The effects of altitude, aspect, grazing and time on the proportion of cyanogenics in neighbouring populations of *Trifolium repens* L. (white clover)

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The proportion of cyanogenic individuals of white clover amongst 200 individuals in each of 32 neighbouring populations in Northumberland, UK were recorded. Sites differed for four altitude zones, two aspects, and were either sheep pasture or ungrazed meadow. The proportion of cyanogenics showed a striking reduction with increasing altitude. Below 100 m, north-facing sites contained more cyanogenics than south-facing sites, but aspect did not affect higher sites. Grassland management had no effect on the proportion of cyanogenics. A 24-year study of one population showed highly significant changes in the proportion of cyanogenics over time, suggesting that a significant turnover of individuals occurs. In some cases, different size class cohorts varied for the proportion of cyanogenics within a year, and the same cohort varied between years. We con-

clude that environment at birth may determine the proportion of cyanogenics for that cohort, so that this proportion persists in that cohort as it matures. Comparisons of the proportion of cyanogenics with mean monthly averages for January minimum temperature, July maximum temperature and August rainfall showed a significant association only with mean January minima $2\frac{1}{2}$ years previously. We hypothesise that the 2 to 3-year cohort may predominate in this population. We suggest that winter cold and summer drought may both select against cyanogenics. Grazing by large herbivores does not favour cyanogenesis, but some invertebrate herbivory may do so. Most selection probably occurs at birth and will be greatest in populations with a high turnover.
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Introduction

The generation of cyanide by damaged leaves of the white clover (*Trifolium repens* L.) is caused by the action of the β-glucosidase linamarase on the related cyanoglucosides linamarin and lotaustralin, controlled respectively by the dominant unlinked alleles *Li* and *Ac* (Corkill, 1942). Almost all wild populations studied were polymorphic for both these genes so that they vary for cyanogenic and acyanogenic phenotypes (Daday, 1954a,b; 1958). Numerous ecological surveys of the distribution of the frequency of cyanogenic clover have been published. At least five of these studies demonstrated an inverse relationship between the frequency of cyanogenics and altitude (Daday, 1954b; De Araujo, 1976; Boersma *et al*, 1983; Till, 1987; Till-Bottraud *et al*, 1988). Daday (1954a,b, 1958, 1965) noted a strong relationship between the frequency of cyanogenics and the mean January isotherm both altitudinally and latitudinally, and suggested that low winter temperatures selected against cyanogenics in this evergreen species.

Numerous other studies have demonstrated that certain invertebrate herbivores preferentially eat acyanogenic clover (summarised in Hughes, 1991). Corkill

(1952) concluded that cows and sheep do not discriminate between cyanogenics and acyanogenics, but that rabbits may do so. Remarkably, this seems to have been the only study of vertebrate grazing apart from a passing comment by Jones (1962) that the vole *Microtus agrestis* selectively eats acyanogenic *Lotus corniculatus*. Adult clover recovers well after grazing, so that selection for cyanogenics by herbivores might be mostly restricted to the seedling phase (Ennos, 1981b, 1982), and so will be greatest at sites where population cycling is common (Horrill and Richards, 1986).

Only one study has investigated the effect of ecological factors independent of those associated with winter temperature and grazing on the distribution of cyanogenic clover. In Derbyshire, sites liable to moisture stress contained fewer individuals with the glucoside gene *Ac* than did comparable moister sites (Foulds and Grime, 1972a). Experimental studies showed that *Ac-* genotypes survived drought stress less well than *acac* plants, flowered less well in these conditions, and were less vegetatively vigorous than *ac* phenotypes in moist sites (Foulds and Grime, 1972b). Consequently, dry sites carried fewer cyanogenic individuals, although no such differential selection was observed between *Li-* and *lili* genotypes. However, Ennos (1981a) observed frequency dependent competitive interactions between *Li-* and *lili* genotypes, and suggested that these might maintain the polymorphism for the *Li* locus. He noted that *Li-* individuals tended to produce larger leaves than *lili* genotypes in one

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wild population, and Ennos (1981b) showed better wild survival of *Li*- seedling transplants than of *lili* transplants. He interpreted the latter result as possibly demonstrating superior survival of cyanogenic seedlings against herbivore attack (Ennos, 1982). He seems not to have considered the possibility of heterozygous advantage at the *Li/li* locus as a hypothesis which would explain all three of his observations.

A comparative study was undertaken of the proportion of cyanogenics in 32 populations within a small area. This pilot study was part of an investigation into selection for sequence-based variation at the *Li* locus. We wished to identify geographically related areas which had selected for high and low levels of cyanogenics. This seems to have been the first time the effects of altitude, aspect and grazing on the proportion of cyanogenics have been compared in a carefully designed survey. We have also included data from a long-term study.

Materials and methods

Sites between Hexham and Corbridge in the central Tyne Valley, Northumberland, UK, were surveyed in July and August 2000, utilising both sides of the valley. The area covered totalled 50 sq km, ranging from (UK grid references) NY92.62. and NY96.57. to NY95.68. and NY98.66. Sites were chosen within each of four altitudinal zones, 20–35 m, 60–90 m, 140–160 m and 200–240 m above sea level. Within each altitudinal zone, aspects were selected as north-facing (bearing 340°–20°) or south-facing (bearing 160°–200°) measured by compass, with average inclines of 10°–25° (judged by eye). The average altitude of north facing slopes was 117.2 m, and of south facing slopes 118.1 m. Within each altitude and aspect, sites were selected as grazed by sheep or ungrazed. Grazed short grassland was permanent pasture managed for sheep throughout the year and had stock present. Ungrazed sites were managed for hay or sileage and in most cases were about to be mown or had been mown recently at the time of sampling. Pseudoreplicated site 'pairs' within 1 km of each other (but different fields) were selected for each altitude/aspect/management type ('treatment') so that 32 sites were surveyed in total.

For each site, 200 plants were sampled by selecting single leaves by eye each more than 1 m distant from the next (6400 plants for all sites). Each leaf was subjected to a modified Guignard picrate test (Corkhill, 1942) which was scored the following day. No ambiguity in scoring was encountered. Leaves testing positive for cyanide production invariably produced a strong orange-red test result. As the sample size did not vary, we were able to use the number of cyanogenic individuals when comparing populations statistically.

We also report findings from a long-term study of the frequency of cyanogenesis in a single population in the Tyne Valley at Town Moor, Newcastle (NZ245655), about 30 km east of the other sites. This is an old level pasture which has been grazed by cattle for several hundred years, 80 m above sea-level. Over 24 years more than 100 plants have been sampled each May, using the method described above. Using Meteorological Office data from Durham, at a similar altitude and distance from the sea and 20 km to the south, we studied associations of the frequency of cyanogenesis with mean January minimum, mean July maximum and mean August rainfall for the year of survey, and also for 1, 2 and 3 years before the year of survey. During March 2000 and April 2001, 300 and 368 individuals respectively were identified within this population by means of distinctive leaf marking characteristics, and the maximum linear dimension of each individual measured. On the basis of non-random size distributions (Figure 4), size cohorts were designated at 0–110 cm, 111–160 cm, 161–250 cm and >251 cm. The frequency of cyanogenic individuals in each cohort was established in each year.

Results

The percentage of cyanogenics in summer 2000 averaged 23.4% across all populations, ranging from 7% to 66% (Figure 1). Overall, the data set was highly heterogenous ($\chi^2_{31} = 436$, $P < 0.0001$). The number of cyanogenics in populations varied significantly with respect to altitude and aspect, but not for grazing. There were no significant interactions between any factors (Table 1).

We observed a marked decrease in the number of cyanogenics with altitude (Table 2). The average percentage for the lowest sites was 31.4%, but that for the highest sites only 10.4%. There was no overlap in the percentage of cyanogenics between any site below 50 m above sea level (minimum 17.5%) and any site above 200 m (maximum 17.0%). Across all sites, the number of cyanogenics was negatively associated with altitude ($r = -0.690$, $P > 0.001$) (Figure 1).

Overall, the number of cyanogenics also differed markedly between aspects (Table 1). The average percentage of cyanogenics on north-facing slopes was 27.7% and on south-facing slopes 18.5%. When aspects were compared for sites below 100 m above sea level the number of cyanogenics was non-overlapping (Figure 2).

There was no significant difference at any altitude or aspect for the number of cyanogenics in ungrazed and grazed sites (both 23.4%).

In the long-term study at Town Moor, Newcastle, the proportion of cyanogenics varied over time ($\chi^2_{21} = 108.2$, $P > 0.0001$) (Table 3). The only significant association found between the proportion of cyanogenics at this locality and monthly climatic averages at Durham was for mean January minimum $2\frac{1}{2}$ years before time of sampling ($r = 0.430$, $P = 0.03$) (Figure 3).
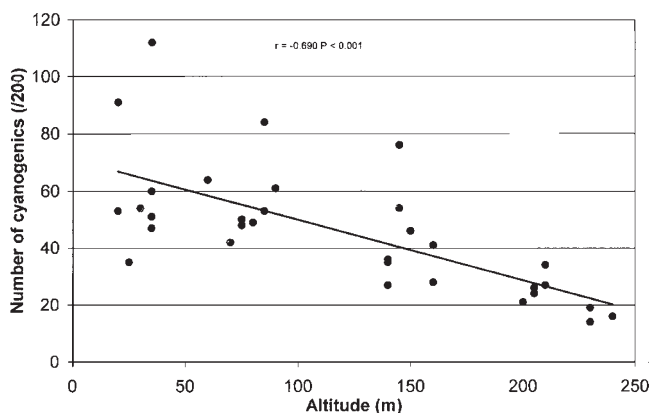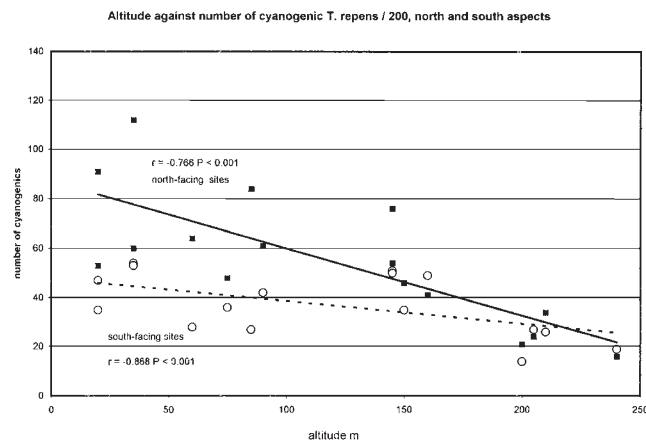

Figure 1 Association between altitude (m) and the number of cyanogenic *Trifolium repens* in samples of 200 in the Tyne Valley, August 2000.

Table 1 Analysis of variance of the effects of altitude, aspect and grazing on the number of cyanogenic white clover in 32 Tyne Valley populations of 200 individuals

Source	Degress of freedom	Sequential sum of squares	Adjacent sum of squares	Adjacent means of squares	F	P
Altitude (alt)	3	8315	8315	2771	14.04	<0.001
Aspect (asp)	3	2397	2397	2397	12.14	0.003
Grazing (gra)	1	7	7	7	0.04	0.853
Alt × asp	3	1218	1218	406	2.06	0.147
Alt × asp	1	555	555	185	0.94	0.445
Asp × gra	1	148	148	148	0.75	0.398
Alt × asp × gra	3	314	314	104	0.53	0.668
Error	16	3159	3159	197	–	–
Total	31	16116	–	–	–	–

Table 2 Average proportion of cyanogenics in populations of white clover in each of four altitude bands in the Tyne Valley

Altitude range (m)	n	Mean proportion cyanogenics (%)	Standard error (%)
20–35	8	31.44	4.46
60–90	8	28.19	2.34
140–160	8	21.44	2.80
200–230	8	10.38	0.95

Figure 2 Association between altitude (m) and the number of cyanogenic *Trifolium repens* in samples of 200, separating samples from north and south aspects, Tyne Valley, August 2000.

Maximum clone dimension at Town Moor appeared to show an uneven distribution with apparent discontinuities at about 110 cm, 160 cm, and 250 cm (Figure 4). In 2001, more cyanogenics occurred in the 111–160 cm cohort than in the 161–250 cm cohort. There were more cyanogenics in the 111–160 cm cohort in 2001 than in 2000, but the proportion of cyanogenics in this cohort in 2000 did not differ from that in the 161–250 cm cohort in 2001 (Table 4).

Discussion

This is the first attempt to partition the effects of several ecological variables on the frequency of cyanogenesis in white clover. The frequency of cyanogenics varied strik-ingly with altitude. This finding agrees with all previous published studies. Daday (1965) suggested that this inverse association results from the relatively poor survival of cyanogenics after low winter temperatures. We found that the annual fluctuation in the proportion of cyanogenics on Town Moor, Newcastle, varied with the minimum temperature $2\frac{1}{2}$ years previously which provides some independent support for this hypothesis. After cold winters, there was a tendency for the proportion of cyanogenics to drop 2 years later, although this association was not highly significant.

If this association is valid we must assume: (i) that most selection for the proportion of cyanogenics surviving in a cohort takes place in the winter following birth; (ii) that $2\frac{1}{2}$ years after birth the plants in this cohort were sufficiently well represented to influence strongly the number of cyanogenics in the population; (iii) that ramets born in subsequent years were still too small to strongly influence the sample; and (iv) ramets whose birth took place 4 years or more before they were sampled were too mature to have been unduly influenced by the winter in question, or were rare in the population.

Despite our evidence for striking changes in the proportion of cyanogenics in this population between some years, we were still unsure whether sufficient turnover of individuals occurred in this population to account for the association of the proportion of cyanogenics with previous winter weather (assumption (ii)). The vegetative demography of this species has been very thoroughly studied. Hamilton and Harper (1989) conclude that 'seedlings of *Trifolium repens* are rarely observed in most temperate pastures…, although in some pastures…it persists as an annual or short-lived perennial'. Turkington *et al* (1979) quantify average regeneration in North Wales at 0.5 seedlings m^2/y and in New Zealand Chapman (1987) obtained values of about 0.2 seedlings m^2/y. Maze and Turkington (1996) show that genet diversity in a pasture declines with age. It is clear that rapid genet turnover normally occurs but that it mostly occurs as a result of ramet birth and death (Hamilton and Harper, 1989). We do not know if the relative rates of establishment of new cyanogenic and acyanogenic ramets of vegetative origin, rather than seedlings, can be differentially affected by invertebrate predation or prolonged frost.

Some support for the hypothesis that the climate two winters previous influences the proportion of cyanogenics came from our discovery that size cohorts within this population sometimes differed significantly for the

Table 3 Variation in the proportion of cyanogenics on Town Moor, Newcastle, in May, and meteorological readings at Durham on that and previous years, over 24 years

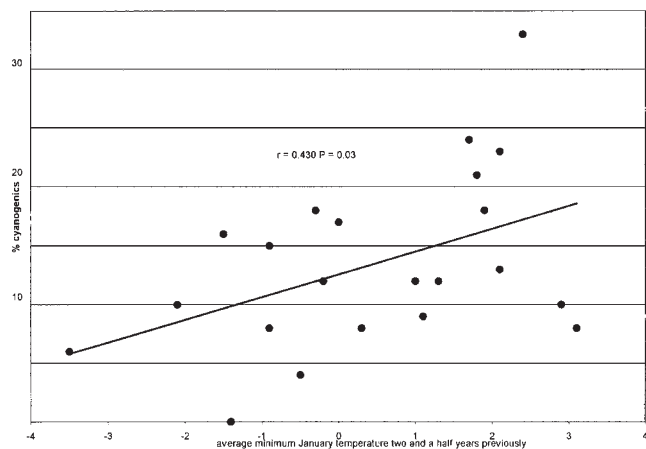| Year	Cyano-genics	Acyano-genics	% cyanogenics	Mean Jan. minimum °C	Mean July maximum °C	Mean August rainfall mm previous year	Mean Jan. minimum °C previous year	Mean Jan. minimum °C 2 years previous	Mean Jan. minimum °C 3 years previous
1976	24	76	24	2.1	22.1	61.5	2.4	1.8	1.7
1978	21	79	21	−0.9	17.8	30.0	2.1	2.4	1.8
1979	33	67	33	−3.5	19.5	92.8	−0.9	2.1	2.4
1982	23	77	23	−2.1	19.2	67.3	−3.5	−0.9	2.1
1983	15	85	15	2.9	22.8	59.5	−2.1	−3.5	−0.9
1984	6	94	6	−0.5	21.2	41.9	2.9	−2.1	−3.5
1985	10	90	10	−1.4	19	55.7	−0.5	2.9	−2.1
1986	10	90	10	−0.3	19.2	81.5	−1.4	−0.5	2.9
1987	4	96	4	−1.5	19.0	169.0	−0.3	−1.4	−0.5
1988	0	100	0	−1.3	17.9	53.7	−1.5	−0.3	−1.4
1989	18	82	18	3.1	22.5	53.7	1.3	−1.5	−0.3
1990	16	84	16	2.1	20.6	61.5	3.1	1.3	−1.5
1991	18	132	12	−0.9	21.0	52.8	2.1	3.1	1.3
1992	12	138	8	0	19.6	21.2	−0.9	2.1	3.1
1993	20	130	13	1.1	18.6	68.8	0	−0.9	2.1
1994	12	138	8	1.0	21.3	89.4	1.1	0	−0.9
1995	25	125	17	0.3	21.6	49.1	1.0	1.1	0
1996	13	117	9	1.9	20.1	14.2	0.3	1.0	1.1
1997	18	136	12	−0.2	20.8	71.8	1.9	0.3	1.0
1998	11	136	8	1.4	18.5	48.1	−0.2	1.9	0.3
1999	29	134	18	1.6	20.8	44.9	1.4	−0.2	1.9
2000	36	264	12	–	–	81.3	1.6	1.4	−0.2

Figure 3 Association between percentage of the population of *Trifolium repens* on Town Moor, Newcastle in May which is cyanogenic and the average minimum January temperature 2½ years previously.

Figure 4 Numbers of *Trifolium repens* on Town Moor, Newcastle, in different size classes, March 2000. Discontinuities in size distribution were perceived at about 110 cm, 160 cm and 250 cm, so that four size cohorts were identified.

proportion of cyanogenics, and that the proportion of cyanogenics for the same size cohort sometimes differed between years. This suggested that a cohort with a distinctive proportion of cyanogenics had increased in diameter between years.

If selection for the proportion of cyanogenics occurs mostly at genet or ramet birth as suggested by Ennos (1981b, 1982) and Horrill and Richards (1986), this finding suggests that the environment at birth may determine the proportion of cyanogenics for that cohort so that this proportion persists in that cohort as it matures. If this interpretation is correct, to account for the relationship between January minima 2½ years previously, we must suppose that the 2 to 3-year-old cohort predominates in
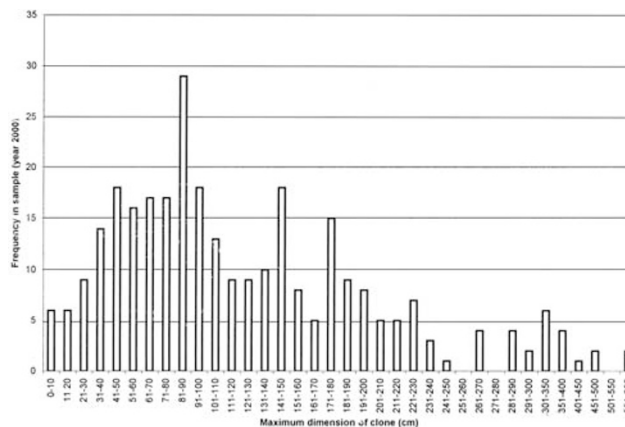
the population. However, we have no information which links size cohorts to age cohorts in the field.

In the summer 2000 survey, we also found that the proportion of cyanogenics varied with aspect. Compared to the relationship with altitude, this finding seemed at first to be counter-intuitive. North-facing sites which are presumed to have been cooler and wetter carried a higher proportion of cyanogenics. However, the effect of aspect on the frequency of cyanogenics was mostly expressed at low altitude sites which are presumed to have been relatively warm in winter. Consequently, we suspect that this relationship reflects the likelihood of stress resulting from water-deficit in summer, which would be greatest for south-facing sites at low altitudes, rather than winter cold. This would correspond with the finding of Foulds

Table 4 Numbers of cyanogenic and acyanogenic individuals of white clover present in four size cohorts in two successive years. χ^2 columns tests the hypothesis that pairs of cohorts of adjacent size do not differ within a year, χ^2 rows tests the hypothesis that the same size cohort does not differ between years, and χ^2 successive cohorts tests the assumption that a size cohort in 2000 does not differ from the next largest cohort in 2001. χ^2_3 tests the assumption that all four cohorts do not differ for the proportion of cyanogenics within a year

Maximum dimension of individual (cm)	March 2000			April 2001			χ^2_1 rows	χ^2_1 successive cohorts
	Cyanogenic	Acyanogenic	χ^2_1 columns	Cyanogenic	Acyanogenic	χ^2_1 columns		
<110 cm	22	141		17	62	–	2.53 NS	–
111–160 cm	3	49	2.18 NS	34	119	0.01 NS	7.1 $P < 0.008$	4.12 $P < 0.05$
161–250 cm	6	54	0.63 NS	8	71	5.14 $P < 0.02$	0 NS	0.77 NS
>251 cm	5	20	1.57 NS	11	46	2.32 NS	0.005 NS	2.03 NS
		$\chi^2_3 = 4.0$ NS			$\chi^2_3 = 5.4$ NS			

NS = not significant.

and Grime (1972a) that dry sites contain fewer individuals with *Ac* than expected. However, this suggestion lacks experimental verification.

We found no indication that a history of grazing by large animals influences the proportion of cyanogenics in a population. This accords with the suggestion of Corkill (1952) that large quadrupeds do not select between cyanogenic and acyanogenic clover. In a future study it would be interesting to compare the effect of rabbit grazing on the proportion of cyanogenics.

Our evidence suggests that a considerable variation in the frequency of cyanogenics in white clover can occur between neighbouring populations and between years within the same population. It seems that low winter temperatures and water-stress may both select against cyanogenics, probably at ramet or genet birth. On the basis of earlier studies, we suppose that differential grazing by some invertebrates on seedlings selects for cyanogenics. It is also possible that heterozygous advantage is expressed at the *Li* locus, and we are currently investigating this hypothesis.

Acknowledgements

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