

THE BIOLOGY OF THE LEAF MARK POLYMORPHISM IN *TRIFOLIUM REPENS* L.

2. EVIDENCE FOR THE SELECTION OF LEAF MARKS BY RUMEN FISTULATED SHEEP

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SUMMARY

It was suggested that the leaf mark polymorphism of *Trifolium repens* might be important in visual selection of diet by sheep. Sheep with rumen fistulae were allowed to graze in restricted areas of permanent grassland and the morph frequency in the rumen was compared with that on offer in the pasture. There was clear preference by the sheep for unmarked leaves and some evidence that suggests apostatic selection between the various forms of mark.

1. INTRODUCTION

THE white leaf marking polymorphism in *Trifolium repens* is controlled by a series of multiple alleles (Brewbaker, 1955; Carnahan *et al.*, 1955; Cahn, 1976), the various forms of marking being dominant to the unmarked condition. The markings are very distinctive and can be used to identify individual clones (Harberd, 1963; Pusey, 1965; Cahn, 1976). Previous studies of the polymorphism have failed to discover selective forces that might maintain and balance this polymorphism (Pusey, 1965). The polymorphism exists as a very fine scale intermingling of morphs within permanent grasslands and there is a conspicuous failure of individual morphs to gain dominance on even a very local scale of a dm² (Cahn and Harper, 1976).

Predation is a strong selective influence in the polymorphism of genes controlling cyanogenesis in *Trifolium repens* and *Lotus corniculatus* (Jones, 1962, 1972, 1973; Crawford-Sidebotham, 1972; Angseesing and Angseesing 1973; Angseesing, 1974) although the largest herbivore that has yet been implicated is the rabbit. The genes controlling cyanogenesis are not on the same chromosome as those controlling white leaf marks.

Wolton *et al.* (1970) showed that sheep preferentially select clover from a mixed grass-clover sward. The sense of smell is relatively unimportant in food selection by sheep (Tribe, 1949) so visual clues such as leaf marks could possibly be important in their recognition of clover. Charles (1968) showed that a marked variety (S100) of *T. repens* survived less well than an unmarked one (S100 no mark) when grazed by sheep. It was therefore decided to investigate the selection of different leaf marks by sheep.

2. MATERIALS AND METHODS

Sheep are reputed to be very selective grazers and can be observed to nibble individual leaves of plants in a sward. Sheep were observed to follow

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similar routes each day when grazing in the field used in this study, however the limits of the vegetation available to each individual are uncertain since one cannot satisfactorily define the limits of the animal's vision. Therefore it was impossible to use free ranging sheep and it was decided to confine the sheep being studied to small areas with defined boundaries. Within these areas it was possible to determine whether or not the sheep selected from among the leaf mark phenotypes of *T. repens* on offer.

Two 6-year-old Border-Leicester \times Welsh Mountain ewes (Golda and Bubbles) were fitted with rumen fistulae in August 1972 and spent the following winter in the experimental field at Henfaes, Aber, Gwynedd.

Seven sites were sampled during the period April-June 1973. At four of these sites the sheep were restricted to localised grazing zones by tethers which allowed each animal access to *c.* 45 m². To check that the behaviour of tethered animals was not anomalous, on the remaining three sites the sheep were confined in pens, each of *c.* 25 m² in area.

Immediately before entry of sheep to the sites a point quadrat sample, the "Prior" sample, was taken. Clover leaves were collected at 30 cm intervals on a square grid and classified according to leaf mark. In all, 500 points were examined within each tethered area and 300 in each pen (600 at site 6).

The sheep spent the night before sampling in a concrete-floored pen with hay and "energy-nuts" as food. After 1 hour of grazing at the experimental sites, samples of *c.* 800 ml were taken from the rumen using a long Tala spoon: a homogeneous mixture of fresh vegetation and hay + energy-nuts was obtained. This suggests that the natural mixing process in the rumen gives an unbiased sampling technique.

Leaf marks in rumen samples remain clear provided that they are examined immediately after the grazing period but become much less distinct once the leaves become flaccid, which usually occurs after about 3 hours. Spoonfuls of rumen content were sorted in tap-water immediately after a grazing period and all the large pieces of leaf were classified. Flaccid leaves and leaf fragments so small that they could have been misclassified were discarded. With this precaution the leaves could be classified accurately into morphs. This technique was refined later and estimates of mark frequency made on whole leaves were compared with those from fragments. No significant bias was introduced by including fragments in the estimates of morph frequency (Cahn, 1976). With the exception of the first sample of all, two samples, one from each sheep were taken after each "Prior" sample.

3. RESULTS

The null hypothesis was made that morph frequency in the rumen samples and in the Prior samples represented unbiased samples from the clover on offer in the experimental plots. Observed morph frequencies in the samples are presented in table 1. Expected morph frequencies were determined from $2 \times n$ contingency tables of rumen and Prior samples against phenotypes (table 2). Observed and expected values were compared by χ^2 . The null hypothesis must clearly be rejected. The evidence strongly suggests that sheep select unmarked in preference to marked leaves. In none of the rumen samples were there fewer unmarked leaves than expected although if selection was random one would expect this to occur in half the samples.

TABLE 1
The number of different leaf mark morphs found in sheep rumen fistula samples

(a)	(b)	(c)	(d)	(e)	(f)	Number of leaves in the fistula sample												(m)	(n)	(p)							
						Successive groups of morphs containing morphs of decreasing frequency*				Unmarked leaves				A							B				C		
Site	Sheep	Date	Enclosure	Total no. clones observed	Prior sample size	S	P	S	P	S	P	S	P	S	P	S	P	S	P	S	P	S	P	χ^2 (Total)†	Degrees of freedom‡ in column (m)	χ^2 (unmarked marked ratio)† (1 d.f.)	
1	Golda	13.4.73	Tether	14	238	23	144	—	—	—	—	—	—	—	—	6	94	10.90	89.10	—	—	—	—	3.95	1	3.95 P < 0.05	
1	Golda	27.4.73	Tether	17	284	18	133	—	—	—	—	—	—	—	—	11	151	15.01	146.99	—	—	—	—	2.44	1	2.44 n.s.	
1	Bubbles	27.4.73	Tether	17	284	34	133	—	—	—	—	—	—	—	—	15	151	24.43	141.57	—	—	—	—	8.51	1	8.51 P < 0.01	
2	Golda	2.5.73	Tether	14	222	16	108	—	—	—	—	—	—	—	—	12	114	14.11	111.89	—	—	—	—	0.83	1	0.83 n.s.	
2	Bubbles	2.5.73	Tether	14	222	12	108	—	—	—	—	—	—	—	—	10	114	11.18	112.82	—	—	—	—	0.27	1	0.27 n.s.	
3	Golda	10.5.73	Tether	18	346	15	34	44	161	15	82	19.85	77.15	—	—	15	69	17.19	66.81	—	—	—	—	5.09	3	3.51 n.s.	
3	Bubbles	10.5.73	Tether	18	346	20	34	35	161	9	82	15.49	75.51	—	—	7	69	12.94	65.06	—	—	—	—	21.93	3	17.56 P < 0.001	
4	Golda	17.5.73	Tether	17	412	63	127	64	119	24	85	32.88	76.12	—	—	27	81	32.59	75.41	—	—	—	—	7.61	3	1.19 n.s.	
4	Bubbles	17.5.73	Tether	17	412	42	127	29	119	18	85	19.95	83.05	—	—	10	81	17.64	73.36	—	—	—	—	7.60	3	4.85 P < 0.01	
5	Golda	25.5.73	Pen	8	230	62	99	24	49	41	39	30.14	49.86	9	32	3	11	15.44	25.56	—	—	—	—	12.92	4	0.08 n.s.	
5	Bubbles	25.5.73	Pen	8	230	103	99	34	49	22	39	27.03	33.97	16	32	8	11	21.27	26.73	—	—	—	—	8.13	4	7.18 P < 0.01	
6	Golda	4.6.73	Pen	9	316	80	160	29	72	24	58	24.67	57.33	—	—	3	26	8.73	20.27	—	—	—	—	6.70	3	2.56 n.s.	
6	Bubbles	4.6.73	Pen	9	316	87	160	36	72	39	58	33.67	63.33	—	—	6	26	11.11	20.89	—	—	—	—	5.01	3	0.06 n.s.	
7	Golda	14.6.73	Pen	9	145	26	24	43	58	22	24	20.15	25.85	9	18	13	21	11.83	15.17	—	—	—	—	3.37	4	1.68 n.s.	
7	Bubbles	14.6.73	Pen	9	145	37	24	38	58	14	24	15.87	22.13	8	18	7	21	10.86	15.14	—	—	—	—	14.04	4	11.85 P < 0.01	
						25.48	35.52	40.10	55.90																		

Expected values are in italics.

* The figures in columns A-D represent the total leaf number from morphs with similar frequencies—thus the frequency of individual clones represented in column A is greater than the frequency of the individual clones in columns B, C and D.

† Contingency χ^2 .

‡ Sheep sample. P = Prior sample.

In two samples the difference between the observed morph frequencies in prior and rumen samples was very slight.

The total contingency χ^2 on the marked and unmarked classes is highly significant ($\chi^2(15 \text{ d.f.}) = 66.52, P < 0.001$) and so selection of these must have contributed heavily to the χ^2 . The heterogeneity χ^2 on these classes is highly significant ($\chi^2_{14} = 38.53, P < 0.001$) indicating that some factor influenced the degree of selection between the morphs at the different sites. This factor was probably the weather which varied during sampling from gale force winds to calm with bright sunshine and which is known to affect sheep behaviour.

The preferential selection of unmarked morphs was also confirmed by the results of an earlier survey which had shown a highly significant increase in the frequency of marked leaves at heavily grazed points in the field (Cahn and Harper, 1976).

TABLE 2
Total number of leaves of different morphs in the samples

Successive groups of morphs of decreasing morph frequency. (See footnote to table 1)	Sheep		Prior (plot)		Total
	Unmarked	Marked	Unmarked	Marked	
	638	(553.90)	1514	(1598.10)	2152
	376	(332.54) <i>300.8</i>	916	(959.46) <i>991.2</i>	1292
	228	(206.95) <i>187.23</i>	576	(597.05) <i>616.77</i>	804
	42	(36.55) <i>33.07</i>	100	(105.45) <i>108.93</i>	142
	153	(307.06) <i>277.90</i>	1040	(885.94) <i>915.10</i>	1193
	1437		4146		5583

$$\chi^2_3 = 130.99 (P < 0.001).$$

The figures in brackets are the expected values based on all leaves. The italic figures are the expected values when only the marked groups are considered. If only the marked leaves are considered (2×4 contingency table) $\chi^2_3 = 112.42, P < 0.001$.

If a 2×2 contingency table is used with only two classes, marked and unmarked, $\chi^2_1 = 27.99, P < 0.001$.

4. DISCUSSION

Selective grazing of clover leaf mark morphs might be explained if the marks were associated with distasteful chemicals. The marks are not linked with the cyanogenic polymorphism but of course some other undetected deterrent chemical might be present. However, although selection of diet by taste and smell is clearly important in some herbivores (Soane and Clarke, 1973; Allen and Clarke, 1968) it does not appear to be so for sheep. If such an association occurred, one might expect only a simple dimorphism—as it would be important for the sheep to form a “specific searching image” (Tinbergen, 1960).

Selective grazing might be explained if leaf mark differences were linked with some other factor of selective value such as leaf size. To test whether selection for leaf size could cause selection for leaf marks, both were recorded in the Prior sample and the Golda rumen sample at site 7. There appeared to have been selection by the sheep of the larger leaves (table 3b). The smaller leaves tend to be younger and/or lower in the canopy and both less visible and accessible. However, the frequency distribution of morphs cannot be related to selection by the sheep for leaf size (table 3a).

It is conceivable that leaf marks are cryptic—breaking up an image of leaf shape and hindering the formation of a search image by the grazing

animal or that the marks make a large leaf look like a small leaf. If there is such a simple choice by sheep of unmarked leaves, maintenance of the polymorphism would require a counter-balancing factor favouring unmarked leaves. Previous work (Cahn, 1976) has shown that clover with no leaf mark has greater resistance to stolon-rotting fungi and this might serve as a balancing factor in a dimorphism, but would not explain such a complex polymorphism as the leaf mark system in clover.

The most likely interpretation of the clover leaf mark polymorphism is apostatic selection (Clarke, 1962) involving the mark as a search image and in which the grazing animal preferentially selects the commoner form on offer. There were insufficient individual samples in which marked phenotypes were

TABLE 3
Selection of different leaf size classes (Golda, Site 7)

(a) Numbers of leaves in different leaflet breadth classes in rumen sample				
	(0.1-5 cm)	(1.5-2 cm)	(> 2 cm)	Total
(a) Unmarked	11 (10.65)	18 (18.27)	14 (14.08)	43
(b) Marks 1	7 (6.45)	10 (11.04)	9 (8.51)	26
(c) 2	9 (5.45)	10 (9.35)	3 (7.20)	22
(d) 3	1 (2.23)	8 (3.82)	0 (2.95)	9
(e) Others	0 (3.22)	2 (5.52)	11 (4.26)	13
Total	28	48	37	113

$$\chi^2_6 = 10.62.$$

Note: Rows (d) and (e) were pooled.

(b) Comparison of number of leaves in different leaflet breadth classes in the sheep and prior samples

Leaf size classes	Sheep sample		Prior sample	Total
0.1-1.5 cm	28	(45.55)	76 (58.45)	104
1.5-2.0 cm	48	(41.61)	47 (53.39)	95
> 2.0 cm	37	(25.84)	22 (33.16)	59
	113		145	258

$$\chi^2_2 = 22.35, p < 0.001.$$

commonest for this hypothesis to be adequately tested for marked against unmarked leaves. However among the marked leaves there was clear evidence of selection by sheep of the commonest morphs—the rarest morphs were consistently under-represented in the rumen samples ($P < 0.001$). This conclusion must, however, carry two caveats (a) if the experimenter himself forms a search image there may be a tendency to classify a rare morph as common more often than the converse and (b) if the sheep showed any tendency to graze the centre of the area more intensively than the periphery, rare morphs, clones whose fringes entered the edges of the plots, would tend to be under-represented in the rumen samples.

These results conflict with those of Charles (1968) who found that unmarked plants survived better in a grazed sward of two similar varieties of *T. repens* one predominantly marked and one unmarked. This could be the result of other fitness differences between the varieties or it could be caused by the strong marks of cultivated varieties which could act as search images

since they are much less varied than those found in wild populations. Marks which are conspicuous to the experimenter have a smaller clonal spread than less conspicuous ones (Cahn, 1976).

The selection of clover morphs by sheep was tested at the time when the marks are most clear (Cahn, 1976)—this is also the season when heavy grazing encourages clover growth by suppressing competing grasses. Protection must be very important to the clover at this time. It is interesting that seedlings of clover which germinate in March-May in the field retain clear marking for the first 3 months after germination after which clarity declines even in constant daylength, under glasshouse conditions. Therefore the marks are at their most distinct at the same time of year in both mature plants and seedlings.

Fistulation techniques are obviously appropriate for this type of study but are best combined with estimates of changes in morph frequency during grazing. Ideally, artificial swards should be sown with known morph frequencies for a critical test of apostatic selection. The interpretations of leaf mark polymorphism suggested above imply that other forms of marking in *T. repens* (e.g. red flecking or tingeing (Pusey, 1965; Corkill, 1971) and in other clover species (Pusey, 1965) may have similar significance and indeed other apparently non-adaptive variegation and morphological variation in plants may be related to predation in ways similar to colour variation in animals (Kettlewell, 1957; Cain and Sheppard, 1954).

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