1   **Title**

2   Urbanization drives parallel adaptive clines in plant populations

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12

13   **Abstract**

14   Urban areas are a new and increasingly dominant feature of terrestrial landscapes that

15   dramatically alter environments. It is unclear whether wild populations can adapt to the

16   unique challenges presented by urbanization. To address this problem, we sampled the

17   frequency of a Mendelian-inherited trait—cyanogenesis—in white clover (*Trifolium*

18   *repens* L.) plants along urbanization gradients in four large cities. Cyanogenesis protects

19   plants from herbivores, but also reduces freezing tolerance. Plants evolved reduced

20   cyanogenesis with increasing proximity to the urban center in three of the four cities. In

21   an experiment, we demonstrate that gradients in herbivore pressure do not cause these

22   clines. Instead, urban areas experience relatively cold minimum winter ground

23   temperatures because of reduced snow cover within cities, which selects against

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24   cyanogenesis. Together, our study demonstrates that wild populations exhibit parallel

25   adaptive evolution in response to urbanization, which likely facilitates the persistence of

26   these plants and promotes pollinator abundance and diversity.

27

28   **Main Text**

29   **Introduction**

30   Cities and urban environments are a novel and increasingly dominant feature of terrestrial

31   landscapes where more than half of the world's human population now reside (United

32   Nations 2014). Relative to non-urban areas, urban environments have dramatically

33   different environmental conditions, such as warmer air temperatures (Oke 1973) and

34   altered ecological communities (Raupp et al. 2010). Whereas urbanization can have large

35   ecological effects on plant and animal communities (Hahs et al. 2009), little is known

36   about whether wild populations are adapting to urban environments (Cheptou et al. 2008;

37   Donihue and Lambert 2014; Alberti 2015; Johnson et al. 2015; Munshi-South and Harris

38   2016). To investigate evolutionary processes in urban ecosystems, we used a globally

39   distributed perennial plant, white clover (*Trifolium repens* L.), as a model to examine

40   adaptive evolution along urbanization gradients.

41   White clover exhibits a Mendelian-inherited polymorphism for the production of

42   hydrogen cyanide (HCN), a chemical defense against herbivores that inhibits cellular

43   respiration and is toxic to both plants and animals. Within populations, cyanogenic

44   genotypes produce HCN following tissue damage, whereas HCN is absent from

45   acyanogenic genotypes (Hughes 1991). The cyanogenesis polymorphism is caused by

46   two genes that each encode a chemical component of cyanogenesis: *CYP79D15*

47   (hereafter *Ac*) and *Li*; Plants must have dominant alleles at both loci to produce HCN

48   (Olsen et al. 2014). The two components are stored separately in cells to avoid self-

49   toxicity, and form HCN when they are brought together following tissue damage.

50   Previous research found that cyanogenesis is most frequent in populations at low latitudes

51   and elevations, where herbivory is often highest and temperatures are warmer (Daday

52   1954a). It has been hypothesized that herbivores select for cyanogenesis in these warm

53   environments (Hughes 1991; Kooyers and Olsen 2012). In cold climates, cyanogenesis is

54   selected against because freezing temperatures lyse cells and trigger HCN release,

55   causing self-toxicity in cyanogenic plants (Daday 1958; Hughes 1991).

56   Environmental change associated with urbanization may alter natural selection on

57   cyanogenesis. Whereas herbivores are not consistently affected by urbanization (Raupp et

58   al. 2010), air temperatures are generally warmer in urban areas relative to non-urban

59   areas (Landsberg 1981). This may cause urban populations to experience fewer freezing

60   events than non-urban populations during winter (Parris and Hazell 2005), and

61   consequently weaken selection against cyanogenesis in cities. Thus, we predicted that

62   cyanogenesis would increase in frequency with increasing proximity to urban centers. We

63   surveyed 2379 plants from 121 populations of white clover along three 50-km transects

64   radiating outward from downtown Toronto, Canada (Fig. S1), and screened them for their

65   ability to produce HCN. To confirm that selection is acting on HCN, and not on *Ac* or *Li*

66   individually for unrelated functions, we also quantified the frequencies of the *Ac* and *Li*

67   alleles.

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3

Results and Discussion

70

71   We found consistent changes in the frequency of cyanogenesis along each transect, but

72   clines were in the opposite direction to our prediction—cyanogenesis frequency

73   decreased with increasing proximity to downtown Toronto at a rate of $0.65 \pm 0.19\%$ km^{-1}

74   ($F_{1, 119} = 30.57$, $P < 0.001$) (Fig. 1A; Fig. S2). The frequency of dominant alleles at both

75   *Ac* and *Li* also decreased toward the urban center, which is consistent with selection

76   acting on cyanogenesis rather than *Ac* or *Li* individually (Kakes 1987) (Fig. S3). Local

77   habitat variables like human population density and percent impervious surface cover did

78   not explain significant variation (all $r^2 \leq 0.06$; all $P > 0.6$) in cyanogenesis frequency in

79   multiple regression models that also included distance from the urban center.

80   Collectively, these results suggest that large-scale biological and/or climatic processes

81   associated with urbanization drive adaptive clines in cyanogenesis.

82   Parallel evolution to independent environmental clines provides some of the

83   strongest evidence for evolution by natural selection (Schluter et al. 2004). To test for

84   parallel evolution to urbanization gradients, we sampled white clover populations along

85   one transect in each of Montreal (969 plants from 47 populations), New York City (946

86   plants from 48 populations), and Boston (876 plants from 43 populations) (Fig. S4-6).

87   The frequency of cyanogenesis decreased toward the urban centers of both New York

88   ($0.34 \pm 0.13\%$ km^{-1}; $F_{1, 46} = 6.88$, $P = 0.01$) and Boston ($0.27 \pm 0.14\%$ km^{-1}; $F_{1, 42} = 4.09$,

89    $P = 0.049$), but there was no cline in Montreal ($-0.14 \pm 0.25\%$ km^{-1}; $F_{1, 47} = 0.33$, $P =$

90   0.57) (Fig. 1B–D). Because we did not observe a cline in Montreal, we hypothesized that

91   selective mechanisms causing clines in the other cities are absent there. We conclude that

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92   adaptation in response to urbanization best explains the observed parallel clines in

93   cyanogenesis across multiple cities.

94   Adaptive clines in cyanogenesis can arise because of spatial gradients in either the

95   defensive benefits or the self-toxicity costs of cyanogenesis (Kooyers and Olsen 2012).

96   We tested two non-exclusive hypotheses that could explain our results: (i) cyanogenesis

97   is more beneficial in rural areas because of increased herbivory, and (ii) cyanogenesis is

98   more costly in urban areas because of colder winter ground temperatures. Urban areas

99   typically have warmer air temperatures than non-urban areas (i.e., urban heat islands),

100   and so it is non-intuitive that urban ground temperatures might be cold relative to non-

101   urban areas. However, relative to non-urban areas, cities typically experience less

102   snowfall and more snowmelt because of their warmer temperatures (Landsberg 1981).

103   Snow cover insulates the ground against freezing temperatures (Goodrich 1982), and thus

104   we explored whether a reduction in urban snow cover may cause plants in urban areas to

105   experience colder winter temperatures than plants in rural areas.

106   To test if the defensive benefits of cyanogenesis change along urbanization

107   gradients, we conducted a field experiment examining spatial variation in herbivory. We

108   established 40 experimental white clover populations, each with equal numbers of

109   cyanogenic and acyanogenic genotypes, on lawns located between downtown Toronto

110   and rural areas west of the city (Fig. S7). Cyanogenic plants received less herbivore

111   damage than acyanogenic plants during the early ($F_{1,45} = 6.57$, $P = 0.01$) and late ($F_{1,45} =$

112   7.85, $P < 0.01$) growing season. However, there was no change in overall herbivory ($\beta =$

113    $-0.13 \pm 0.13\%$, $F_{1,36} = 1.04$, $P = 0.31$) or relative herbivory on cyanogenic plants

114   compared to acyanogenic plants ($\beta = -0.001 \pm 0.001\%$, $F_{1,36} = 1.07$; $P = 0.31$) along the

115   urbanization gradient (Fig. 2). Thus, the defensive benefits of cyanogenesis are unlikely

116   to explain the observed clines.

117   To test whether clines in cyanogenesis could be caused by spatial variation in

118   winter freezing temperatures, we placed temperature probes along an urbanization

119   gradient in Toronto during the winter of 2015 (Fig. S8). We found that minimum ground

120   surface temperatures were often coldest in urban areas (Fig. 3A). Using data for regional

121   snow depth during this period, we found that ground temperatures in urban areas were

122   colder than in non-urban areas when there was deep snow in the region, and warmer

123   when there was little or no snow in the region ($F_{1,57} = 32.1$; $r^2 = 0.44$, $P < 0.01$) (Fig. 3B).

124   High regional snow cover likely provides the necessary conditions for clines in snow

125   depth—and thus ground temperature—to arise along urbanization gradients. To test

126   whether the white clover populations we sampled in the field occur along a snow cover

127   gradient, we used remote sensing to quantify the normalized-difference snow index

128   (NDSI) (Shimamura et al. 2006) for our sampled populations. We found that NDSI

129   decreased (i.e., less snow) toward the urban center in each city ($\beta = 3.70 \times 10^{-3} \pm 1.02 \times$

130   10^{-3}, $F_{1, 228} = 30.03$; $P < 0.01$) (Fig. 4A). These observations collectively suggest that

131   clines in winter minimum ground temperatures along urbanization gradients cause clines

132   in cyanogenesis due to higher self-toxicity in urban habitats.

133   We sought to explain why clines in cyanogenesis occur in Toronto, New York

134   City, and Boston, but not Montreal. Because insulation provided by snow cover could

135   weaken selection against cyanogenesis, we predicted that urban plants are most insulated

136   by snow cover in Montreal. We found that mean NDSI was significantly higher in

137   Montreal (i.e., more snow) than all other cities (pairwise contrasts, all $P < 0.02$), which

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138   did not differ from each other in mean NDSI (pairwise contrasts, all $P > 0.5$) (Fig. 4A).

139   Using weather station data from each city, we calculated daily snow depth and minimum

140   temperature between 1980 and 2005. We found that Toronto, Boston, and New York City

141   have more days with < 0 °C temperatures and no snow cover than Montreal (Tukey HSD,

142   all $P < 0.01$) (Fig. 4B). On < 0 °C days, snow is > 5 cm deeper in Montreal than the other

143   three cities (Tukey HSD, all $P < 0.01$) (Fig. 4C). Moreover, cyanogenesis frequency is

144   highest in Montreal (Fig. S9). These results are consistent with greater snow cover in

145   Montreal protecting plants from freezing and relaxing selection against cyanogenesis.

146   Our results suggest that urban-rural gradients in winter ground temperature, which

147   arise due to snow cover gradients, drive parallel adaptive clines in cyanogenesis. These

148   conclusions are consistent with several studies of cyanogenesis in *T. repens* that

149   experimentally demonstrate reduced cyanogenesis as an adaptation for freezing tolerance

150   (Daday 1965; Dirzo and Harper 1982b; Caradus and Eerens 1989; Hughes 1991; Olsen

151   and Ungerer 2008), and hypothesize that high snow cover may prevent the evolution of

152   clines in cyanogenesis (Till-Bottraud and Gouyon 1992). Genetic variation for

153   cyanogenesis is likely maintained by a balance between gene flow and spatial variation in

154   selection (Supplementary Text S2-3).

155   Although adaptation to urban environments can reduce the risk of extinction in

156   cities, it is currently unclear whether wild populations are adapting in cities (Cheptou et

157   al. 2008; Donihue and Lambert 2014; Johnson et al. 2015; Munshi-South and Harris

158   2016). Our study demonstrates that wild plant populations are capable of rapidly adapting

159   to the unique environmental conditions caused by urbanization. White clover is the most

160   abundant source of nectar for pollinators in many temperate ecosystems (Baude et al.

161   2016). Thus, the ability of white clover to rapidly adapt to urban ecosystems may not

162   only contribute to its own dominance in cities, but could also facilitate diverse and

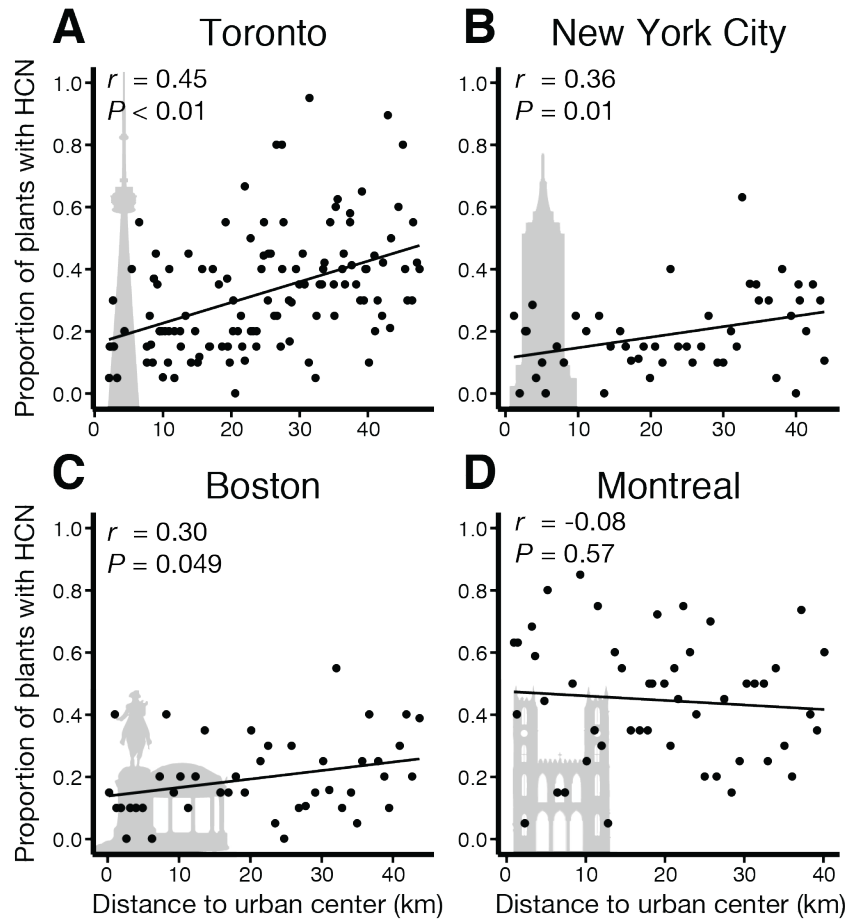163   abundant pollinator communities in urban ecosystems.

164   **Figures**

165

166

167   **Fig. 1.** Urban populations of white clover, *Trifolium repens*, have evolved reduced

168   cyanogenesis relative to non-urban populations in three cities. We observed parallel

169   clines in cyanogenesis in: (**A**) Toronto, (**B**) New York City, and (**C**) Boston, but not in

170   (**D**) Montreal. Each point represents the proportion of plants within a population that

171   tested positive for cyanogenesis (mean *n* per population = 19.7 plants). Statistics were

172   calculated using linear regression.

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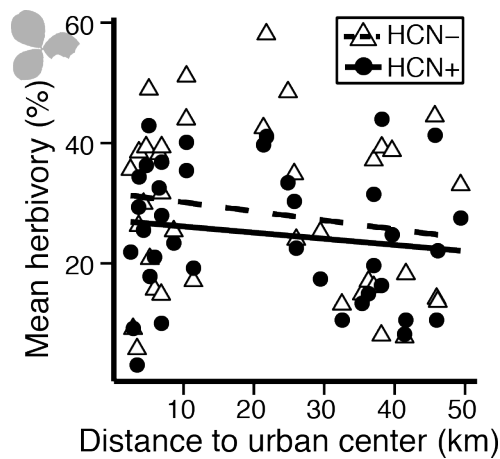
Fig. 2. The benefits of cyanogenesis as a defense against herbivores do not explain the

observed clines. Our field experiment revealed that cyanogenic plants (HCN+) received

less leaf herbivory than acyanogenic plants (HCN−) overall ($P < 0.05$, ANOVA), but

mean herbivory on both cyanotypes within experimental populations (each $n = 38$)

changed little along an urbanization gradient in Toronto (both $P > 0.1$, ANOVA).
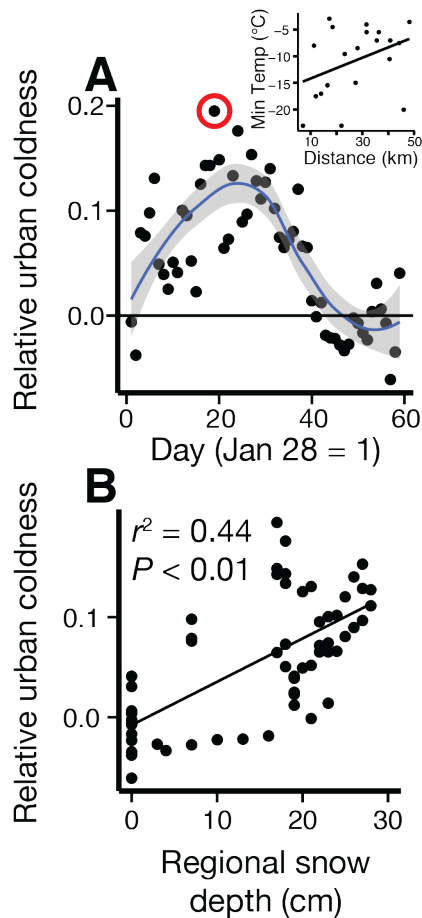
Damage scores are the mean of early and late season herbivory.

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10

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Fig. 3. Urban ground temperatures are often colder than non-urban ground temperatures.

(**A**) Analysis of daily relative urban coldness values reveal that it was often colder in

Toronto during winter 2015 than in non-urban areas (curve is a 95%-CI loess-smoothed

surface). We hypothesized that relatively cold urban ground temperatures are caused by

urban-rural snow depth gradients, and consistent with this expectation we found (**B**) a

significant positive correlation between the daily relative urban coldness index value and
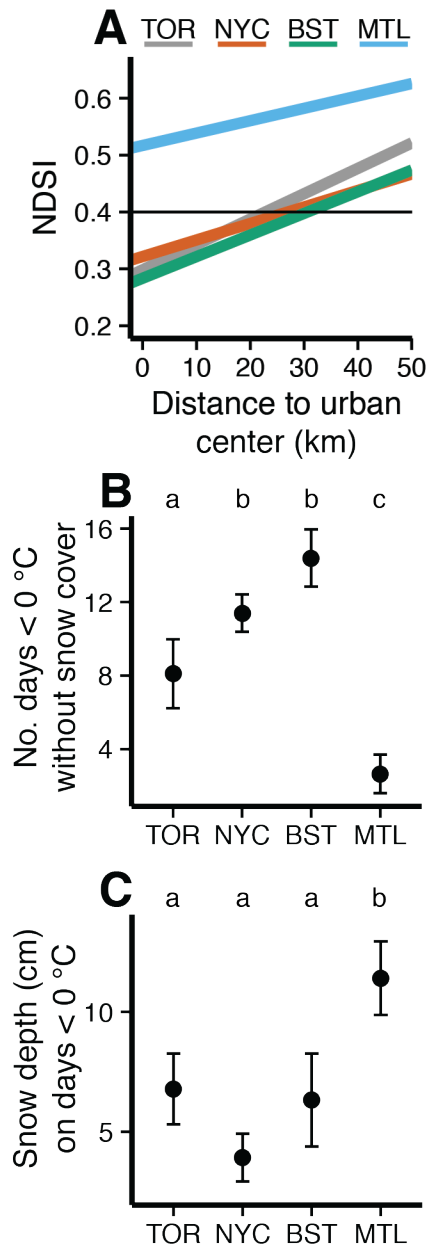
regional snow depth on those days.

190

Fig. 4. Historic differences in January-February snow cover and temperature across the four cities sampled in this study. (**A**) The normalized-difference snow index (NDSI) decreased toward the urban center of each city. The horizontal line at NDSI = 0.4 represents the threshold value for the presence of snow (Shimamura et al. 2006). (**B**) Montreal (MTL) has fewer < 0 °C temperature days without snow cover (± 1 SE), and (**C**) deeper snow cover (± 1 SE) on days where temperatures are < 0 °C, than Toronto

198   (TOR), New York City (NYC), and Boston (BST). Different letters represent significant

199   differences at $P < 0.05$ (Tukey HSD).

200 **Methods**

201 *Surveys of natural populations*

202 Field sampling

203 We sampled 128 populations (2509 plants) of *T. repens* along three transects in the

204 Greater Toronto Area (GTA) (Fig. S1). Transects extended west, north, and east of

205 Toronto. For both the east and west transects, we attempted to maintain a constant

206 distance from Lake Ontario. We stopped approximately every 1 km to sample

207 populations of *T. repens* along each transect. For each population, we recorded GPS

208 coordinates and elevation, and collected stolon cuttings from up to 20 plants; cuttings

209 were approximately 4 cm in length and had at least two leaves. To reduce the likelihood

210 of sampling a single genotype twice, we ensured that there was at least 1.5 m between all

211 collected stolons. We placed all stolons from a population in a sealed bag, and stored

212 them in a cooler with ice. At the lab, we put one leaf from each plant into wells of 96-

213 well plates and stored the rest of the tissue in microcentrifuge tubes. We stored plates and

214 tubes at –80 °C. Seven populations had coarse netting above the soil and were excluded

215 from the analysis because they may have been seeded in recent years as a soil stabilizer;

216 our conclusions do not change when these populations are included in the analysis. To

217 determine if parallel clines in cyanogenesis occur in other cities, we sampled one 50-km

218 transect in each of Montreal, QC, Boston, MA, and New York City, NY, between 26 July

219 and 1 Aug 2015 (Fig. S4-6). Sampling and tissue storage methods were identical to those

220 outlined above.

221

222 Cyanogenesis analysis

223 To determine the frequency of cyanogenesis in natural populations of *T. repens*, we

224 tested each plant for the presence of HCN using Feigl-Anger assays. Feigl-Anger assays

225 determine the presence or absence of HCN in a sample using a simple colour change

226 reaction (Thompson and Johnson 2016). We removed the 96-well plates that contained

227 leaves from the freezer, allowed them to thaw, and added 80 μL of water to each well.

228 We used pipette tips to macerate the tissue in the wells, and secured Feigl-Anger test

229 paper over the plates, taking care not to let any water or tissue contact the paper. We

230 incubated the plates for 3 hr at 37 °C, and then scored each well for cyanide (i.e.,

231 cyanotype *AcLi*), which is indicated by a blue colour. We recorded the proportion of

232 plants that were cyanogenic in each population.

233 To confirm that clines were due to selection on HCN and not *Ac* or *Li*, we

234 screened the frequency of *Ac* and *Li* from acyanogenic plants collected from the three

235 Toronto transects. There was insufficient leaf tissue to screen every plant at both loci, so

236 we screened all acyanogenic plants in a particular population for either *Ac* or *Li*,

237 alternating the locus assayed with every sequential population. Screening was conducted

238 as above but instead of 80 μL of H_2O, we added either 30 μL of 10mM linamarin (Sigma-

239 Aldrich 68264) and 50 μL H_2O to each well to screen for linamarase (cyanotype *acLi*), or

240 80 μL of 0.2 EU/mL linamarase (LGC Standards CDX-00012238-100) with 20 μL of

241 H_2O to each well to screen for linamarin (cyanotype *Acli*). Our earlier study confirmed

242 that Feigl-Anger assays correspond with the presence/absence of the *Ac* and *Li* gene

243 sequences (Thompson and Johnson 2016).

244

15

245   Statistical analysis

246   We used linear regression to determine if the frequency of cyanogenesis was associated

247   with urbanization. We first used the haversine formula—which calculates the distance in

248   kilometers between two sets of co-ordinate—to determine the distance from each

249   sampled population to the urban center (Table S1):

250
$$D = 2R\arctan\left(\frac{\sqrt{hav(\theta)}}{\sqrt{1 - hav(\theta)}}\right),$$

251
$$\text{where } hav(\theta) = \sin^2\left(\frac{\delta_1 - \delta_2}{2}\right) + \cos\delta_1 \cos d_2 \sin^2\left(\frac{\alpha_1 - \alpha_2}{2}\right),$$

252   (latitude, longitude) co-ordinates of the two points are (α_1, δ_1) and (α_2, δ_2) in radians, and

253   R is the radius of earth (6,371 km). Next, we regressed the frequency of cyanogenesis in

254   populations against the distance of those populations from the urban center. All models

255   had the proportion of plants in a population that tested positive for HCN as the response

256   variable and distance to the urban center as a predictor. All statistical analyses were

257   conducted using R 3.0.3 (R Core Team 2014).

258   While distance to an urban center might explain adaptive patterns, it is important

259   to distinguish large-scale factors associated with urbanization from small-scale local

260   factors. To accomplish this, we retrieved 2015 population density estimates from the

261   NASA GPW4 dataset (resolution = 1 km) (CIESIN 2015). We also retrieved data for

262   percent impervious surface cover (resolution = 30 m)

263   (https://coast.noaa.gov/dataregistry/search/collection/info/nlcd-impervious), which has

264   recently been shown to predict evolutionary patterns in urban environments (Munshi-

265   South and Harris 2016). To determine if these variables explained variation in the

16

266   frequency of cyanogenesis, we fit generalized linear mixed effects models as before, but

267   added a term for population density (all cities) or percent surface imperviousness (New

268   York City and Boston only). We found that in all cities, population density and percent

269   surface imperviousness did not significantly explain variation in cyanogenesis when

270   distance to the urban center was included in the model (all $r^2 < 0.06$; $P > 0.6$). On its own,

271   population density was a significant predictor of cyanogenesis frequency in Toronto and

272   New York City, but in both cases its explanatory power (r^2) was less than half of that of

273   distance to the urban center. Percent impervious surface cover did not significantly

274   predict cyanogenesis frequency. Thus, we concluded that large-scale factors associated

275   with urbanization are more important for driving the evolution of cyanogenesis clines

276   than fine-scale local factors.

277

278   *Field Experiment*

279   Experimental design

280   We conducted a field experiment in the Greater Toronto Area (GTA) to test if herbivory

281   is associated with urbanization, and if this could explain clines in cyanogenesis along

282   urbanization gradients. We created forty small populations of *T. repens* on private lawns

283   that spanned from downtown Toronto to suburban and rural areas west of the city (Fig.

284   S7). The strongest individual cline in cyanogenesis was observed along this transect (Fig.

285   S2A). From 4–6 June 2015, we placed 24 *T. repens* plants into solid plastic flats on a 1.5

286   m^2 area of each lawn. We used solid flats to allow rainwater to collect below the plants

287   and to prevent plants from accessing the belowground environment. Trays had spouts 1.5

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288   cm above the base. During dry periods we watered all populations. Two populations were

289   destroyed during the first month of the experiment and excluded from analysis.

290   In the field, each population contained twelve cyanogenic (*AcLi*) and twelve

291   acyanogenic (4 *Acli*, 4 *acLi*, 4 *acli*) plants. Plants were initially propagated from stolons

292   sourced from a group of 48 genotypes with cyanotypes determined using methods

293   outlined above, except plants were screened at least twice for HCN, or *Ac* and *Li* gene

294   products. We propagated stolons that were 5 cm long with 2–5 leaves into 6" pots

295   (AZE06000, ITML, Brantford, ON) filled with pro-mix potting soil (Sunshine Mix #1;

296   Sun Gro Horticulture, Agawam, MA). Plants were saturated with water and kept in a

297   growth chamber set to 15.3 h of 500 μmol light, with a 25:20 °C day:night temperature

298   cycle. Approximately 0.5 g of slow-release fertilizer (Nutricote Total 13-13-13 With

299   Minor Nutrients, Florikan, Sarasota, FL) was added to each pot. We randomly assigned

300   plants to populations such that: (i) each genotype did not occur more than once in a

301   population, (ii) each genotype occurred in 20 of 40 populations, and (iii) each population

302   satisfied the cyanotype ratio described above.

303   We estimated herbivore damage on each plant twice during the experiment, early

304   (July 9–13) and late (August 19–23) in the growing season. To measure herbivory, we

305   selected 5 of the oldest non-senesced leaves from each plant and visually quantified the

306   proportion of the leaf surface area eaten. Previous field experiments with *T. repens*

307   determined that this method is sufficient to demonstrate differences in herbivory between

308   cyanogenic and acyanogenic plants (Thompson and Johnson 2016). During each visit, we

309   collected all ripe inflorescences. Halfway through the experiment, we harvested the

310   aboveground biomass of each plant. This harvesting mimicked the intermittent mowing

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311   that plants typically experience on lawns, and prevented plants from rooting in adjacent

312   pots. We collected all inflorescences that were clipped in this process. We dried

313   harvested biomass for 72 hr at 70 °C and weighed it to the nearest 0.1 g.

314   We harvested all plants in late August. We recorded the total lifetime number of

315   inflorescences that each plant produced, and weighed the floral and vegetative tissue

316   separately. After weighing and counting the inflorescences, we ground them through 1

317   mm and 0.5 mm sieves in series to isolate the seeds, which were weighed to estimate

318   maternal fitness.

319

320   <u>Statistical analysis</u>

321   To assess whether cyanogenesis reduced herbivory, we conducted a linear mixed-effects

322   model with HCN as a fixed effect factor, and population and genotype as discrete random

323   effects. We fit separate models for both the early- and late-season surveys, using the

324   average damage recorded across all measured leaves as a response variable. The full

325   statistical model used in our analyses was:

326

327   Herbivory = intercept + HCN + genotype + population + error

328

329   To ensure that differences in herbivory were due to HCN and not *Ac, Li*, or linked genes,

330   we fit separate models for a dataset that consisted of acyanogenic plants. These models

331   were similar to those described above but instead of HCN, the predictor variable was the

332   presence or absence of functional *Ac* or *Li* alleles.

19

333   To determine if herbivory and selection on cyanogenesis changed across the

334   urbanization gradient, we calculated the mean herbivory in each population, and both

335   relative fitness and relative herbivory for each plant within its own population. We used

336   both lifetime and final vegetative biomass, and lifetime seed biomass as fitness

337   components. To determine if these variables changed along an urbanization gradient, we

338   regressed the population-level estimates of mean herbivory, and relative fitness and

339   herbivory, against the distance of each population from the urban center of Toronto. To

340   determine if herbivory and selection on cyanogenesis changed across the gradient, we

341   took the mean value of relative herbivory, and relative fitness, for all cyanogenic plants in

342   a population and regressed it against distance from the urban center. These values

343   represent the relative fitness (or relative herbivory) of the *AcLi* genotype in a given

344   population. Models all took the same form; as an example, the full statistical model for

345   relative fitness of cyanogenic plants was:

346

347                $w_{i(HCN)}$ = intercept + distance to urban center (km) + error

348

349   *Ground temperature data*

350   During Winter 2015, we recorded ground temperature data across an east–west transect

351   in the GTA (Fig S8). On 27 Jan 2015, we placed 20 iButton DS1921G thermochron

352   devices (Maxim Integrated™ , San Jose, USA), housed inside sandwich bags, at twenty

353   locations approximately 5 km apart. We placed the iButtons underneath trees and bushes

354   so they were not directly exposed to sunlight. We retrieved all iButtons on 28 March

355   2015. We extracted daily values for mean, maximum and minimum temperature, and

356   standard deviation of temperature.

357   We used the temperature data to calculate a daily index of relative urban coldness.

358   This index was calculated as the slope of minimum daily temperature versus the distance

359   from the urban center of Toronto (Fig. S9). Positive index values indicate that minimum

360   temperatures were colder in urban areas relative to non-urban areas, whereas negative

361   values indicate that minimum temperatures were warmer in urban areas relative to non-

362   urban areas. We treated each index value ($n = 58$) as an independent observation and fit a

363   95% CI locally weighted scatterplot smoothing function to visually evaluate if

364   temperatures were significantly colder or warmer in the city during winter (Fig. 3A).

365   Because we observed that urban areas were often colder than non-urban areas, we

366   hypothesized that this effect was due to differences in snow cover between the downtown

367   core and outside non-urban areas. We retrieved snow depth data for the central Toronto

368   weather station (WMOID: 71508; Lat: 43.6667, Long: -79.4000, Elevation: 113 m). This

369   weather station is located approximately 2 km from our urban center reference point. We

370   used a simple linear model with the relative urban coldness index value as the response

371   and snow cover on the same day as the predictor. We found that snow cover was highly

372   correlated with the relative urban coldness (Fig. 3B) and to a lesser extent maximum

373   temperature and the standard deviation of daily temperatures (data not shown). The

374   conclusions do not change when index values from all days are used.

375

376   *Landsat Data*

377   To assess whether our study populations occurred along a gradient of snow cover, we

21

378   used LandSat satellite data collected by the United States Geological Survey (USGS;

379   earthexplorer.usgs.gov). We selected all images that met the following criteria: (i) image

380   captured in January or February, (ii) no cloud cover above study area; (iii) images

381   captured between 1980 and 2015; (iv) snow present along the transect (maximum NDSI

382   ≥ 0.4; see below); (v) < 10 cm snowfall in the previous five days to allow any effects of

383   urbanization on snow melt and/or removal to accumulate. We focused our analyses on

384   January and February because January isotherms have previously been implicated as

385   major factors driving the evolution of clines in *T. repens* cyanogenesis (Daday 1954b;

386   Kooyers and Olsen 2012), and there were insufficient images from January alone for

387   robust analyses. We note that the general pattern of decreasing NDSI toward urban

388   centers is statistically significant when data from January alone are analyzed ($P < 0.01$).

389   For images that met these criteria (list will be archived online), we extracted the

390   normalized-difference snow index (NDSI) for each of our study populations using the

391   point sampling tool in QGIS 2.12. NDSI is a quantitative measurement of snow cover

392   derived using the unique reflective and absorptive properties of snow in the green and

393   short wave infrared (SWIR) wavelengths. It is calculated as (Green – SWIR) / (Green +

394   SWIR). These wavelengths correspond to bands 2 and 5 for the Landsat 4, 5 and 7

395   satellites, and bands 3 and 6 for the Landsat 8 satellite. We maintained the standard 30 m

396   cell resolution throughout our analysis because any resampling would bias urban areas

397   toward lower NDSI due to a high density of artificial and paved surfaces, and bias rural

398   areas toward higher NDSI for the opposite reason.   Although Landsat data is at a

399   resolution of 30 m × 30 m, we often sampled populations that were within 30 m of roads

400   and were much smaller than the Landsat 30 m cells—especially in urban areas. Thus,

22

401   using the GPS co-ordinates of populations would often cause us to include roads,

402   sidewalks, or buildings in our analysis, which could bias our data toward detecting low

403   NDSI values in cities. Because of this issue, we manually moved all of our points to the

404   nearest large park or field before extracting NDSI data. Parks were larger than the NDSI

405   grid cell size. A statistically significant, though weaker, associations remain between

406   NDSI and urbanization remains when original locations are used.

407   We used linear mixed-effects models to calculate the change in snow cover vs.

408   distance of populations to the urban center. We fit the following model to the data:

409

410   NDSI = intercept + distance + city + distance × city + month/year + population + image +

411   error

412   where distance (from the urban center) is a continuous independent variable, city is a

413   categorical fixed effect, month and year as nested random effects, and population

414   (sampling location) and image both as categorical random effects. We analyzed the

415   model using ANOVA with type-III sums of squares and the Kenward-Roger denominator

416   degrees of freedom approximation to test the significance of fixed effects. We fit separate

417   models for each city to extract the slope of NDSI vs. distance, and the y-intercept at

418   distance = 0 km. These lines are plotted in Fig. 4A of the main text. To determine if mean

419   NDSI was significantly different across cities, we fit the same model four times, but with

420   the City 'reference' level changed across models. That is, one model compared Montreal

421   to other cities, the next compared Toronto to other cities, *etc*. Because we had a small

422   number of Landsat images in Toronto ($n = 16$), New York ($n = 19$) and Boston ($n = 26$),

423   we did not correct for multiple testing when comparing mean NDSI across cities. When

23

424   we conduct a Tukey HSD test, mean NDSI in Montreal is significantly different from the

425   other three cities at $P < 0.06$, while mean NDSI does not differ between the other three

426   cities ($P > 0.9$).

427

428   *Weather Station Data*

429   To compare snow cover and snowfall across cities, we used data from weather stations.

430   All data were retrieved through the National Climatic Data Center, which is operated by

431   the US National Oceanic and Atmospheric Administration (NOAA;

432   www.ncdc.noaa.gov/cdo-web/datatools/normals). We retrieved data for daily snow depth,

433   snowfall, maximum temperature, and minimum temperature for the period of 1980 –

434   2010. We retrieved data from one airport weather station per city: Pierre Trudeau

435   International Airport in Montreal, Lester B. Pearson International airport in Toronto,

436   Logan International Airport in Boston, and Laguardia Airport in New York City. We

437   excluded dates where these data were not available for all four cities and those that were

438   flagged for quality. We treated all 'trace' values as zeroes.

439   After data were filtered, we had data for 3545 days in each city, which

440   represented 20 years (1980–1992, 1994, 1996, 1999–2002, 2004). To test the hypothesis

441   that plants in the four cities would differ in their exposure to snow cover, we calculated

442   the cumulative number of days in January and February where temperatures were $< 0\ °C$

443   and there was no snow cover. This metric is strongly correlated with mean cyanogenesis

444   frequency across the four cities ($t_2 = 6.74$, $r = 0.97$, $P = 0.02$). We recorded this value for

445   each year. Because the depth of snow influences its insulation properties (Goodrich

446   1982), we also compared the depth of snow on days where temperatures were $< 0\ °C$.

24

447   Lastly, we compared snowfall between the cities, and found that Montreal has higher

448   snowfall than the other three cities (Tukey HSD, all $P < 0.01$), which do not differ from

449   one another (Tukey HSD, all $P > 0.2$). All models were simple mixed effects models that

450   had the following form:

451

452   *Snow variable = intercept + city + year + error*

453

454   where city was a categorical fixed effect and year was a random effect.

455

456   **<u>References</u>**

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635

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644

645   **Author contributions**

646   K.A.T. and M.T.J.J. designed the study. All authors conducted fieldwork. K.A.T.

647   conducted lab work and remote sensing analysis. K.A.T. analyzed the data with input

648   from M.T.J.J. K.A.T. wrote the first draft of the manuscript, and K.A.T. and M.T.J.J.

649   jointly revised the manuscript.

650   **Supplementary Figures**

651

652
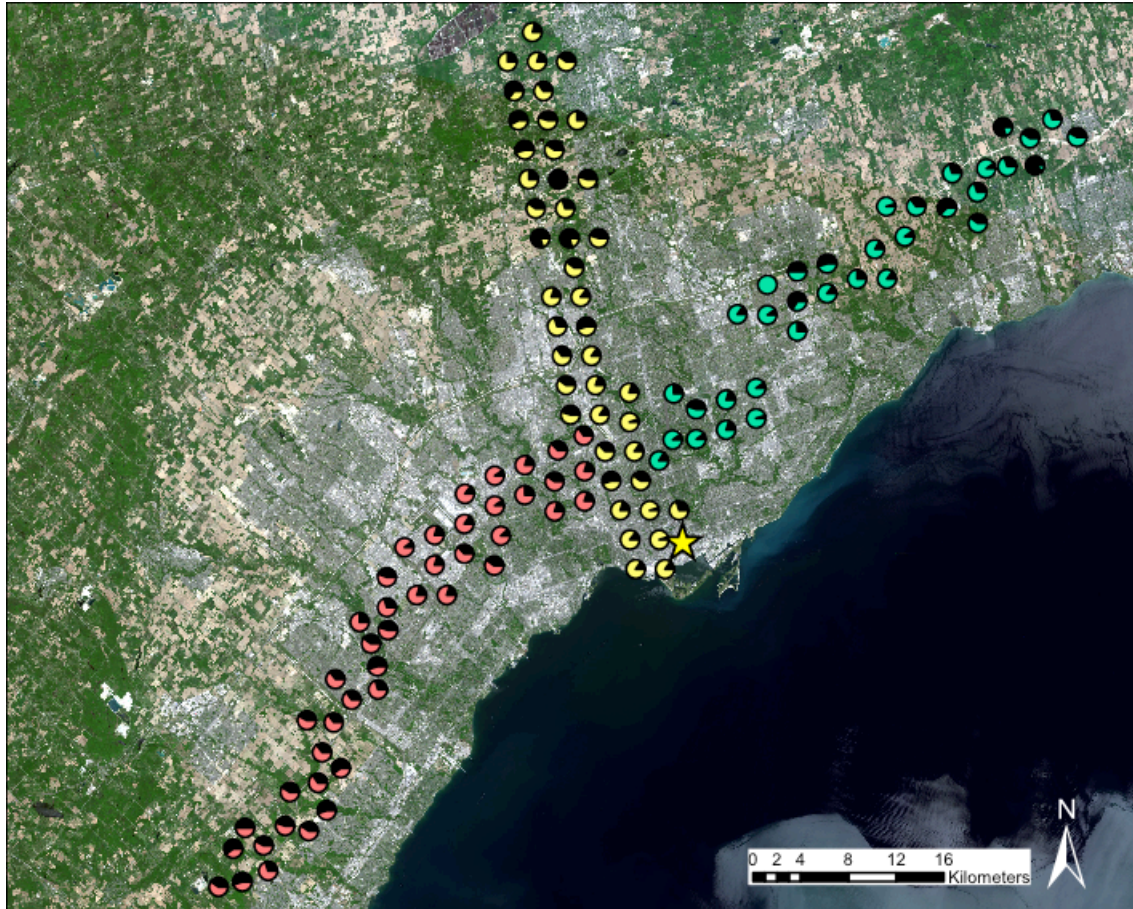
653   **Fig. S1.** Map showing the location and cyanogenesis frequency of populations sampled in

654   Toronto along transects that extended west (pink and black circles, $n = 44$), north (yellow

655   and black circles, $n = 43$) and east (blue and black circles, $n = 34$) of the city center (total

656     $n = 121$). The proportion of black colouration in pie charts reflects the frequency of

657   cyanogenesis in that population. Pie chart locations are jittered to avoid overlap. We

658   defined the urban center of Toronto as Yonge-Dundas Square (star symbol). The image is

659   from the Landsat 8 satellite and was captured on 31 May 2014.
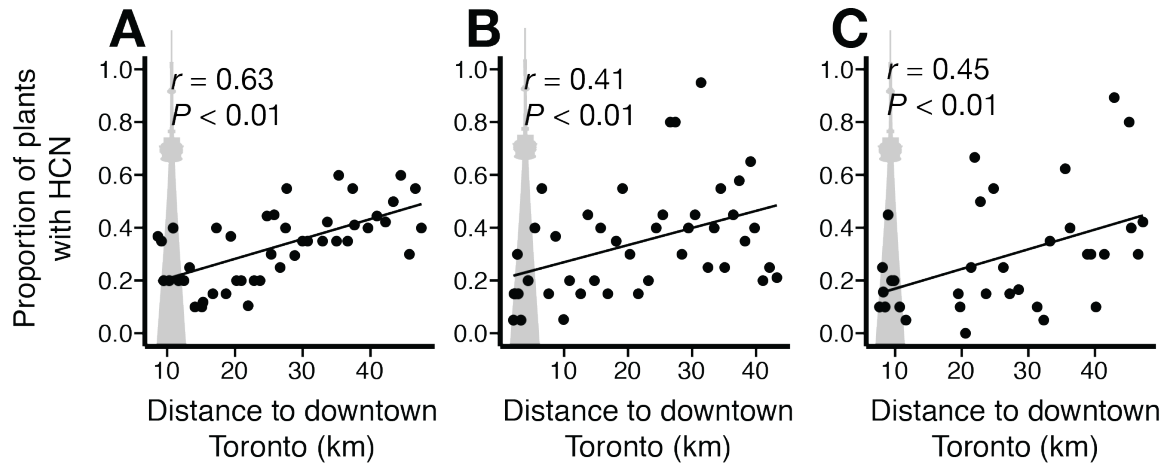
Fig. S2. Regression of HCN frequency in natural populations of *Trifolium repens* along rural-urban transects extending: (**A**) west (*n* plants = 856, *n* populations = 44), (**B**) north (*n* plants = 843, *n* populations = 43), and (**C**) east (*n* plants = 680; *n* populations = 4 of downtown Toronto, Canada. We observed that the frequency of cyanogenesis decreased toward the urban center along each transect. Statistics were calculated using linear regression.
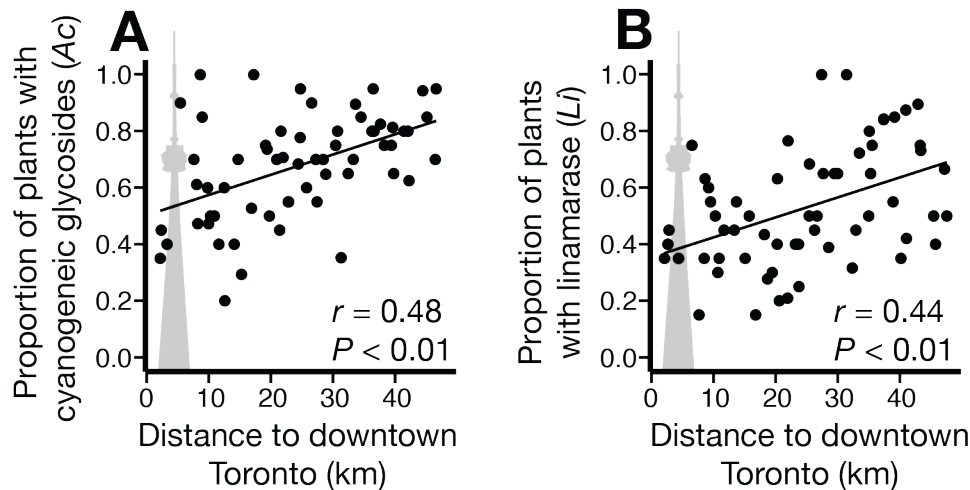
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670 **Fig. S3.** The frequency of functional alleles at: (**A**) *CYP79D15* (*Ac*) (0.71 ± 0.17% km^{-1};

671 $\chi^2_1 = 9.45$, $P < 0.01$) and (**B**) *Li* (0.71 ± 0.19% km^{-1}; $\chi^2_1 = 12.00$, $P < 0.01$) increase

672 toward the urban center of Toronto. The dominant allele *Ac* encodes the primary step in

673 cyanogenic glycoside biosynthesis, and the dominant allele at *Li* encodes the enzyme,

674 linamarase. Data are pooled across all three transects. These results are consistent with

675 the hypothesis that the epistatic interaction of the two loci (i.e., cyanogenesis) is

676 responsible for urban-rural cyanogenesis clines, rather than either gene individually or

677 linked genes. Statistics were calculated using linear regression.

678

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680
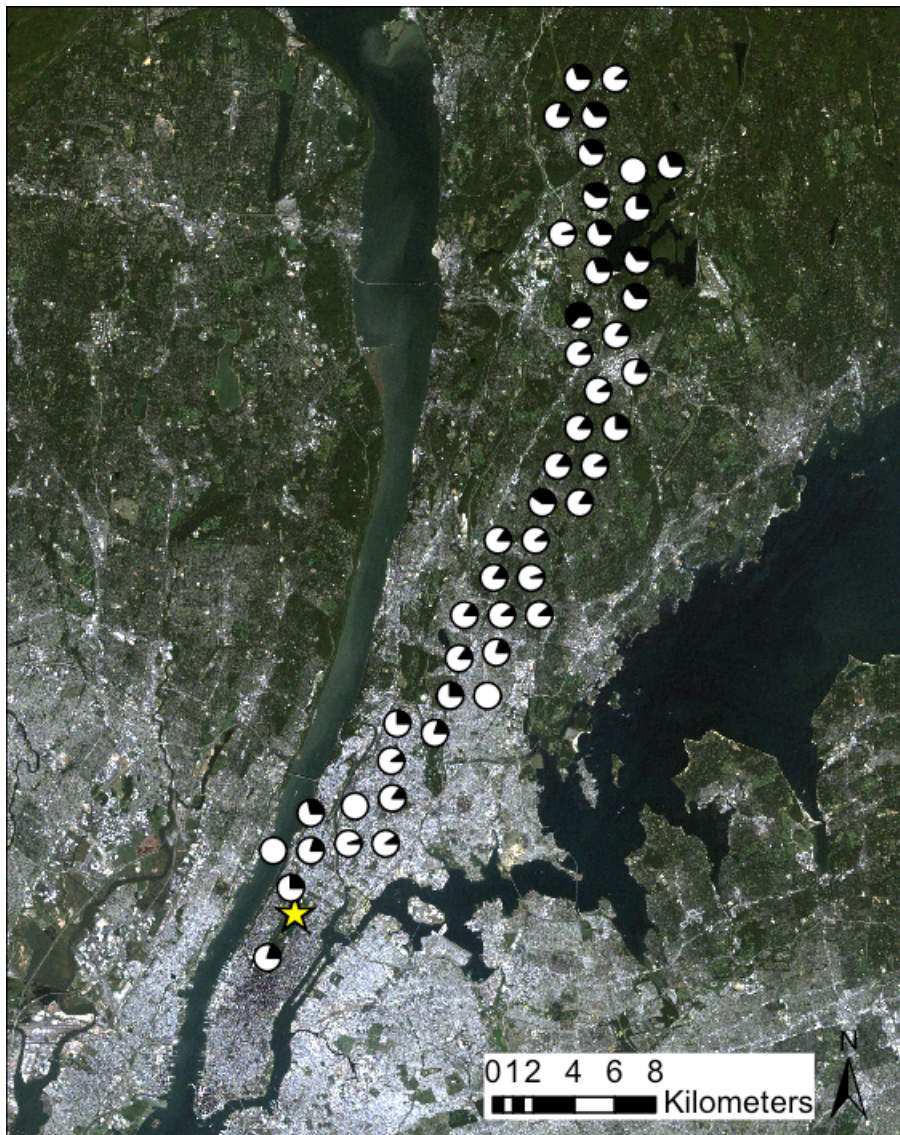
Fig. S4. Map depicting the location and HCN frequency in populations sampled in New York City, USA ($n = 48$). The proportion of black colouration in pie charts reflects the frequency of HCN in that population. Pie chart locations were jittered to avoid overlap. We defined the urban center of New York as the northern border of Central Park (star symbol). The image is from the Landsat 5 satellite and was captured on 22 September 2010.
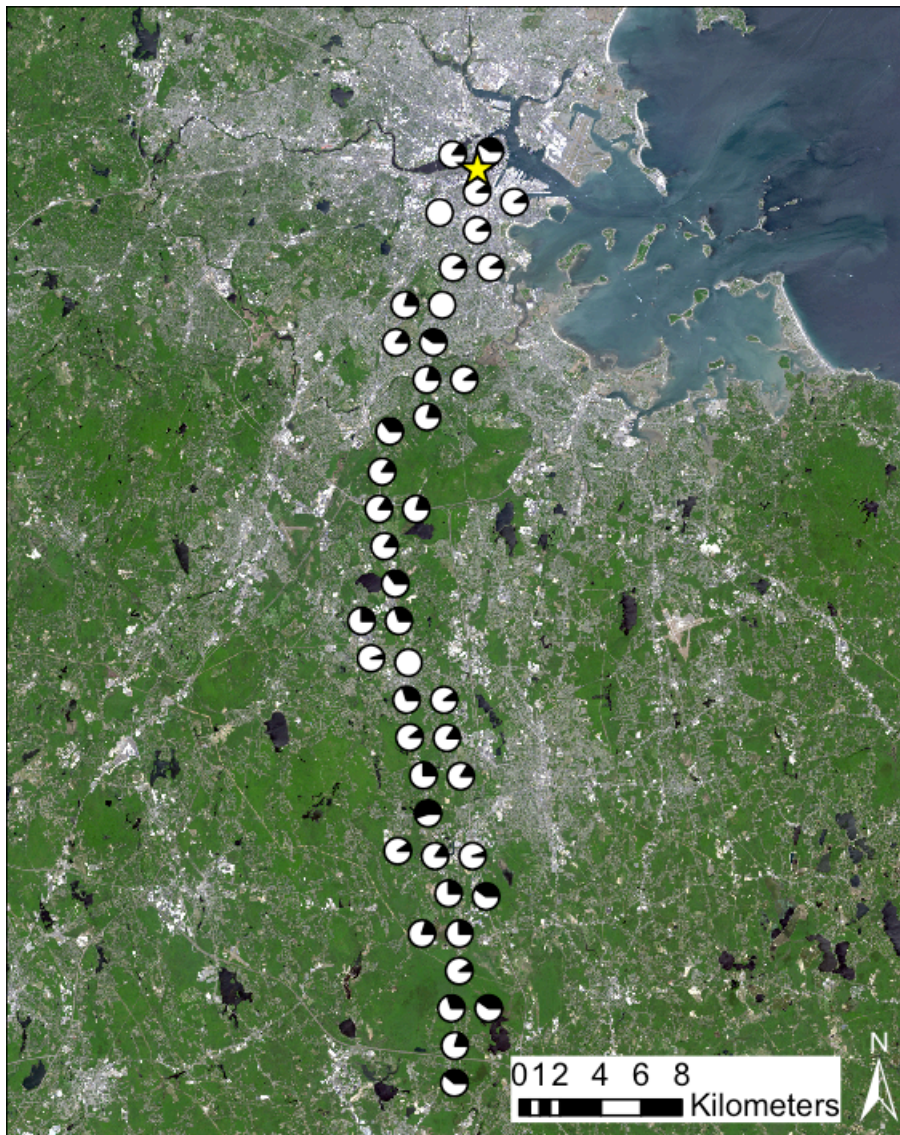
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Fig. S5. Map depicting the location and HCN frequency in populations sampled in

Boston, USA ($n = 43$). The proportion of black colouration in pie charts reflects the

frequency of HCN in that population. Pie chart locations were jittered to avoid overlap.

We defined the urban center of Boston as Boston Common, indicated by the star symbol.

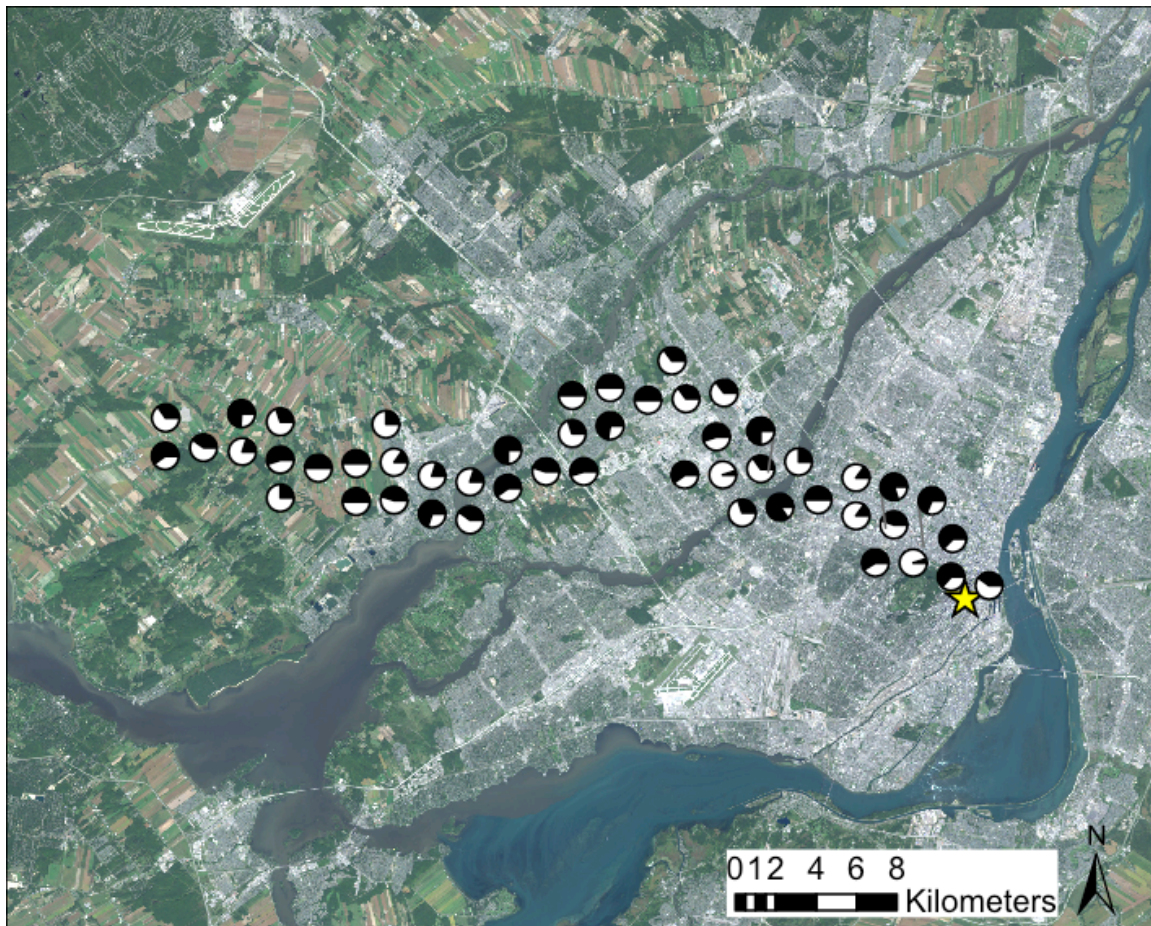The image is from the Landsat 8 satellite and was captured on 24 May 2015.

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696 **Fig. S6.** Map depicting the location and HCN frequency in populations sampled in

697 Montreal, Canada ($n = 49$). The proportion of black colouration in pie charts reflects the

698 frequency of HCN in that population. Pie chart locations were jittered to avoid overlap.

699 We defined the urban center of Montreal as the intersection of Boulevard Robert-

700 Bourassa and Boulevard René-Lévesque Ouest, indicated by the star symbol. The image

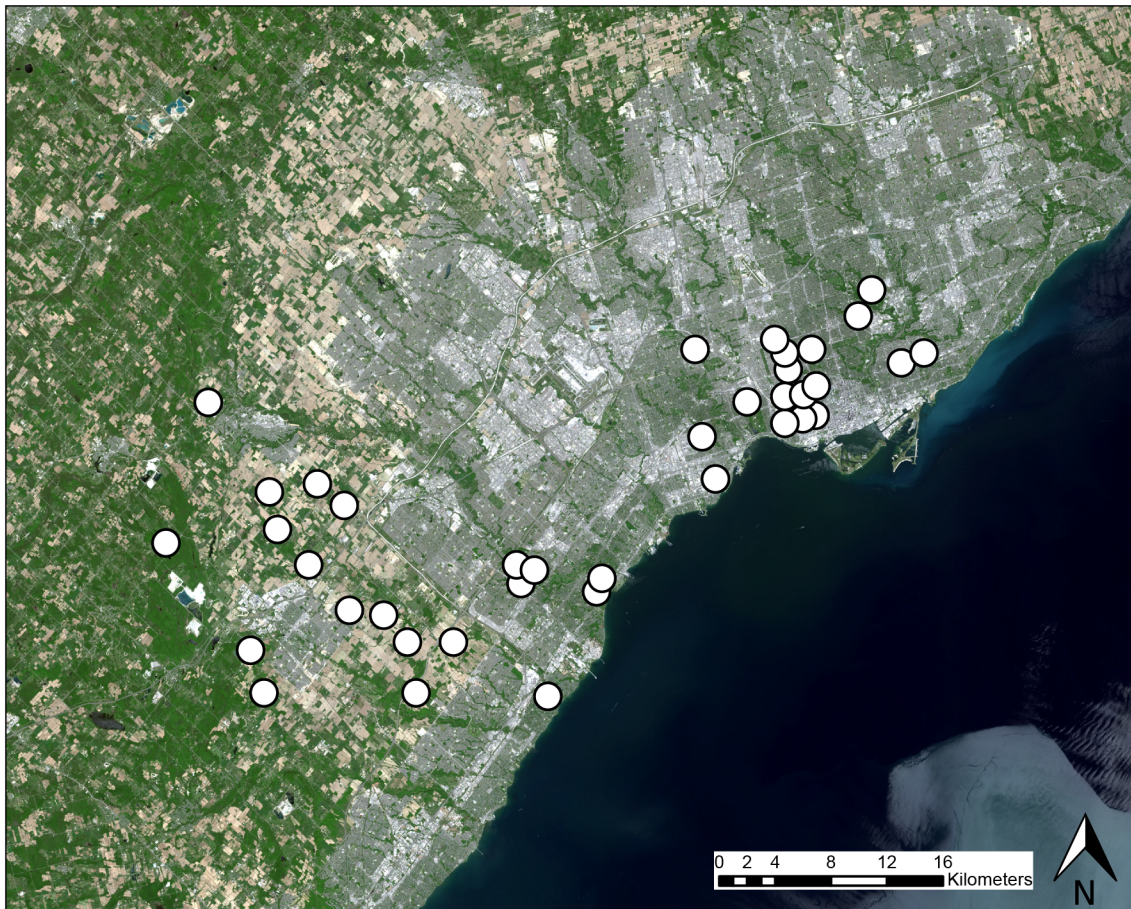701 is from the Landsat 8 satellite and was captured on 18 September 2015.

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Fig. S7. Map showing the location of experimental clover populations in our field

experiment in the Greater Toronto Area during summer 2015 ($n = 38$). The image is from

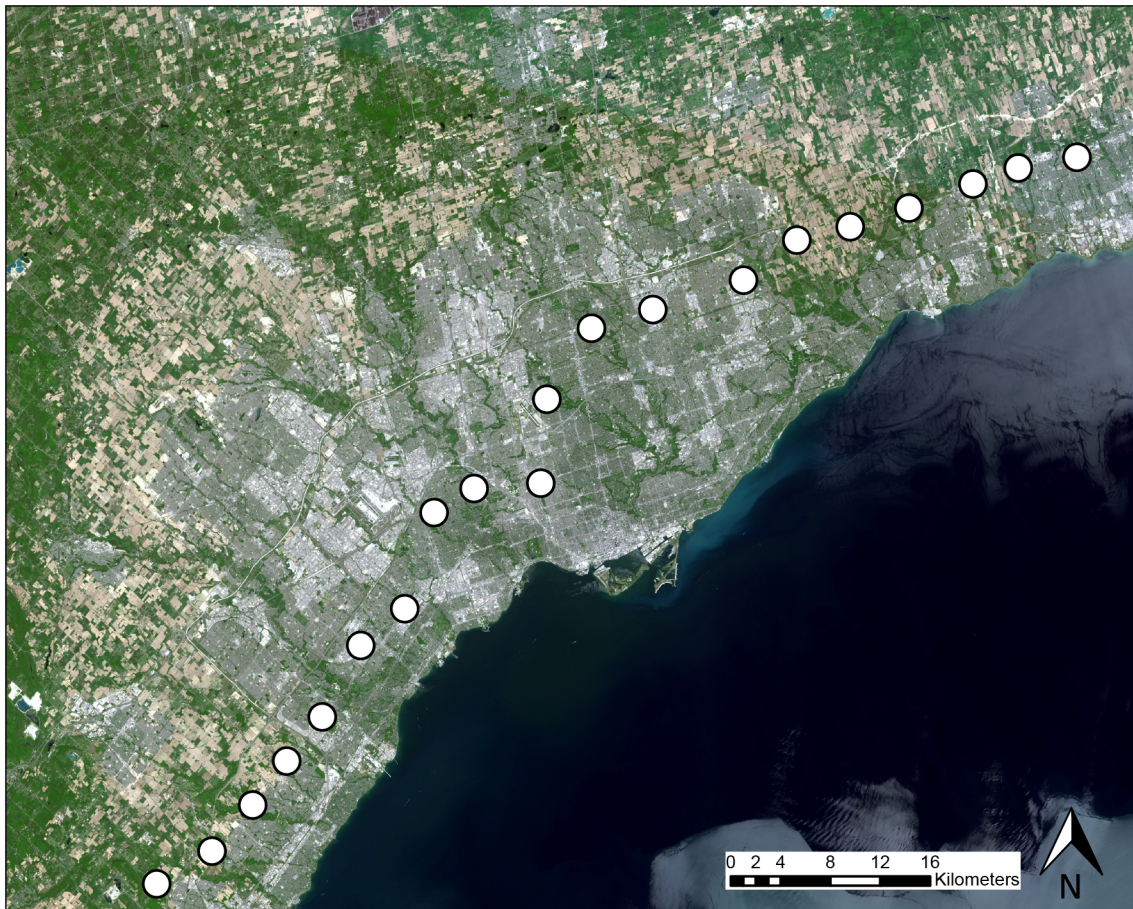the Landsat 8 satellite and was captured on 31 May 2014.

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710   **Fig. S8.** Map showing the locations of iButton temperature probes that were dispatched

711   across the Greater Toronto Area during winter 2015 (n = 20). iButtons recorded ground

712   temperature measurements every hour from 28 Jan to 27 Mar 2015. The image is from

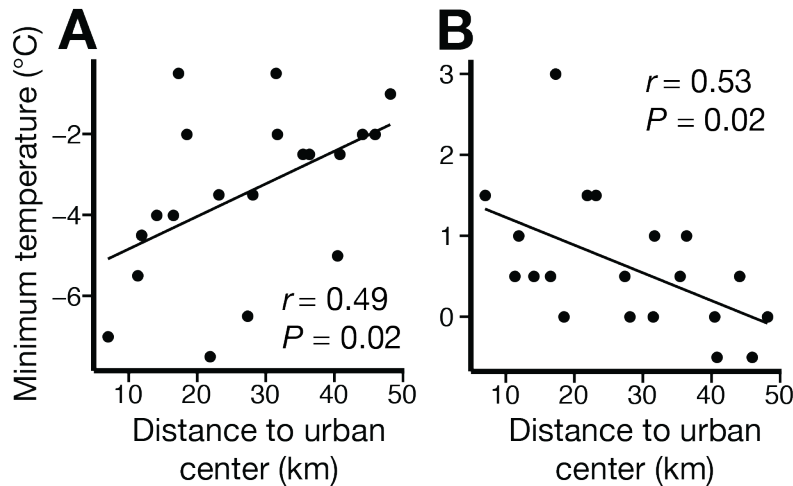713   the Landsat 8 satellite and was captured on 31 May 2014.

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717 **Fig. S9.** Representative images of: (**A**) positive and (**B**) negative relations between

718 minimum temperature and distance from the urban center. The 'relative urban coldness'

719 index values for the left and right panels are 0.081 and –0.034, respectively, which was

720 equivalent to the slope. When the index is positive, temperatures are colder in the city

721 than outside of the city (the winter urban cold island effect) and when the index is

722 negative, temperatures are warmer inside the city than outside of the city (the urban heat

723 island effect). Minimum temperature values were taken from iButton temperature probe

724 data. The data in panel (**A**) were captured on 4 March 2015, and the data in panel (**B**)

725 were captured on 26 March 2015. Statistics were calculated using linear regression.
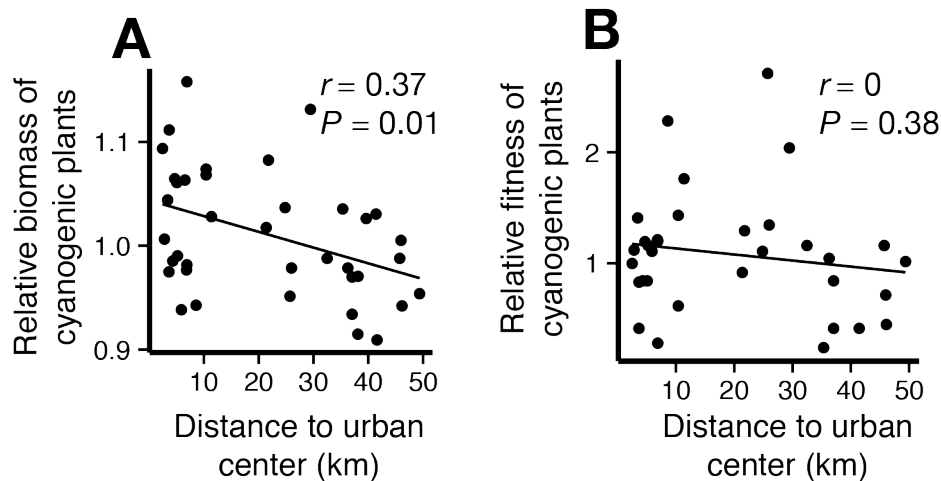
726

727

728   **Fig. S10.** Selection on cyanogenesis during the field experiment in summer 2015. (**A**)

729   Relative to cyanogenic plants, cyanogenic plants had higher final biomass in urban areas

730   and lower final biomass in non-urban areas. We interpreted this result as evidence of

731   natural selection for cyanogenesis in the urban populations. This result contrasts the

732   results of our natural population surveys. Whereas we observed selection operating

733   through final biomass, (**B**) there was no selection on cyanogenesis via maternal fitness

734   (seed mass) across the urbanization gradient. Statistics were calculated using linear

735   regression.

43

736   **Supplementary information**

737   Supplementary text S1: *Study System*

738   *Trifolium repens* L. (Fabaceae), white clover, is a globally distributed perennial plant

739   common to lawns and disturbed areas. Native to Eurasia, white clover has spread globally

740   because of human introduction as a nitrogen-fixing pasture crop and soil stabilizer (Baker

741   and Williams 1987). Self-fertilization is rare in *T. repens* due to a gametophytic self-

742   incompatibility system (Douglas and Connolly 1989). While individual plants produce

743   many seeds, seed recruitment in wild populations is rare (Barrett and Silander 1992).

744   Clover is highly clonal, and produces trailing stolons that root at the nodes (Kemball and

745   Marshall 1995).

746   In wild populations of white clover, some plants produce hydrogen cyanide

747   (HCN)—a general toxin that inhibits enzymes containing heavy metals, including

748   cytochromes in the electron transport chain (Nahrstedt 1985; Leavesley et al. 2008). The

749   cyanogenesis polymorphism in *T. repens* has been studied for over a century (Mirande

750   1912; Armstrong et al. 1913) and is regulated by alleles at two independently segregating

751   diallelic loci—*CYP79D15* and *Li*. *CYP79D15* (hereafter *Ac*) regulates the initial step of

752   cyanogenic glycoside biosynthesis, and *Li* encodes the enzyme linamarase (Olsen et al.

753   2007, 2008). Cyanogenic glycosides (linamarin and lotaustralin) are stored in vacuoles

754   (Poulton 1990), and linamarase is stored in the cell wall (Kakes 1985). Following tissue

755   disruption, these two components are brought into contact to release free HCN. A single

756   copy of the dominant allele at both loci (*Ac* and *Li*) is required for a plant to produce

757   HCN (cyanotype *AcLi*). Plants without one or both dominant alleles are acyanogenic

758   (cyanotypes *Acli*, *acLi*, and *acli*). Dominant alleles confer the presence of the

44

759 corresponding nucleotide sequence, and recessive alleles confer the compete absence of

760 the genes due to complete deletions at *Ac* and *Li* (Olsen et al. 2013).

761   Early work examining the distribution of cyanogenesis in wild populations of *T.*

762 *repens* found that cyanogenesis decreases in frequency toward high latitudes (Daday

763 1954b) and elevations (Daday 1954c). Many authors have since reported similar patterns

764 (de Araújo 1976; Brighton and Horne 1977; Till 1987; Till-Bottraud et al. 1988; Ganders

765 1990; Caradus and Forde 1996; Pederson et al. 1996; Richards and Fletcher 2002;

766 Majumdar et al. 2004; Kooyers and Olsen 2013; Oliveira et al. 2013; Shrestha Malla

767 2014), and several have explored their mechanistic basis. In warm climates, cyanogenic

768 plants are hypothesized to outperform acyanogenic plants because of increased herbivore

769 pressure and aridity. In addition to the present study, several studies have shown that

770 cyanogenic plants are less damaged by herbivores than acyanogenic plants (Angseesing

771 1974,Dirzo and Harper 1982a; Horrill and Richards 1986; Kakes 1989; Pederson and

772 Brink 1998; Saucy et al. 1999; Thompson and Johnson 2016), but the evidence for

773 differential tolerance to aridity is mixed (Foulds and Grime 1972; Kooyers et al. 2014).

774   Cold winter climates are hypothesized to select against cyanogenesis because

775 cyanogenic plants experience self-toxicity when free HCN is released following freezing-

776 induced tissue damage (Stout et al. 1980, 1986; Gleadow and Møller 2014). Several

777 studies have shown that cyanogenic *T. repens* plants are more damaged by freezing

778 temperatures than acyanogenic plants (Daday 1958,Dirzo and Harper 1982b; Caradus and

779 Christie 1998; Olsen and Ungerer 2008), and artificial selection for frost hardiness in *T.*

780 *repens* causes selection against cyanogenesis (Caradus and Eerens 1989). Perhaps to

781 avoid frost damage, *T. repens* decreases the production of cyanogenesis components

45

782   when temperatures drop below 8°C (Collinge and Hughes 1982). Physiological studies in

783   *Lotus corniculatus*, another legume with a cyanogenesis polymorphism, demonstrated

784   that cyanogenesis irreversibly inhibits leaf respiration following exposure to freezing

785   temperatures (Brighton and Horne 1977). Additional studies have found that freezing

786   causes HCN release in live tissue, and that highly cyanogenic plants across multiple

787   species have reduced freezing tolerance (Lebeau 1966; Stout et al. 1981; Kuti and

788   Konoru 2006).

789

790   *Supplementary text S2: Opposing natural selection on cyanogenesis across seasons*

791   We observed significant natural selection favouring cyanogenesis in cities during the

792   summer ($\beta = -1.5 \times 10^{-3} \pm 5.6 \times 10^{-4}$, $F_{1, 36} = 7.24$, $P = 0.01$) (Fig. S10A). Relative to

793   acyanogenic plants, the final biomass of cyanogenic plants was larger in urban

794   populations and smaller in non-urban populations. This pattern only held for *AcLi*

795   genotypes; relative fitness did not change across an urbanization gradient for *Acli* ($\beta =$

796    $1.0 \times 10^{-4} \pm 1.4 \times 10^{-2}$, $F_{1, 36} = 0.005$, $P = 0.94$) or *acLi* ($\beta = 6.1 \times 10^{-4} \pm 1.3 \times 10^{-3}$, $F_{1, 36} =$

797   0.22, $P = 0.64$) genotypes, indicating that the cyanogenic phenotype *per se* is responsible

798   for the pattern. This observation is consistent with our original prediction that

799   cyanogenesis would be favoured in cities. Recent experimental studies have linked

800   cyanogenesis with tolerance to dry soils and hypothesize that water stress may be

801   partially responsible for the evolution of clines in cyanogenesis (Kooyers et al. 2014).

802   While we did not quantify soil moisture during our experiment, urban populations may

803   have experienced increased water stress because of warmer temperatures and increased

804   impervious surface cover (Whitlow et al. 1992). One possible problem with this

46

805 hypothesis is that previous studies have linked dominant alleles at *CYP79D15* to water

806 stress tolerance (Kooyers et al. 2014), but we did not find a fitness advantage of *Acli*

807 genotypes. We note that selection was only acting through final biomass, and not lifetime

808 biomass or sexual fitness (Fig. S10).

809

810 *Supplementary text S3: Alternative explanations for cyanogenesis clines along*

811 *urbanization gradients*

812 S3.1—*Mowing and fertilization experiment*

813 S3.1-1—*Experimental design*

814 Urbanization can be associated with clines in human activities such as lawnmower use

815 and fertilizer application, both of which have the potential to differentially affect

816 cyanogenic and acyanogenic plants. We tested whether a differential response to mowing

817 and fertilizer could explain the observed urban-rural cyanogenesis clines using a growth

818 chamber experiment. We took four stolon cuttings from each of 89 plants for a total of

819 356 plants. Cyanotypes of these 89 plants were: 35 *AcLi*, 20 *Acli*, 14 *acLi*, and 20 *acli*.

820 Cyanotypes were determined as in the field experiment described above. All plants were

821 grown from seed collected from different genotypes in the experiment described by

822 Thompson and Johnson (2016). All cuttings weighed between 0.5 g and 0.8 g. We

823 replaced cuttings that did not survive transplanting within the first four days of the

824 experiment. Two plants died after this period and thus our final analysis consisted of 354

825 plants. Cuttings were propagated into 500 mL pots (STD0400, ITML, Brantford, ON)

826 filled with soil (Sunshine Mix #2, Sun Gro Horticulture, Agawam, USA). Pots were

827 randomized into a growth chamber with day:night temperatures of 25:22 °C, with 16

47

828   hours of 500 μmol/m^2/s light. All pots were contained in small plastic cups to prevent

829   mixing of water between pots, which were held in solid plastic trays.

830   We applied damage and fertilization treatments in a 2 × 2 fully factorial design.

831   The damage treatment consisted of undamaged control plants and plants that were

832   damaged in a manner that simulated lawnmower damage on lawns. We housed plants

833   beneath a wooden plant with a hole in the center that was level with the upper rim of the

834   pots, and removed all tissue that was greater than 1.5 cm above the upper rim of pots.

835   Damage treatments were applied three times, on the 20th, 30th, and 40th day of the

836   experiment. Our fertilizer treatment included control plants (no fertilizer) and fertilized

837   plants, which received 1.5 g of slow-release fertilizer at the beginning of the experiment

838   (Nutricote Total 13-13-13 With Minor Nutrients, Florikan, Sarasota, FL). The experiment

839   was terminated on day 58, at which time we harvested the aboveground biomass, dried

840   the tissue for 72 hr at 60 °C and then weighed the tissue to the nearest 0.1 g.

841   The ability for plants to regrow following damage represents an estimate of

842   tolerance. Because of issues with interpreting tolerance estimates when multiplicative and

843   additive data are used (Wise and Carr 2008), we conducted our analysis using log-

844   transformed data because the amount of tissue removed was related to the size of plants.

845   Our conclusions about whether HCN influences tolerance do not change if untransformed

846   data are used.

847

848   S3.1-2—*Statistical analysis*

849   Because damage and fertilization treatments influenced plant biomass to very different

850   degrees, we analyzed the main effects of each in a mixed-effects one-way ANOVA with

48

851   one treatment as a fixed categorical variable, and genotype and the other treatment as

852   random effects. We used a mixed-effects three-way ANOVA to analyze whether

853   cyanogenesis influenced the response to damage or fertilization. Our full statistical model

854   was:

855

856   *W = intercept + damage + fertilization + HCN + damage × fertilization + damage ×*

857   *HCN + fertilization × HCN + damage × fertilization × HCN + genotype + error*

858

859   where *W* is the log-transformed fitness component (final vegetative biomass or lifetime

860   floral biomass), damage and fertilization were factors representing experimental

861   treatments, HCN is the presence (cyanogenic) or absence (acyanogenic) of HCN, and

862   genotype was a categorical random effect.

863

864   S3.1-3—*Results*

865   Mowing and fertilization both had significant effects on fitness components. In univariate

866   mixed-effects models, damaged plants had approximately 20% lower final vegetative

867   biomass ($F_{1,264} = 64$, $P < 0.01$) and produced 20% less lifetime sexual biomass than

868   undamaged plants ($F_{1,264} = 36$, $P < 0.01$). Similarly, unfertilized plants had approximately

869   52% lower final vegetative biomass ($F_{1,264} = 692$, $P < 0.01$) and produced 72% less

870   lifetime sexual biomass ($F_{1,264} = 995$, $P < 0.01$) than fertilized plants. There was no

871   difference in vegetative biomass between cyanogenic and acyanogenic plants ($P > 0.5$),

872   but acyanogenic plants produced 18% more sexual biomass than cyanogenic plants ($F_{1,87}$

873   $= 5.06$, $P = 0.03$).

49

874   Cyanogenesis and fertilization did not influence the ability of plants to tolerate

875   simulated mowing treatments (all interactions: $P > 0.20$), and cyanogenesis did not

876   influence plant response to fertilization ($P = 0.73$). That is, cyanogenesis did not

877   influence plant fitness in response to any treatment or treatment combination. We

878   conclude that differential tolerance to mowing and/or fertilization does not cause clines in

879   cyanogenesis along urbanization gradients.

880

881   S3.2.2—*Salinity and clines in cyanogenesis*

882   Recent studies have shown that soil salinity can upregulate HCN production in *T. repens*

883   (Ballhorn and Elias 2014). Several additional studies have shown that soil salinity is

884   higher in urban areas relative to non-urban areas (Cunningham et al. 2008). Given that

885   highly cyanogenic plants are the most susceptible to freezing damage (Caradus et al.

886   1989), urban plants may be more highly damaged from freezing than non-urban plants

887   because of higher HCN concentration in their tissue. This would be exaggerated under a

888   winter urban cold island scenario. The link between soil salinity, cyanogenesis, and

889   freezing tolerance in *T. repens* requires further investigation.

890

891   S3.2.3—*Aridity and cyanogenesis clines*

892   Clines in cyanogenesis are often associated with aridity, with higher frequencies of

893   cyanogenic plants in arid habitats (Kooyers and Olsen 2013). Arid soils are often

894   associated with urban environments (Whitlow et al. 1992), and so if aridity was

895   responsible for cyanogenesis clines then we expect cyanogenesis to be most frequent in

896   cities. While our field experiment is consistent with natural selection favouring

897   cyanogenesis in the summer in urban habitats (Fig. S10A), if aridity were the primary

898   driver of cyanogenesis cline evolution, the clines would likely be in the opposite direction

899   than we observed (i.e., higher frequency of cyanogenesis in urban environments). Given

900   that this is not the case we conclude that aridity is unlikely to explain the observed clines.

901

902   <u>S4. Additional acknowledgements</u>

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