THE ROLE OF SLUGS AND SNAILS IN THE MAINTENANCE OF THE CYANOGENESIS POLYMORPHISMS OF *LOTUS CORNICULATUS* AND *TRIFOLIUM REPENS*

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1. INTRODUCTION

IT has long been known that *Lotus corniculatus* L. and *Trifolium repens* L. are polymorphic for the presence or absence of cyanogenic β-glucosides. Plants which evolve hydrocyanic acid gas (HCN) after mechanical damage to the stems or leaves are cyanogenic; those not possessing this property are acyanogenic.

In *T. repens*, cyanogenesis is controlled by two unlinked loci; the dominant allele at one locus determines the presence of the glucosides, and the dominant allele at the other locus determines the presence of a β-glucosidase. After mechanical damage the latter controls the hydrolysis of the glucosides, leading to the evolution of free HCN gas. Of the nine possible genotypes, only four are cyanogenic. The situation is essentially similar in *L. corniculatus*, but is complicated by the presence of tetrasomic inheritance. The details of these balanced polymorphisms have been reviewed recently by Jones (1967, 1972).

Two types of selection influencing the frequency of the phenotypes have been proposed. Jones (1966) suggested that an advantage is conferred upon cyanogenic *L. corniculatus* because the evolution of HCN deters herbivores, for example molluscs. Daday (1965), on the other hand, has suggested that cyanogenic *T. repens* is at a disadvantage in colder climates because low temperatures activate the β-glucosidase, leading to the production of HCN in sufficient quantities to cause tissue death. These two explanations need not be mutually exclusive.

Bishop and Korn (1969) failed to find selective eating of *T. repens* by molluscs. They argued that it is incorrect to extrapolate from *L. corniculatus* to *T. repens*. It was decided, therefore, to design a series of simple laboratory experiments to determine quantitatively the extent to which the different phenotypes of both plants are eaten by several species of mollusc.

2. MATERIALS AND METHODS

Thirteen species of molluscs were employed in the experiments: *Agriolimax caruanae* Poll., *Agriolimax reticulatus* (Müll.), *Arion ater* (L.), *Arion hortensis* Fér., *Arion subfuscus* (Drap.) and *Milax budapestensis* (Hazay) (slugs); *Arianta arbustorum* (L.), *Cepaea hortensis* (Müll.), *Cepaea nemoralis* (L.), *Helicella virgata* (da Costa), *Helix aspersa* Müll., *Monacha cartusiana* (Müll.) and *Theba pisana* (Müll.) (snails).

For each species of mollusc parallel experiments were run, one with *L. corniculatus* and the other with *T. repens*, thus making a total of 26 experiments. To allow comparisons between the results of different experiments,

each analysis was based upon 30 individuals. Since mortality was a problem, particularly with the slugs, an excess of individuals was introduced to each experiment, and the results of only the first 30 to finish were used in the analyses.   Each mollusc retained an individual identity throughout an experiment.

Four different feeding situations were presented to each animal in the following order: (1) acyanogenic plant only; (2) acyanogenic plant and carrot; (3) cyanogenic plant and carrot; (4) cyanogenic plant only. Each individual mollusc, assuming that it lived until the end of the experiment, completed two cycles of these feeding trials, the order being unchanged in the second cycle. Four days were required to complete each feeding trial and, therefore, each mollusc spent a total of 32 days in the experiment.

The first feeding trial, *i.e.* acyanogenic plant only, constituted a pilot trial in which 10 individuals were used. If less than two of these molluscs produced plant faeces the experiment was discontinued.

Each feeding trial, irrespective of the food to be presented, was conducted as follows. On the first day the molluscs were placed in $33 \times 22 \cdot 9 \times 8 \cdot 9$ cm. polythene lunch boxes, the bottoms of which had been covered with damp tissue paper. Two piles of bran were placed in each of the lunch boxes which were kept in a constant environment room maintained at 15° C. with continuous illumination. On the following morning the bran was removed and the tissue replaced. The molluscs were thus starved until the afternoon of the third day, when each was placed in a separate $9 \cdot 5$ cm. diameter petri dish on a circle of damp filter paper. The appropriate food was added and the petri dishes were placed in the constant environment room in boxes to exclude the light. The food remains were removed from the petri dishes on the following morning (the fourth day) and it was found that cyanogenic leaves were still capable of releasing HCN. The petri dishes were returned to the constant environment room and placed in the light for a further 24 hours. On the fifth day the molluscs were removed from the petri dishes and replaced in polythene lunch boxes with damp tissue paper and bran. Thus the fifth day of a particular feeding trial was also the first day of the succeeding trial.

Leaves of *L. corniculatus* or *T. repens* were cut from plants which had been grown under glasshouse conditions. Five leaves (nine for *H. aspersa*) of the appropriate plant, *i.e.* species and phenotype, were placed in each petri dish. If alternative food was also to be presented, a slice of carrot was cut into quadrants and these were placed around the edge of the petri dish, between the leaves. On account of the size of *H. aspersa* the feeding trials were conducted in petri dish bottoms, using inverted glass crystallising dishes as lids.

Mollusc faeces retain the colour of the food eaten. Thus, leaves and carrot produce green and orange faeces, respectively, and these are easily distinguished from the brown or white faeces produced after feeding on bran or paper. It had been established previously that faeces were produced between 8 and 24 hours after feeding, irrespective of species, under these experimental conditions. The time between successive feeding trials was, therefore, sufficient to allow all faeces to be eliminated. The green and orange faeces produced by each mollusc were left to dry in filter-paper cones for at least 24 hours before being weighed. The weight of faeces provided a measurement of the amount of food of the appropriate type consumed.

3. Results

Full details of the raw data and the statistical analyses are available elsewhere (Crawford-Sidebotham, 1971). Nine experiments were discontinued after the pilot feeding trial. In the remaining 17 experiments the first cycle of four feeding trials, one of each type, was considered as the first block, and the second cycle of four as the second block. A three-way analysis of variance was conducted upon the total weights of the faeces (plant and carrot) produced by the 30 molluscs in each experiment during the four types of feeding trial within each block. All three main effects, *i.e.* between blocks, feeding trials and experiments, were considered " fixed ". The amount of food consumed differed both between experiments and between the four different feeding trials and, further, the differences between feeding trials were not consistent over experiments. There was no difference between blocks, and the block interactions were not significant. Thus there were no sequential effects upon the amount of food eaten as a result of the length of time that the animals had been subjected to the experimental conditions. Also, feeding upon cyanogenic food in trial four of the first block did not appear to affect the feeding behaviour of the molluscs in the subsequent block.

The results of each separate experiment were subjected to individual mixed model analyses of variance so as to investigate more closely whether the species of molluscs under consideration ate different quantities of cyanogenic and acyanogenic plants. Four main effects were considered in the analyses: the difference between blocks; the difference between plant phenotypes; the effect of the presence of alternative food; and the differences between individual molluscs. This last main effect was considered " random ", the others as " fixed ". It was not feasible to include true replicates in the design of the experiments and, therefore, there was no true estimate of σ^2. Consequently, there was no correct test mean square for the between individuals' main effect and interactions. The third-order interaction is, however, almost certainly a suitable test for the three second-order interactions involving individuals. Of these 51 tests, no more were significant than would be expected by chance alone. For each experiment Bartlett's test showed that these three second-order interactions and the third-order interaction were homogeneous and would provide, when pooled together, a suitable test mean square for those items without a proper test. These pooled estimates of σ^2 were not, however, independent of the mean amount of faeces produced per trial and comparisons cannot be made between mean squares for particular items from different experiments. The percentages of the total variation that are attributable to each significant item may, however, provide useful information on general patterns, and these are summarised for each experiment in table 1.

The quantity of *T. repens* eaten by *A. arbustorum* was greater in the second block than in the first. These snails were collected from the high limestone region of Derbyshire during an early autumn cold-spell and so it is possible that many of the snails were still in an inactive state at the beginning of the experiment. As the experiment progressed, the warmer conditions in the laboratory stimulated greater activity. Because of these block differences it is not possible to arrive at any meaningful conclusions from the results of this experiment.

TABLE 1

The proportion (%) of the total variation attributable to each significant item in each experiment

Mollusc	Plant	B	P	A	I	B×P	B×A	P×A	P×I	A×I	B×P×A	Error
A. reticulatus	L. corniculatus	—	14	24	4	—	—	13	—	—	—	45
	T. repens	—	—	47	—	—	—	4	—	—	—	49
A. arbustorum	T. repens	6	—	20	—	—	9	—	—	—	—	66
A. ater	L. corniculatus	—	8	33	—	—	—	12	—	—	—	48
	T. repens	—	6	26	—	—	—	12	—	8	—	48
A. hortensis	L. corniculatus	—	—	14	—	—	—	—	—	—	—	86
	T. repens	—	8	41	—	—	—	13	—	—	—	37
A. subfuscus	L. corniculatus	—	2	26	9	—	—	6	—	12	—	44
C. hortensis	L. corniculatus	—	9	7	—	—	—	13	—	—	—	71
	T. repens	—	5	11	6	—	—	9	—	10	—	59
C. nemoralis	L. corniculatus	—	7	8	7	—	—	12	—	9	—	57
	T. repens	—	3	4	7	3	6	6	7	15	8	42
H. virgata	T. repens	—	6	7	9	—	—	8	—	9	—	60
H. aspersa	L. corniculatus	—	12	32	4	—	—	14	8	—	—	30
	T. repens	—	4	48	—	—	—	9	—	—	—	40
T. pisana	L. corniculatus	—	3	16	8	—	—	4	—	—	—	69
	T. repens	—	8	22	—	—	—	16	—	—	—	54

B = Between blocks. P = Between phenotypes (*i.e.* differential eating).
A = Effect of alternative food. I = Between individuals.

There is some evidence for differential eating of the two plant pheno-
types in the *C. nemoralis/T. repens* experiment, but the proportion of the
total variation for which this effect accounts is small compared with many
of the interactions. Furthermore, this effect was inconsistent both over
blocks and also from one individual to another. The evidence for differential
eating in this experiment must be considered as unreliable.

The remaining 15 experiments may now be considered in more general
terms. Two experiments (*A. reticulatus/T. repens* and *A. hortensis/L. cornicu-
latus*) provided no evidence for differential eating. In relation to its weight
A. reticulatus ate large quantities of *T. repens*, irrespective of phenotype. *A.
hortensis* ate more acyanogenic *L. corniculatus* but the quantities involved were
too small to provide a significant comparison. Differential eating was
demonstrated in the remaining 13 experiments, seven with *L. corniculatus*
and six with *T. repens*. With six molluscs (*A. reticulatus*, *A. ater*, *A. subfuscus*,
C. hortensis, *C. nemoralis* and *H. aspersa*) the differential eating effect was more
marked in the *L. corniculatus* than in the *T. repens* experiments. Only three
molluscs (*A. hortensis*, *H. virgata* and *T. pisana*) showed a more marked effect
in the *T. repens* experiments.

It would seem in general, therefore, that molluscs do eat differentially
both *L. corniculatus* and *T. repens*, the cyanogenic plants being eaten to a
lesser extent than the acyanogenic. The different molluscs, however, do
not behave in the same way. Some species of mollusc differentially eat
neither of the plant species, while others eat more of the acyanogenic pheno-
type of one plant species but not of the other. The remainder differentially
eat both species of plants. Generally, it would appear that with the molluscs
investigated in these experiments, differential eating of the two phenotypes
is more a feature of *L. corniculatus* than of *T. repens*. But the components of
variation upon which these interpretations are based are subject to large
standard errors. These conclusions, therefore, should not be treated as
exact for any one mollusc/plant combination, but rather all the experiments
should be considered together to demonstrate patterns.

A feature of each experiment is the relatively large proportion of variation
attributable to the effect of the presence or absence of carrot as an alternative
food. In the presence of carrot the quantity of plant consumed, irrespective
of phenotype, was greatly reduced, so much so that the differential eating
effect was often lost, or even reversed so that more cyanogenic plant was
eaten than acyanogenic. Hence the significant phenotypes × alternative food
interactions.

In seven of the 15 experiments molluscs showed individual differences
in the total amount of plant consumed during the experiment, but in only
one (*H. aspersa/L. corniculatus*) were individuals inconsistent in the degree
to which they exhibited differential eating of the two plant phenotypes. In
five experiments individual molluscs differed in the degree to which they
ate carrot in preference to plant, irrespective of phenotype.

4. Discussion

These experiments differ from other investigations in one important
detail. Jones (1966) and Bishop and Korn (1969) studied the actual
selective eating by offering to the molluscs both plant phenotypes simul-
taneously. The animals were in a position to choose whichever of the

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plants they preferred to eat, and any differences in the quantities eaten of acyanogenic and cyanogenic plants provided a measure of "selective eating". In the present experiments the two different phenotypes were offered separately. Thus molluscs did not actually make any choice between the phenotypes of the plants. Any differences in the quantities of acyanogenic and cyanogenic plants eaten in these experiments have been referred to, therefore, as " differential eating ".

Jones (1966) showed that *A. reticulatus* would selectively eat acyanogenic *L. corniculatus*, but once the acyanogenic plant had been completely eaten, then the slugs began to eat the cyanogenic plant. The present experiments have shown that if only one phenotype is available, then some species of mollusc will still eat more acyanogenic than cyanogenic plant, and it is reasonable to conclude that if both phenotypes were present then selective eating would occur.

Bishop and Korn (1969) found no selective eating of *T. repens* by 50 *A. reticulatus*. The results of the present experiments concur with this conclusion. Similarly, they found no evidence for selective eating of *T. repens* by *H. aspersa*. Twenty-four snails were employed in their experiments, but of these only six had not been subjected to an ommatophorical surgical operation, and these were not considered individually, but rather as a single group. The extent to which a plant had been eaten was measured as the difference in the numbers of leaves before and after a feeding trial. A re-analysis of their data (Table 2, Bishop and Korn, 1969) using a model I four-way analysis of variance shows that the greatest proportion of the variation (25 per cent.) was attributable to differences between the two scorers.

Jones has not investigated selective eating in *T. repens* but the results which he obtained with *L. corniculatus* (Jones, 1966), with the exception of *A. arbustorum*, agree with those presented here. It has already been suggested that the results obtained with *A. arbustorum* in the present experiment cannot be used to provide any evidence for or against selective eating by this snail in either species of plant.

It is likely that carrot segments are more attractive to molluscs than the alternative foods normally found in the wild. Certainly the carrot was eaten in preference to the green plants offered in these experiments. Even if carrot can be regarded as an entirely artificial alternative food, it is clear that interactions between plant phenotypes and alternative food in the natural situation cannot be dismissed as trivial.

Jones (1966) has suggested that the distribution of the animals which eat *L. corniculatus* and *T. repens* may be controlled by the January mean temperature. If animal populations were more dense in those environments with a higher temperature, then the correlations between glucoside and enzyme frequencies and the January mean temperature obtained by Daday (1954) for *T. repens* might be secondary. Cyanogenic plants are at a selective disadvantage in colder areas, Daday (1965) argues, because low temperatures activate the enzyme. This leads to the production of HCN which irreversibly inhibits the respiratory system of the leaves. Bishop and Korn (1969) suggest that this makes Jones' theory seem unlikely, presumably because when molluscs nibbled cyanogenic plants the HCN evolved would lead to tissue death and, thus, no advantage would be conferred upon the cyanogenic plants. If this be so, why is there such a high frequency of cyanogenic plants in some populations, particularly in warmer climates?

The stage of development at which predation by herbivores would most greatly reduce the fitness of plants is at the seedling stage. It seems likely that the fitness of mature plants would be only marginally reduced, unless the mollusc populations were extremely dense. A seedling that was acyanogenic, however, would be at a much greater selective disadvantage compared with cyanogenic seedlings, because only a small degree of predation would greatly reduce its fitness. Furthermore, the time of year at which most plants pass through the seedling stage is probably during the spring, and in warmer environments at that time of the year mollusc populations are larger and more active than they are in colder areas.

It would appear that there is now sufficient evidence for selective eating in both plants to warrant attempts at the determination of quantitative estimates of the magnitude of the selective forces involved. The next step is an ecological study of natural populations of plants and the molluscs which live within the same habitat.

5. SUMMARY

1. Leaves of acyanogenic and cyanogenic *Lotus corniculatus* L. and *Trifolium repens* L. were presented to 13 species of slugs and snails. An estimate of the quantity of leaves consumed in each feeding trial was obtained from the dry weight of the plant faeces produced.

2. Evidence is presented to support the hypothesis that molluscs selectively eat acyanogenic plants and, therefore, that the cyanogenic character acts as a defensive mechanism against herbivores.

3. Species of mollusc differ greatly in the degree to which they exhibit differential eating and, in general, the phenomenon appears to be more a feature of *L. corniculatus* than of *T. repens*.

4. It is suggested that this form of selection is more likely to operate upon seedlings, rather than upon established plants.

6. REFERENCES

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