression coefficient for the jth genic class, Class_j is the genic class of the ith SNP.

There are some chromosomal inversions segregating in the populations we studied, and they are known to contribute to adaptation (Wright and Dobzhansky 1946; García-Vázquez and Sánchez-Refusta 1988; Kapun et al. 2014). We tested whether the relationship between clinal and seasonal variation is stronger at SNPs that were identified to be markers of inversions in Kapun et al. (2014). To do so, we performed a regression that takes the form:

$$\beta_i^{\text{clinal}} = \alpha + \gamma_i \beta_i^{\text{seasonal}} \times \text{Inversion}_j + \epsilon_i$$

where Inversion_j is the inversion status of the SNP, and γ_i is the regression coefficient for the jth status. We considered SNPs surrounding the inversion breakpoints in the same manner (Corbett-Detig and Hartl 2012). All statistical analyses were performed in R 3.5.0 (R Core Team 2018) and can be found at gitlab.com/mufernando/clinal_sea.git.

Enrichment tests

259 We tested for enrichment of genic classes using our sets of clinal and seasonal SNPs using
260 Fisher's exact test for each genic region and statistic. To control for confounders, such as
261 read depth variation, we computed P-values based on 2,000 permutations. In each iteration,
262 we shuffled the season and latitude labels and reran the generalized regressions. Using the P-
263 values obtained from regressions in which season and latitude labels were shuffled, we
264 defined for each iteration the top clinal and seasonal SNPs. Then, we calculated the
265 enrichment of each genic class using Fisher's exact test. To obtain a P-value for an
266 enrichment of a given genic class, we compared the observed odds ratio in the actual dataset
267 to the distribution of odds ratios observed for datasets in which season and latitude labels
268 were shuffled.

269 **Mitigating the impact of linkage disequilibrium**

270 Selection at one site affects genetic variation at nearby, linked neutral sites (Smith and Haigh
271 1974). Because we assume that sites are independent in our models, the indirect effects of
272 selection can inflate the magnitude of the patterns we investigated. To test the effect of
273 linkage disequilibrium (LD) in our outlier analyses, we computed for all SNPs how their
274 distance to a top SNP affects their P-value. We then smoothed the scatterplot using cubic
275 splines as implemented in ggplot2 (Wickham 2016). To test the effect of linkage on the
276 relationship between clinal and seasonal variation, we implemented a thinning approach.
277 Sampling one SNP per L base pairs one thousand times, we constructed sets of SNPs with
278 minimized dependency, where L ranged from 1 to 20kb. For each of these sets for a given L,
279 we regressed clinal against seasonal slopes, and compared the distribution of the thinned
280 regression coefficients to the coefficients we obtained using all SNPs.

281 **Inferring proportion of selected SNPs**

282 Although a negative, significant relationship between clinal and seasonal variation indicates
283 that a subset of the polymorphisms is under selection, it is difficult to measure how a given

slope is related to a biological parameter, such as the proportion of SNPs that are driven by selection. Most of the observed polymorphisms should be neutral and only a few under direct selection, therefore we expect the relationship between clinal and seasonal change to be minor (i.e., for most SNPs clinal and seasonal change should be unrelated). To gain some intuition of how the proportion of SNPs with uncoupled clinal and seasonal change (e.g., neutral polymorphisms) would impact our measure of interest, the regression coefficient between clinal and seasonal variation, we performed a set of simple simulations.

The polymorphisms most affected by the environment are going to be the ones that change most consistently with seasons and latitude. In this thought experiment, we will assume that the rank order of clinal and seasonal change should be the same (e.g., for SNPs under selection, the linear relationship between clinal and seasonal change will be assumed to be exactly -1). Biologically, this would happen if the environment changed in the same ways with latitude and seasons. This assumption is most likely not true, but it renders our thought experiment conservative (Fig. S4). Note the coefficient cannot be less than -1, because the two variables being regressed were z-normalized and thus have same variance.

We simulated datasets with different proportions of points falling along a line ($y = -x$), representing selected SNPs and points without a linear relationship, representing SNPs that are not under spatial and seasonal selection. For each simulation replicate, we calculated the linear relationship between two variables with varying proportions of points falling on the 1:-1 line to independent points. Each value was drawn from the combined distribution of observed z-normalized regression coefficients for season and latitude. By calculating the observed slope between the regression coefficients of each of our simulated datasets, we could establish a relationship between observed slope and the expected proportion of SNPs that fall along 1:-1 line (or that are under selection). We then used Approximate Bayesian Computation using a sequential sampling scheme (as implemented in the R package

309 EasyABC; Jabot et al. 2013) to estimate the posterior distribution of proportion of SNPs
310 under spatial and seasonal selection that could explain our observed relationship between
311 clinal and seasonal change (using our thinned estimate to alleviate the effects of linkage
312 disequilibrium).

313 **Results**

314 We assembled 21 *D. melanogaster* population samples collected from seven localities across
315 multiple years in the east coast of the United States. All of these samples are the result of a
316 collaborative effort of many researchers from a consortium, the DrosRTEC (Bergland et al.
317 2014; Machado et al. 2018). Seven of our samples span from Florida to Massachusetts and
318 together comprise our clinal set. The seasonal samples were collected in Pennsylvania in the
319 spring (6 samples collected in June or July) and in the fall (7 samples collected in September,
320 October or November). For each sample, a median of 55 individuals (with a range of 33 to
321 116) was pooled and resequenced to an average 75x coverage (ranging from 17 to 215). We
322 also used four clinal samples from the Australia (Anderson et al. 2005; Kolaczkowski et al.
323 2011). More details about the samples can be found on Table S1 (also see Machado et al.
324 2018; Bergland et al. 2014). After all the filtering steps, we identified 797,792 common
325 autosomal SNPs, which were used in our downstream analyses.

326 **Allele frequency changes with latitude, seasons and years**

327 Latitude explains much of allele frequency variation along the surveyed populations, as there
328 is an excess of low GLM P-value SNPs (Fig. 1A). The mean absolute difference in allele
329 frequency between the ends of the clines is 9.2%. Seasons, on the other hand, explain less of
330 the variation in allele frequency. There is only a minor excess of low GLM P-value SNPs
331 (Fig. 1B) and the mean absolute difference in allele frequency between seasons is 2.6%. We
332 also found that year of sampling is a good predictor of allele frequency change (in

Pennsylvania), more so than seasons, given there is a huge excess of low GLM P-values (Fig. 1C).

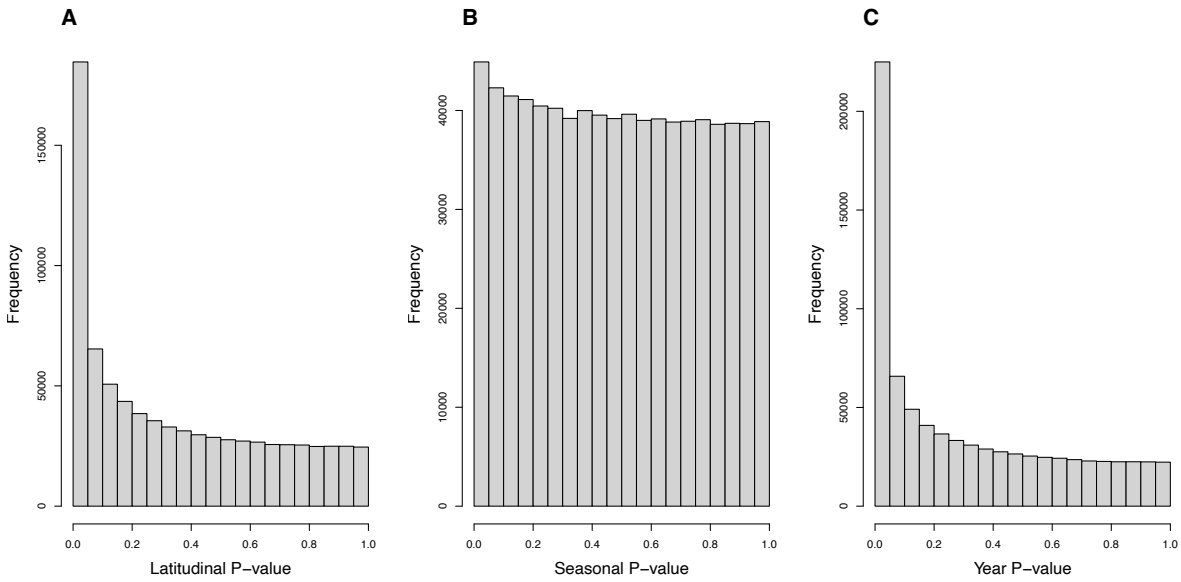
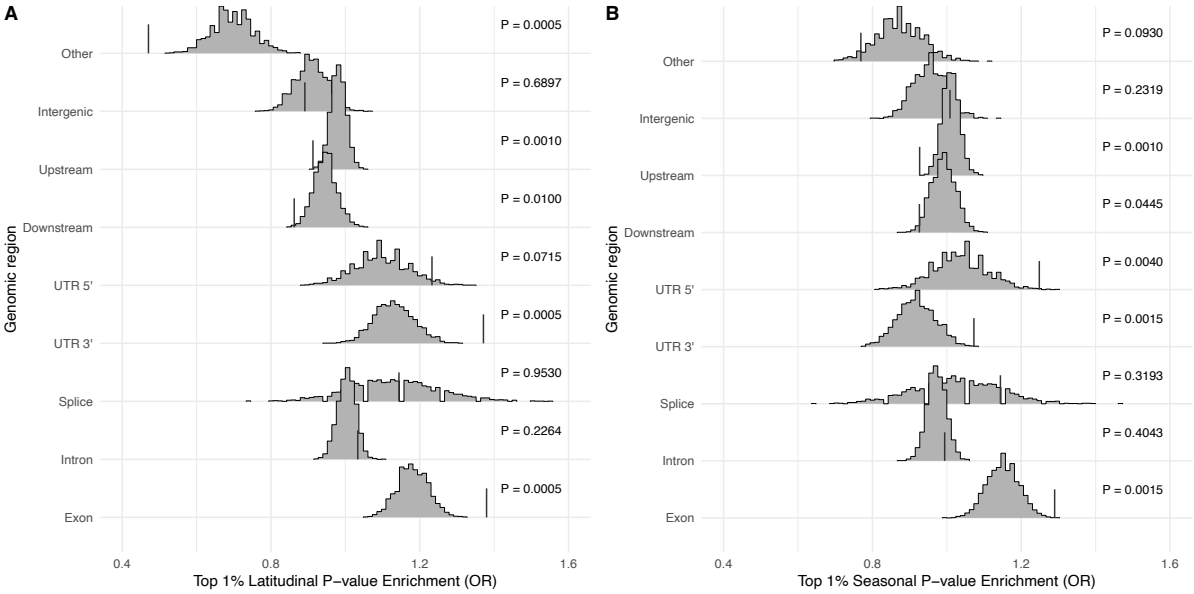


Figure 1. Distribution of P-values from the generalized linear models of allele frequency and latitude, and allele frequency and seasons/years.

Our generalized linear models do not account for dependency between samples, which can be a problem when regressing allele frequency on seasons. To investigate whether this could be an issue, we performed Durbin-Watson tests for autocorrelation in the residuals of the seasonal regressions using Julian days as the time variable. We found a minor excess of low P-values (Fig. S1), and the season P-values are weakly, but negatively correlated with the Durbin-Watson test P-values (Spearman $\rho = -0.004, P = 0.0001$). This indicates that the assumption of independency is being met for most variants, and that autocorrelation is not artificially creating patterns of seasonality in allele frequency.

Given we do not have enough information to build an appropriate null distribution to calibrate our P-values, we sought to demonstrate that top significant clinal and seasonal SNPs are enriched for functional variants, which are more likely to contribute to adaptation. Latitudinal SNPs are more likely to be in exonic and UTR 3' regions (Fig. 2A), whereas seasonal SNPs are enriched for exonic, UTR 3’ and UTR 5’ regions (Fig. 2B). Further, top

352 latitudinal and seasonal SNPs seem to be underrepresented within upstream and downstream
353 regions (Fig. 2). The enrichment of top latitudinal SNPs among functional genic classes has
354 been reported by previous studies which used different latitudinal samples (Fabian et al.
355 2012; Kolaczowski et al. 2011, Machado et al. 2016). Enrichment of top seasonal SNPs
356 among some functional regions have been observed before (Bergland et al. 2014; Machado et
357 al. 2018). Using a 5% cutoff, our results with respect to enrichments is largely replicated
358 (Fig. S2).



359

360 **Figure 2.** Top SNPs are enriched for functionally relevant classes. Enrichment of top 1%
361 SNPs in each genic class for A) latitudinal P-value and B) seasonal P-value. The histograms
362 show the distribution of odds ratios when latitude and season labels were permuted, and the
363 vertical bars show the observed odds ratios.

364 **Clinal variation is related to seasonal variation**

365 A clinal pattern can arise solely as a result of demographic processes, such as isolation by
366 distance and admixture (Duchen et al. 2013; Kao et al. 2015; Bergland et al. 2016). Although
367 seasonality is less affected by such processes, seasonal change is less pronounced and more
368 subject to stochastic changes in the environment, making it harder to detect seasonal change
369 with precision. Here, we integrate both clinal and seasonal change estimates across a large
370 number of SNPs in the genome (about 790,000 SNPs). We expect the overall pattern that

371 emerges to be informative of the relative role of natural selection, because selection is a
372 plausible process to produce a pattern of clinal variation mirroring seasonal variation (Cogni
373 et al. 2015). With higher latitudes, cold-favored alleles rise in frequency, whereas from spring
374 to fall, the frequency of cold-favored alleles decreases. Conversely, with higher latitudes,
375 warm-favored alleles decrease in frequency, whereas from spring to fall, the frequency of
376 warm-favored alleles increases.

377 To assess if clinal variation and seasonal variation are related, we performed a linear
378 regression of the slopes of clinal and seasonal allele frequency change (see Methods). We
379 find a significant negative linear relationship between clinal and seasonal regression
380 coefficients (Fig. 3A, Table S2). The relationship is strongest for SNPs within exons, and the
381 weakest for unclassified SNPs and those within intergenic regions (Fig. 3A; Table S3).
382 Nonetheless, the relationship is different than zero for all classes (except for the unclassified),
383 what would be consistent either with pervasive linked selection or widespread distribution of
384 variants that are important for adaptation, even within non-coding regions. Our results are
385 robust to modelling assumptions, as they are significant when compared to the datasets with
386 permuted of latitude and season labels (Fig. 3C). Qualitatively similar results were replicated
387 using a different minor allele frequency cutoff and using samples from Maine, which were
388 sampled in the summer (Fig. S3A-B).

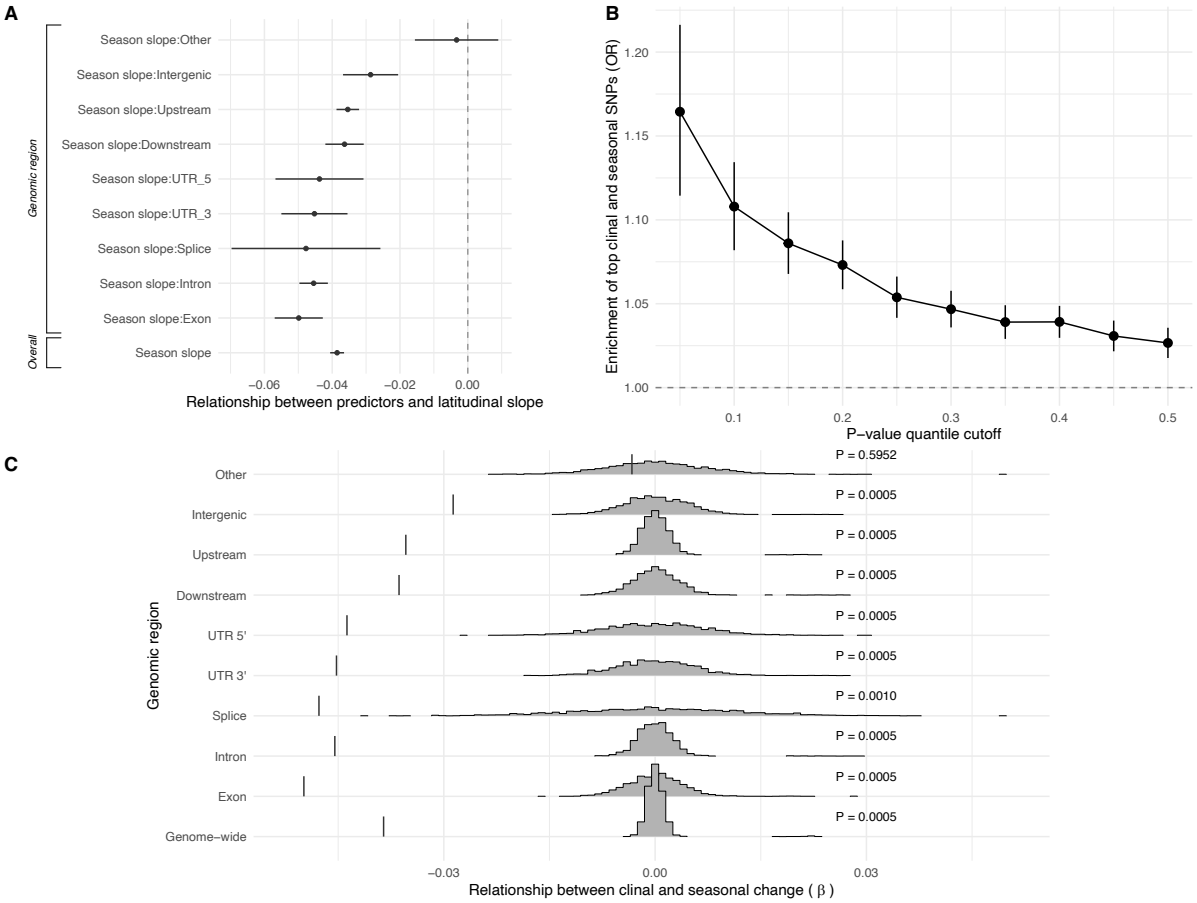


Figure 2. Clinal change parallels seasonal change. A) Linear relationship between latitudinal change and seasonal change for each genic class. B) Association between top latitudinal and seasonal variants for different P-value cutoffs. C) Null distribution of the regression coefficient between latitudinal and seasonal change for each genic class (after permuting the latitude and season labels). Vertical bars represent the observed values.

Given that previous studies have demonstrated the importance of cosmopolitan inversions in climatic adaptation (Kapun et al. 2016), we looked at how clinal and seasonal variation are related within common cosmopolitan inversions. In our dataset, we found only 16 out of hundreds of marker SNPs identified in Kapun et al. (2014). It is unclear whether this is due to lifting over their annotations to a more recent version of the reference genome or if these variants are truly absent within the samples used in our study. We found that the relationship between clinal and seasonal change for those markers is high but did not reach significance due to small number of variants (Table S4). Most of the divergence (and differentiation) between flies with and without inversions should be located around the

405 breakpoints (Kapun et al. 2014, Kapun et al. 2018). Thus, we checked whether the pattern of
406 parallelism between clinal and seasonal variation is stronger surrounding common
407 cosmopolitan inversions (considering SNPs within 2Mb of the breakpoints identified in
408 Corbett-Detig and Hartl, 2012). We found that clinal and seasonal change are strongly
409 coupled surrounding the inversions (Table S5). Nevertheless, the pattern is still strong outside
410 these regions, indicating our main results are not purely driven by frequency changes of
411 inversions.

412 Another way of testing for parallelism between clinal and seasonal change is by
413 testing if clinal SNPs are more likely to be seasonal (and vice-versa). We observed that clinal
414 SNPs are enriched for seasonal SNPs (Fig. 2B). The enrichment increases with more
415 stringent lower P-value quantile cut-offs, as we would expect if even strictly non-significant
416 variants were informative of the role of selection.

417 We also confirmed our main finding, that clinal and seasonal change are related using
418 samples from Australia. To measure clinal change in Australia, we only used four low
419 coverage samples in Australia, two for each of low and high latitude locations. There is a
420 genome-wide relationship between clinal variation in Australia and seasonal variation in
421 Pennsylvania that, although minor, is significant (Fig. S3C). We also found a relationship
422 between latitudinal regression significance and seasonal significance using the Australian
423 samples (Fig. S3C).

424 **The effects of linkage disequilibrium on clinal and seasonal variation**

425 Variation at one site is linked to variation at other sites, and selection will increase this
426 dependency (Smith and Haigh 1974). First, we assessed if latitudinal and seasonal P-values,
427 were dependent on how distant a SNP was from our top 1% SNPs. We show that both
428 statistics are dependent on distance from the outlier SNPs (Fig. 3A-B), but the effect virtually
429 disappears after 5kb.

430 Our regression analysis of clinal and seasonal variation assumes that SNPs are
431 independent. Thus, we assessed the impact of linkage on the relationship between clinal and
432 seasonal change by implementing a thinning approach. First, we tested how the genome-wide
433 regression estimate varied with changing window sizes. The effect of non-independency of
434 variants on the relationship between clinal and seasonal change is rather small (Fig. 3C).
435 Nevertheless, we chose a conservative window length of 5kb for downstream analyses. The
436 strength of the genome-wide relationship between clinal and seasonal variation decreased
437 when randomly sampling one SNP per 5kb, but it still remained significantly different from
438 zero (P= 0.001; Fig. 3D). Thus, it seems the signal is widespread across many loci. The
439 thinning did not significantly impact the signal for many regions, but the strength of the
440 signal within splicing, UTR 5', upstream, downstream and intergenic regions decreased and
441 is not significantly different from 0 (Fig. 3D). It may be that most of the signal coming from
442 those regions are due to the linked effects of selection.

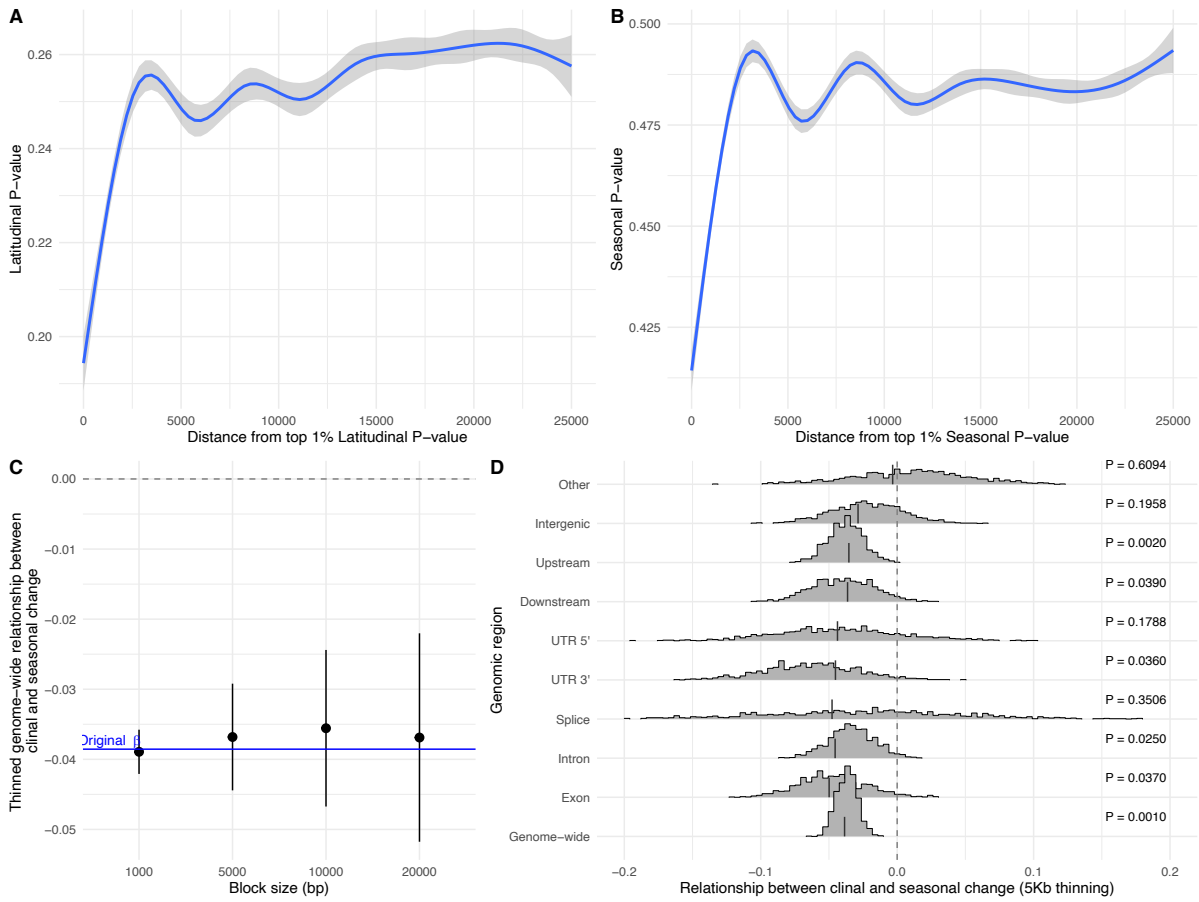


Figure 3. Effects of linkage disequilibrium. The mean (A) latitudinal P-value and (B) seasonal P-value depend on distance to the respective top 1% outliers. The relationship between clinal and seasonal variation is affected by dependency among SNPs. C) the strength relationship between clinal and seasonal variation changes with the size of the thinning window. D) comparison among original estimates (bar) and values obtained after thinning using a window size of 5kb (histogram). Histograms show the distributions across sampled thinned datasets.

Selection accounts for a substantial proportion of genome-wide clines

Although we identified a significant negative relationship between clinal and seasonal variation, the magnitude of the regression coefficient appears small (5kb bootstrap estimate $\beta = 0.037$). It is hard to measure the biological relevance of such coefficient, and so we designed a simple simulation approach that translates our statistic of interest into a biological parameter, that is the proportion of selected sites. We assume that: (i) for the subset of variants that are under selection, we would expect to see a perfectly linear relationship between z-normalized clinal and seasonal regression coefficients; (ii) for the variants that are

460 not contributing to adaptation, then we would expect absolutely no relationship between
461 clinal and seasonal variation; (iii) variants are not linked. Assumption (i) renders our test
462 conservative, as the relationship would not necessarily be negative one because some variants
463 may be strongly seasonal but not necessarily strongly latitudinal and vice-versa. In Figure S4,
464 we show that if the true slope for selected SNPs is not -1, we would be severely
465 underestimating the proportion of selected SNPs. Accounting for linkage disequilibrium
466 (assumption iii) is notoriously complicated, especially because we cannot accurately measure
467 LD from pooled sequencing (Feder et al. 2012). However, we show that most of the effects of
468 autocorrelation can be accounted for with our thinning approach, thus we used the observed
469 relationship between clinal and seasonal change after 5kb thinning.

470 We asked what the expected relationship between clinal and seasonal regression
471 coefficients would be if some of the variants were subject to spatially and seasonally varying
472 selection but most were neutral. To assess this, we built datasets with varying proportion of
473 adaptive variants (with linear relationship between clinal and seasonal variation) to neutral
474 variants (no relationship between clinal and seasonal variation) and calculated the expected
475 linear relationship between the two variables.

476 We found that the observed regression coefficient is consistent with approximately
477 3.9% of the common variants being under selection (Fig. 4), with 95% of the posterior
478 estimates ranging from 3.7% to 4.2%. Thus, our analysis suggests that a sizable fraction of
479 common, autosomal SNPs changes due to selection. Note this proportion is in line with
480 previous estimates for the proportion of clinal SNPs (3.7% in Machado et al. 2015) and
481 seasonal variants (~4% in Machado et al. 2018).

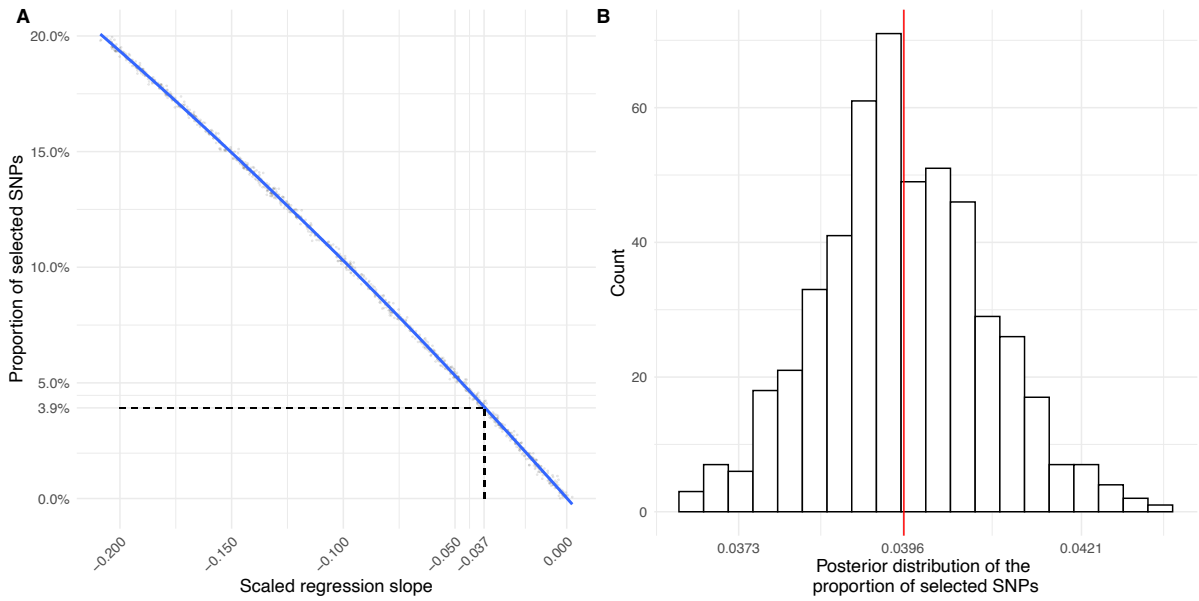


Figure 4. Proportion of SNPs that should be under selection to explain the genome-wide relationship between clinal and seasonal variation. A) background points represent each simulation, and the line is the fitted loess curve. Dashed lines relate the observed regression coefficient after thinning (5kb) to the expected proportion of selected SNPs. B) posterior distribution of the proportion of selected SNPs, given the observed relationship between clinal and seasonal variation (5kb thinned), with horizontal line highlighting the mean.

Discussion

Clinal patterns have been observed in both phenotypic and genotypic traits in many different species (Hancock et al. 2008; Baxter et al. 2010; Adrion et al. 2015). Especially in systems in which there is collinearity between the axis of gene flow and environmental heterogeneity, disentangling the contribution of selection and demography in producing clines is not trivial. Detecting seasonal cycling in allele frequencies is also challenging, mostly because the effect size is likely to be small and the environment may change unpredictably within seasons. We relied on integrating patterns of clinal and seasonal variation, since variation over short periods is independent of most confounding demographic processes. Parallel environmental change and the differences in the onset of seasons with latitude can create parallelism between clinal and seasonal variation. Natural selection would be the process driving the coupling between latitudinal and seasonal change in both scenarios, thus investigating it can

502 help discern the role of natural selection. Here, using *D. melanogaster* samples from the east
503 coast of the United States and Australia, we show that (i) there is a genome-wide pattern of
504 clinal variation mirroring seasonal variation, (ii) this is consistent with the action of natural
505 selection, and (iii) a significant number of common variants may be being driven by
506 selection.

507 We show that clinal and seasonal change are related, as there is a significant negative
508 relationship between clinal and seasonal regression coefficients in the *D. melanogaster*
509 genome (Fig. 2A), and there is an enrichment for seasonal SNPs among clinal SNPs (Fig.
510 2B). The relationship between clinal and seasonal regression is stronger for genic when
511 compared to intergenic regions (Fig. 2A,C). Demographic processes would impact the
512 genome as a whole, but variants in genic regions are more likely to impact fitness (Andolfatto
513 2005). Further, we estimate that about 3.9% of common, autosomal SNPs should be under
514 selection to explain the genome-wide pattern of clinal and seasonal concordance uncovered
515 (Fig. 4).

516 Because we expect selection to intensify linkage disequilibrium, the relationship
517 between clinal and seasonal variation could be mostly driven by a few large effect loci.
518 However, we show that our results cannot be fully explained by linked selection (Fig. 3).
519 When accounting for autocorrelation along the chromosomes, the relationship between clinal
520 and seasonal change disappears for variants within intergenic regions, but not for exons (Fig.
521 3D). The effects of linkage disequilibrium are important in this short time scale, but it decays
522 rapidly, and returns to background levels after 5kb (Fig. 3A-C). We also investigated whether
523 our results could be due to differences in inversion frequencies, whichh are known to underlie
524 much of climatic adaptation in *D. melanogaster* (Kapun et al. 2016). The coupling between
525 clinal and seasonal change surrounding common inversions is generally high, but in isolation
526 it cannot explain the genome-wide patterns we observed. Therefore, our results support a

527 scenario in which the genome-wide pattern of clinal variation mirroring seasonal variation is
528 a result of selection acting on many sites across the genome.

529 An important mechanism that can cause clinal patterns has been neglected from
530 recent discussions of clinal variation in *Drosophila*. The latitudinal variation on the onset of
531 seasons can produce clines, a phenomenon termed "seasonal phase clines" (Roff 1980;
532 Rhomberg and Singh 1986). Under this model, a correlation between clinal and seasonal
533 change is expected. Our latitudinal samples were all collected within one month of difference
534 (during the spring), and so our observations could be partially explained by differences in the
535 seasonal phase. Our data does not allow for proper disentangling of seasonal phase clines
536 from parallel environmental change but change on the onset of seasons alone cannot explain
537 our results. We found that latitude is usually a much better predictor of allele frequency
538 differences (Fig. 1A-B), and the magnitude of change along the cline is much greater than
539 what we found within a population across seasons (9.2% vs. 2.64%). We show that including
540 Maine samples (which were sampled in the fall, in contrast to all other samples that were
541 sampled in the spring) in our analyses does not meaningfully change our main results (Fig.
542 S3). However, the rate of change (per degree of latitude) in allele frequency from Florida to
543 Maine is smaller than the rate from Florida to Massachusetts. This could be due to
544 differences in sampling year, but we found that the rate of change in frequency between
545 Virginia and Maine is also smaller than the rate between Virginia and Massachusetts (FL and
546 ME were sampled in 2009, whereas VA and MA were sampled in 2012; Fig. S5). We
547 believe these differences are due to the shift in seasonal phase, as the samples from Maine
548 were collected in the fall, but all other samples were collected in the spring.

549 Importantly, our results should be robust to differential admixture from Europe and
550 Africa to the ends of the clines (Duchen et al. 2013; Kao et al. 2015; Bergland et al. 2016).
551 Given variation over seasonal time scales are less affected by broader scale migration

552 patterns, it is not plausible that secondary contact can create parallel clinal and seasonal
553 variation. Furthermore, the evidence for secondary contact in Australia is quite weak (see
554 Bergland et al. 2016), but we show that clinal variation in Australia is related to seasonal
555 variation in Pennsylvania (Fig. S3). Secondary contact may have contributed ancestral
556 variation, that have since been selectively sorted along the cline (Flatt 2016). Consistent with
557 this interpretation, the proportion of African ancestry is lower in low recombination regions,
558 suggesting selection is mediating admixture in *D. melanogaster* (Pool 2015).

559 Population substructure and migration could cause seasonal variation in allele
560 frequency in *D. melanogaster*. One possibility is that rural populations of *D. melanogaster* in
561 temperate regions collapse during the winter and recover from spring to fall. However,
562 reproductive diapause cycles in orchards and reaches high frequencies early in the spring,
563 whereas its frequency in urban fruit markets in Philadelphia is much lower (Schmidt and
564 Conde 2006). Another possibility is that seasonal variation is produced by migration of flies
565 from the south in the summer, and from the north in the winter. There is little evidence of
566 long-range migration in *D. melanogaster*, though this process seems important in *D. simulans*
567 (Bergland et al. 2014; Machado et al. 2016). Importantly, flies have been shown to survive
568 and reproduce during winter season in temperate regions, so rural flies can withstand a harsh
569 winter season and be subject to selection (Mitrovski and Hoffmann 2001; Hoffmann et al.
570 2003, Rudman et al. 2019). These seasonal patterns have been replicated in many populations
571 across North America and Europe (Machado et al. 2018), bolstering the argument for
572 seasonal selection. Given the patterns we uncovered here are the result of subtle, but
573 repeatable changes across multiple seasons, it is hard to imagine that selection is not the main
574 causing force, even if it's acting to maintain cryptic population structure within each location.

575 A recent study suggested the temporal changes in allele frequency reported in
576 Bergland et al. (2014) is only weakly consistent with seasonal selection (Buffalo et al. 2019).

577 Consecutive spring-fall pairs showed some signal of adaptation, but the effect was small and
578 disappeared at larger timescales (same season but across different years). Similarly, Machado
579 et al. 2018 found that when they flipped the season labels of some samples the seasonal
580 model fit was greatly improved. Consistent with these observations, we found there is strong
581 temporal structure across years (Fig. 1C). Though we use a different set of samples, we show
582 that the distribution of seasonal P-values is only slightly enriched for low P-values (Fig. 1B).
583 Importantly, top seasonal SNPs are enriched for functional genic classes, when compared to
584 datasets in which the season labels were permuted (Fig. 2B). These results highlight how
585 difficult it is to find truly seasonal SNPs, likely because change in allele frequency is
586 generally much smaller and natural populations are subject to stochastic changes in the
587 environment not related to seasons. Once we have larger time series data, we will not need to
588 rely on seasons as a proxy for environmental heterogeneity, and instead it will be possible to
589 perform a proper causal analysis.

590 A few previous studies have investigated the relationship between clinal and seasonal
591 variation (Bergland et al. 2014; Cogni et al. 2015; Kapun et al. 2016; Machado et al. 2018),
592 but here we further explore parallelism between clinal and seasonal variation to tease apart
593 the role of selection. Cogni et al. (2015) focused on a few SNPs in metabolic genes, and so
594 they could not draw robust conclusions about processes that impact most of the genome. Our
595 work is also fundamentally different from that of Machado et al. (2018), which revolves
596 around identifying seasonal variation in *D. melanogaster* by aggregating data from many
597 locations in North America and Europe. Here, we only included seasonal samples from
598 Pennsylvania. This location had the greatest number of years surveyed (7 years, as opposed
599 to 2 years for most of the other locations; see Machado et al. 2018), and it is the population
600 for which we have the most information about seasonal variation in relevant phenotypes,

601 such as diapause (Schmidt and Conde 2006; Behrman et al. 2015; Rajpurohit et al. 2017;
602 Behrman et al. 2018).
603 Many species occur along spatially structured environments and show clinal variation
604 in traits, so a question that remains open is: what is the role of selection in producing and
605 maintaining these patterns? Seasonal variation is also ubiquitous, especially in temperate
606 environments, so seasonal change could be an important feature of organisms that have
607 multiple generations each year (Behrman et al. 2015). Here, we demonstrate that by
608 integrating clinal and seasonal variation, we can discern the contributions of selection in
609 driving allele frequency changes with the environment. This approach could potentially be
610 applied to other multivoltine species that occur along environmental gradients, including
611 invasive species, which are known to often have short generation times and to reproduce
612 quickly (Sakai et al. 2001).
613

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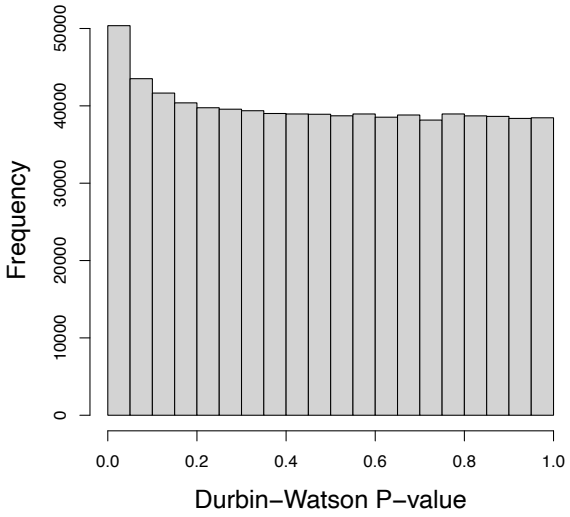
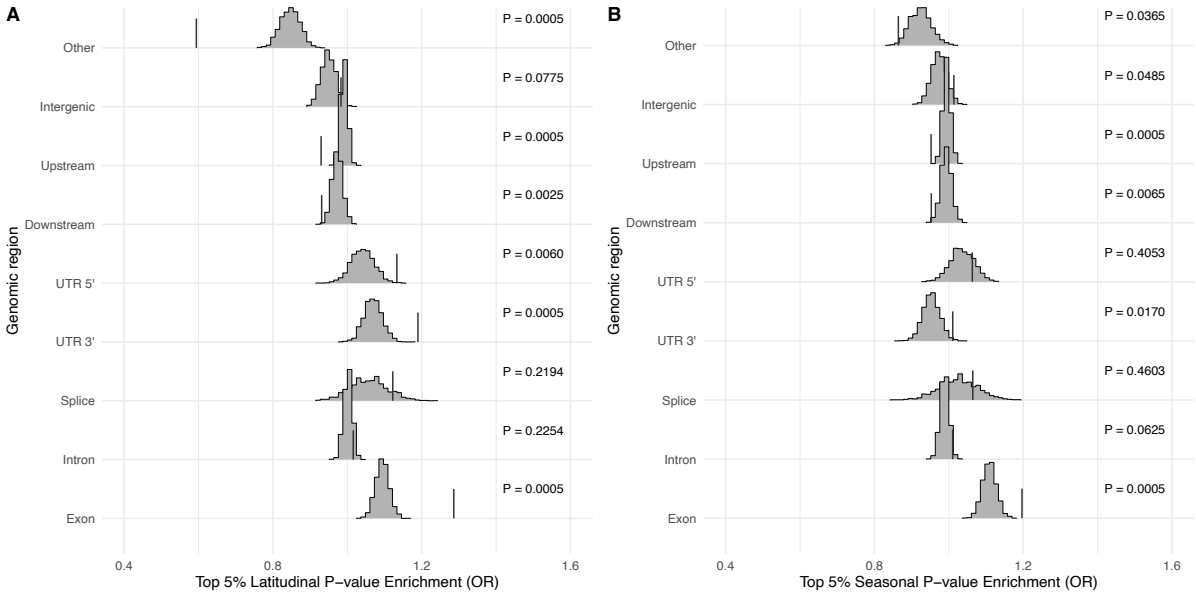
845 **Supplementary information**



846

847 **Figure S1**. Distribution of Durbin-Watson P-values. We tested whether there is
848 autocorrelation in the residuals of the seasonal generalized linear models.
849



850

851 **Figure S2.** Enrichment of top 5% SNPs in each genic class for A) latitudinal P-value, B)
852 seasonal P-value. Histograms show the distribution of odds ratios when season labels were
853 permuted, and the vertical bars indicated the observed odds ratio.

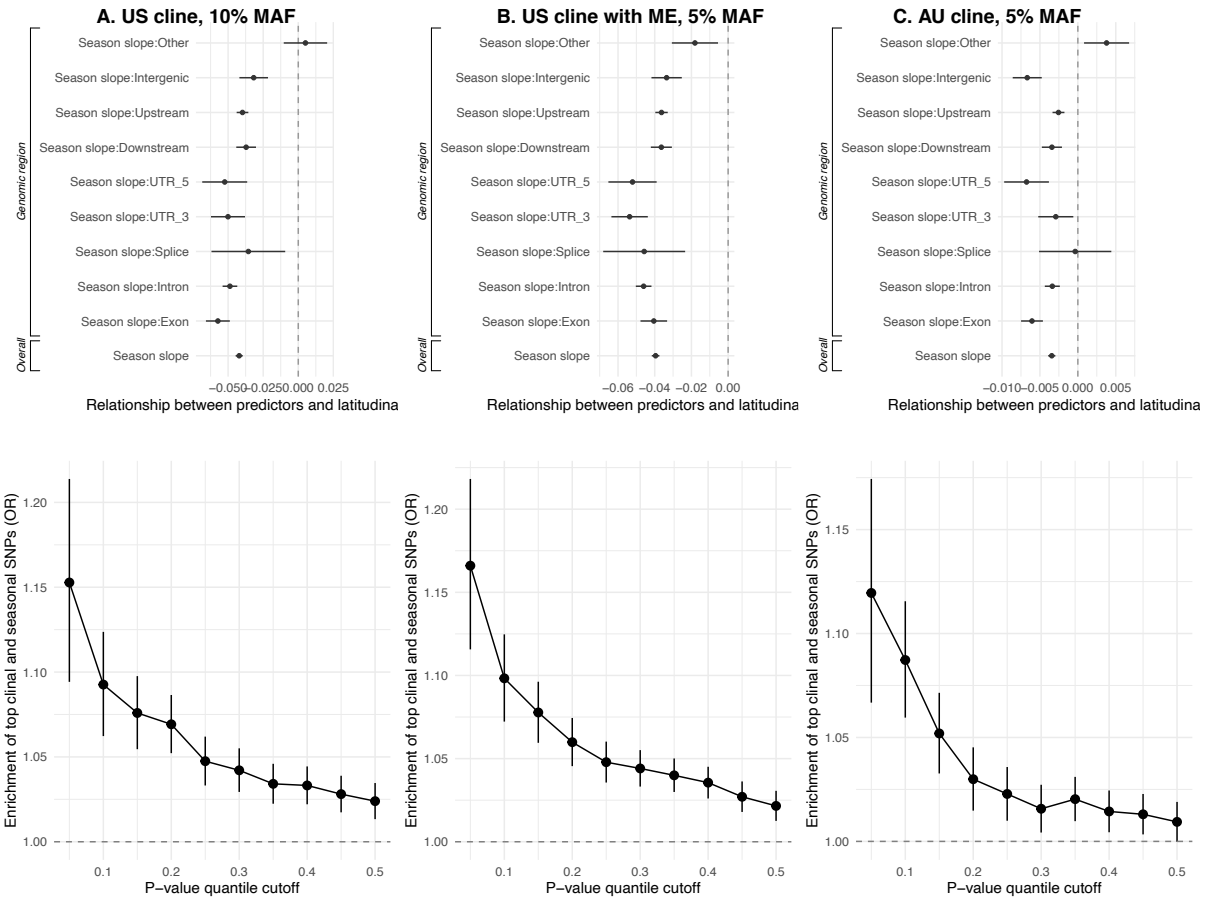


Figure S3. Relationship between clinal and seasonal change using A) a more stringent minor allele frequency cutoff, B) latitudinal samples from Maine, which were collected in the summer and C) samples from Australia to calculate latitudinal change.

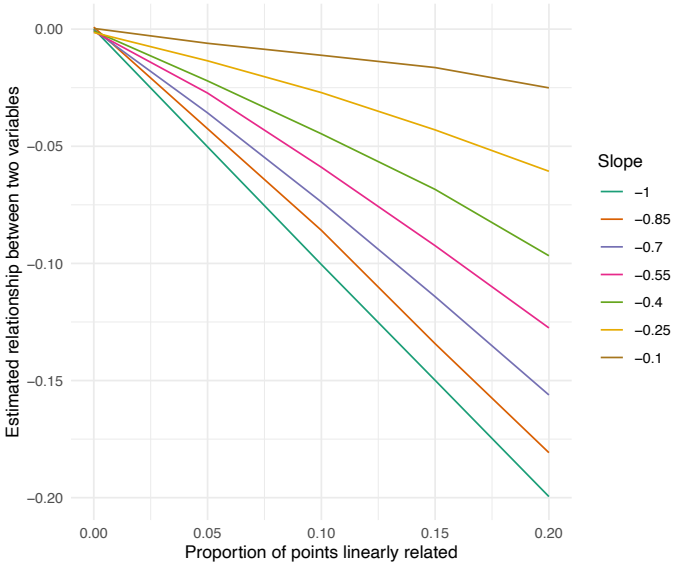
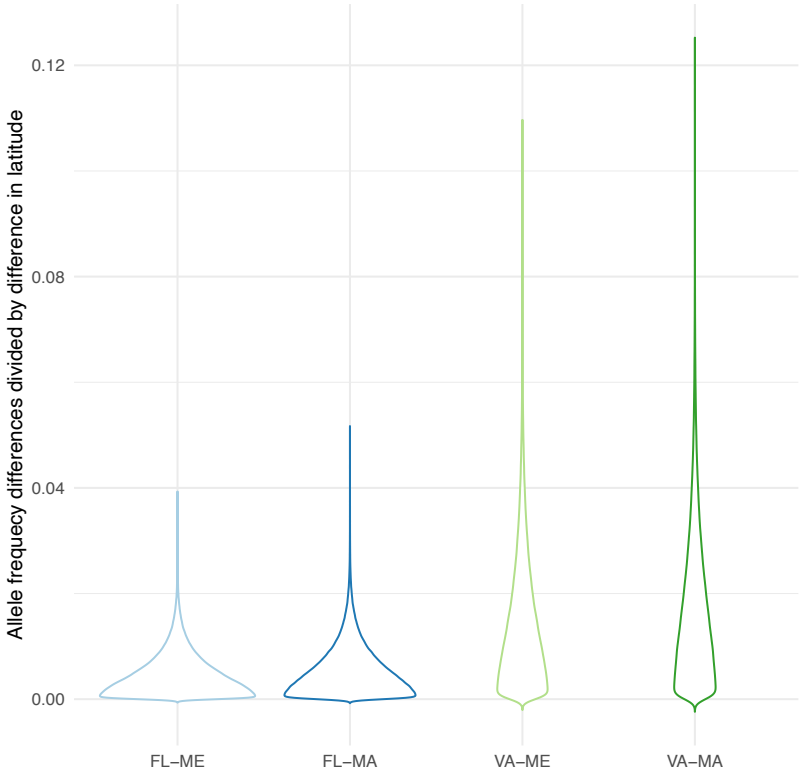


Figure S4. Relationship between proportion of points linearly related (in comparison to points that are not related) and the expected relationship between two z-normalized variables. Each color represents a different assumed degree of linear relatedness (ranging from -1 to -0.1). Note how for a given estimated relationship between two variables, the slope we assume

863 (-1) results in the smallest possible proportion of points linearly related, demonstrating that
864 our test is conservative.

865



866

867 **Figure S5.** Rate of allele frequency differences between two populations (normalized by
868 difference in latitude). Note the rates are smaller for comparisons involving the samples from
869 Maine. FL: Florida (July 2008 and 2010), ME: Maine (October 2009), MA: Massachusetts
870 (July 2012), VA: Virginia (July 2012).

871

872

873 **Table S1.** Information of the samples used in this study.

Accession #	Sample name	Location	Latitude	Collection date	# Flies	Median depth	Month	Season	Seasonal set	Clinal set
SRR1177951	AU_LO1	Queensland, Australia	16.90	2004	17	10				
SRR1177952	AU_LO2	Queensland, Australia	16.90	2004	17	12				
SRR1177953	AU_HI1	Tasmania, Australia	42.77	2004	15	10				
SRR1177955	AU_HI2	Tasmania, Australia	42.77	2004	15	14				
SRR1525685	FL1	Homestead, FL	25.47	Jul-08	39	59	7	Spring	0	1
SRR1525694	FL2	Homestead, FL	25.47	Jul-10	48	37	7	Spring	0	1
SRR1525698	ME1	Bowdoinham, ME	44.02	Oct-09	75	86	9	Fall	0	0
SRR2006283	ME2	Bowdoinham, ME	44.02	Oct-09	75	22	9	Fall	0	0
SRR1525695	GA	Hahira, GA	30.99	Jul-10	51	101	7	Spring	0	1
SRR1525696	SC	Euatwville, SC	33.40	Jul-10	48	83	7	Spring	0	1
SRR3590551	VA_07_2012	Charlotttesville, VA	38.03	Jul-12	69	70	7	Spring	0	1
SRR3939095	PA_06_2013	Linvilla, PA	39.88	Jun-13	54	37	6	Spring	0	1
SRR3590557	MA_07_2012	Lancaster, MA	42.46	Jul-12	90	51	7	Spring	0	1
SRR1525768	PA_07_2009	Linvilla, PA	39.53	Jul-09	55	186	7	Spring	1	0
SRR1525769	PA_11_2009	Linvilla, PA	39.53	Nov-09	74	66	11	Fall	1	0
SRR1525770	PA_07_2010	Linvilla, PA	39.53	Jul-10	116	17	7	Spring	1	0
SRR1525771	PA_11_2010	Linvilla, PA	39.53	Nov-10	33	76	11	Fall	1	0
SRR1525772	PA_07_2011	Linvilla, PA	39.53	Jul-11	75	53	7	Spring	1	0
SRR1525773	PA_10_2011	Linvilla, PA	39.53	Oct-10	47	74	10	Fall	1	0
SRR3590560	PA_10_2012	Linvilla, PA	39.53	Oct-12	100	25	10	Fall	1	0
SRR3590561	PA_07_2012	Linvilla, PA	39.53	Jul-12	115	59	7	Spring	1	0
SRR3590563	PA_9_2012	Linvilla, PA	39.53	Sep-12	50	55	9	Fall	1	0

SRR3939096	PA_10_2014	Linvilla, PA	39.88	Oct-14	50	103	10	Fall	1	0
SRR3939097	PA_06_2014	Linvilla, PA	39.88	Jun-14	92	68	6	Spring	1	0
SRR3939098	PA_10_2015	Linvilla, PA	39.88	Oct-15	52	97	10	Fall	1	0
SRR3939099	PA_07_2015	Linvilla, PA	39.88	Jul-15	74	215	7	Spring	1	0

Table S2. Summary of a regression of z-normalized latitude regression coefficients against z-normalized season regression coefficients genome-wide. CI stands for 95% confidence interval.

	Latitude slope		
Predictors	*Estimates*	*CI*	*p*
Intercept	0.013	0.011 – 0.015	**<0.001**
Seasonal slope	-0.039	-0.041 – -0.037	**<0.001**
Observations	797792		

Table S3. Summary of a regression of z-normalized latitude regression coefficients against z-normalized season regression coefficients for each genic class. CI stands for 95% confidence interval.

	Latitude slope		
Predictors	*Estimates*	*CI*	*p*
Intercept	0.013	0.011 – 0.015	**<0.001**
Seasonal slope:Intergenic	-0.029	-0.037 – -0.021	**<0.001**
Seasonal slope:Exon	-0.050	-0.057 – -0.043	**<0.001**
Seasonal slope:Intron	-0.045	-0.050 – -0.041	**<0.001**
Seasonal slope:Downstream	-0.036	-0.042 – -0.031	**<0.001**
Seasonal slope:Upstream	-0.035	-0.039 – -0.032	**<0.001**
Seasonal slope:UTR_3	-0.045	-0.055 – -0.035	**<0.001**
Seasonal slope:UTR_5	-0.044	-0.057 – -0.031	**<0.001**

Seasonal slope:Splice	-0.048	-0.070 – -0.026	**<0.001**
Seasonal slope:Other	-0.003	-0.016 – 0.009	0.600
Observations	797792		

Table S4. Summary of a regression of z-normalized latitude regression coefficients against z-normalized season regression coefficients for each inversion status. CI stands for 95% confidence interval.

	Latitude slope		
Predictors	*Estimates*	*CI*	*p*
Intercept	0.013	0.011 – 0.015	**<0.001**
Seasonal slope:Outside	-0.039	-0.041 – -0.036	**<0.001**
Seasonal slope:Inside	-0.174	-0.607 – 0.258	0.429
Observations	797792		

Table S5. Summary of a regression of z-normalized latitude regression coefficients against z-normalized season regression coefficients for each SNPs surrounding inversion breakpoints. CI stands for 95% confidence interval.

	Latitude slope		
Predictors	*Estimates*	*CI*	*p*
Intercept	0.013	0.011 – 0.015	**<0.001**
Seasonal slope:In(2L)t	0.004	-0.003 – 0.010	0.248
Seasonal slope:In(2R)NS	-0.019	-0.026 – -0.012	**<0.001**
Seasonal slope:In(3L)P	-0.068	-0.074 – -0.061	**<0.001**
Seasonal slope:In(3R)P	-0.170	-0.177 – -0.163	**<0.001**
Seasonal slope:Outside	-0.030	-0.033 – -0.028	**<0.001**
Observations	797792		