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Enzyme protein polymorphism in the slug *Arion ater*

By BARRIE BURNET
Department of Genetics, University of Sheffield

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SUMMARY

Populations of the slug *Arion ater rufus* sampled from several localities in England and the Netherlands are polymorphic for different electrophoretic variants of digestive-gland esterase and salivary-gland tetrazolium oxidase. Samples of *Arion ater ater*, which is endemic to the British Isles, were found to be monomorphic for these enzymes. Localized populations of *A. a. rufus* in Britain could have originated from specimens imported accidentally from continental Europe. There is evidence for gene flow between overlapping populations of the two subspecies. In one of the English populations of *A. a. rufus*, studied in detail, polymorphism for esterase and tetrazolium oxidase is shown to be balanced. Heterosis may be an important factor contributing to the maintenance of balanced polymorphism for tetrazolium oxidase in this population.

1. INTRODUCTION

The successful application of gel-diffusion techniques to the study of genetic variation in enzyme proteins has yielded new insight into the proportion of polymorphic gene loci, and average level of heterozygosity per individual in different species populations (Selander *et al.* 1970). Such information has a direct bearing on what is called the 'genetic load problem' (Wallace, 1970). Studies on enzyme protein polymorphism can also yield information at a different level, namely the relationship between populations which on conventional criteria are of uncertain taxonomic status. Manwell & Baker (1970) cite instances of the way in which comparison of gene frequencies in different populations has revealed the existence of sibling species, or lead to the detection of introgressive hybridization between related species. In the study reported here, gene frequency data are used to investigate the breeding structure and detect gene flow between two subspecies of slugs belonging to the genus *Arion*.

Cain & Williamson (1958) have studied pigmentation and genital anatomy in populations of slugs referable to *Arion ater*. Their findings, which amply illustrate the difficulties which exist in dealing with this genus, indicate that there are two subspecies. *A. a. ater* is indigenous to the British Isles and Scandinavia, whereas *A. a. rufus*, which occurs in continental Europe south of Scandinavia, is found in Britain as an accidental import in gardens and on cultivated land. A key to the two subspecies, which are thought to hybridize on contact, is given by Quick (1960). Genetic variation in electrophoretic mobility of two enzymes, an esterase

and a tetrazolium oxidase, in populations of these slugs is described here for the first time. Gene frequency data for the two enzymes confirm the subspecific status presently accorded on the basis of colour variation and genital morphology.

2. MATERIALS AND METHODS

(i) *Collection of specimens*

Slugs were collected in the late evening during wet weather or following rain. Juveniles were ignored and only those individuals exceeding 3 g body weight were normally used for zymograms. Individuals were examined morphologically and stored at −20 °C until homogenization.

Collections of *A. a. rufus* from the Netherlands were made on grass or rough herbage beside drainage channels bordering cattle grazing or horticultural land. Each collection for a named locality was made in an area of between 500 and 1000 square yards.

The collections of *A. a. ater* from Britain were made on open sheep grazing or from grass and rough herbage bordering country lanes. The area of search was similar to that used for the Netherlands collections. The yield of *A. a. ater* in a given area is generally much lower than for corresponding populations of *A. a. rufus*.

Two populations of *A. a. rufus* in England are described. The first of these occurs in gardens on the south-west side of the City of Sheffield. These gardens border on to a meadow of rough turf and mixed herbage which is cut once annually. *A. a. ater* were collected from this meadow. These two populations overlap and the changeover from *rufus* to *ater* takes place along a narrow zone a few yards wide. Specimens of *rufus* are often found on garden rubbish dumped into the meadow. *A. a. ater* were also collected from a second location (Sheffield 2) about 1 mile from the meadow in open country where there are no *rufus*. The second population of *A. a. rufus* occurs in gardens, and on mixed herbage bordering the pathway leading to the Peak Cavern at the village of Castleton in Derbyshire. *A. a. ater* are found on the adjoining sheep grazing in Cave Dale. The two populations of *ater* and *rufus* overlap. Collections from each of these two populations were from areas separated by a distance of approximately 150 yards.

(ii) *Colour polymorphism*

Animals were assigned to colour classes subjectively, immediately after collection, according to their appearance to the author when alive and in an extended state in daylight.

(iii) *Electrophoresis*

Slugs were homogenized individually in four volumes of ice cold gel buffer. The resulting homogenate was then centrifuged at low speed and allowed to stand at 5 °C for 1 h. A sample of the homogenate was removed by pipette, from beneath the upper frothy layer which forms on standing, and applied to 1 × 0·5 cm Whatman

3 mm sample carriers. Horizontal starch gels in 18 × 21 × 0·5 cm trays were used for electrophoresis at 5 °C. The buffer systems used were as follows. (i) Gel pH 8·65, 0·076 M tris, 0·005 M citric acid; tray 0·3 M boric acid, 0·05 M sodium hydroxide (Poulik, 1957). (ii) Gel pH 7·0, 0·0126 M tris, 0·0037 M citric acid; tray 0·378 M tris, 0·141 M citric acid (Syner & Goodman, 1966). (iii) Gel pH 8·7, 0·9 M tris, 0·5 M boric acid, 0·02 M EDTA, 1:20 dilution used in starch gel; 1:7 dilution used in the anodal tray; 1:5 dilution in the cathodal tray (Market & Faulhaber 1965).

(iv) *Reaction mixtures*

Esterase: 0·3 M phosphate buffer pH 6·0, α-naphthyl acetate 0·05%, fast blue BB salt 0·1%. Tetrazolium oxidase: 0·1 M Tris buffer pH 8·6, nicotinamide adenine dinucleotide 0·06%, phenazine methosulphate 0·01%, nitro blue tetrazolium 0·02%, incubated in darkness. Leucine aminopeptidase: 0·2 M Tris maleate buffer pH 5·2, L-leucyl-α-naphthylamide 0·02%, fast black K salt 0·05%.

3. RESULTS

(i) *Colour variation in* Arion ater rufus *and* A. a. ater

Arion a. ater is typically black or grey with a grey foot sole and grey or dirty yellow foot fringe. Dark brown specimens occur in which the foot sole and foot fringe are brown. White or cream individuals with a more brightly coloured foot fringe also occur. An account of the genetics of body colour variation in this subspecies is given by Williamson (1959). Black melanin pigmentation (M) is dominant to brown melanin pigmentation (m). Longitudinal juvenile banding (U) is dominant to the unbanded condition (u). Uniform adult pigmentation (F) is dominant to lateral streaking (f^s) and both are dominant to white (f), in which melanin is restricted to the tentacles and lineolations of the foot fringe. The sample from Skibbereen, Eire, included five specimens showing the streak phenotype resembling the var. *albolateralis* illustrated in Taylor (1902). No white specimens were found in any of the samples of *A. a. ater*.

A. a. rufus is typically brown, fawn or orange, with a bright orange foot fringe. The foot sole may be cream, brown or orange. Rufine, the pigment which is present in orange specimens, is also secreted with the slime. The amount which is present, and consequently the colour of these individuals, varies depending on dietary and other factors. Without exception, specimens of *A. a. rufus* collected from the Netherlands had the bright orange foot fringe. Body colour was dark brown, sometimes nearly black, in specimens collected in the northern provinces of Friesland and Groningen. Samples from the southern provinces are highly polymorphic for body colour, and more than half of the specimens in the sample from Limburg were bright orange or red.

The distribution of colour varieties in adjacent populations of *A. a. ater* and *A. a. rufus* is shown in Tables 1 and 2. The Sheffield garden population is polymorphic for colour with the conspicuous orange foot fringe characteristic of continental *rufus* populations. The adjoining meadow population consists of black

Table 1. *Colour variation in samples collected in the same evening from the garden population of slugs and in the adjacent meadow population at Sheffield*

	Body colour					
Foot fringe	Cream	Orange	Fawn	Brown	Grey	Black
			Gardens			
Cream	4	—	—	—	—	—
Orange	—	26	25	19	4	—
Brown	—	—	—	—	6	—
Grey/black	—	—	—	—	—	3
			Meadow			
Cream	—	—	—	—	—	—
Orange	—	—	—	3	—	—
Brown	—	—	—	9	1	—
Grey/black	—	—	—	—	7	72

Table 2. *Colour variation in samples collected in the same evening from the slug population at Castleton village and in the adjacent sheep grazing in Cave Dale*

	Body colour			
Foot fringe	Fawn	Brown	Grey	Black
		Village		
Orange	26	80	15	2
Brown	—	8	—	—
Grey/black	—	—	—	3
		Dale		
Orange	—	—	—	—
Brown	—	1	4	—
Grey/black	—	—	21	126

specimens typical of *A. a. ater*. Three specimens with brown body colour and bright orange foot fringe were undoubtedly *rufus*. The population of *A. a. rufus* at Castleton is generally darker than that at Sheffield, with the bright orange foot fringe. No cream or orange specimens were present in the sample for which data are given in Table 2. *A. a. ater* on the sheep grazing in Cave Dale are black or grey with occasional brown individuals. The foot fringe is always grey/black or brown. With respect to their genital morphology the two English populations of *rufus* are broadly similar to continental *rufus*. However, there is considerable individual variation in this respect especially in the Sheffield *rufus* population in which many specimens could not confidently be assigned to either subspecies with genital morphology as the sole criterion (cf. Quick, 1960).

(ii) *Isoenzyme variation in* Arion ater ater *and* A. a. rufus

Esterase enzymes of *Arion* capable of utilizing α-naphthyl acetate as a substrate are tissue specific. The results to be described refer to a single enzyme (esterase 1) present in the gut and digestive gland of the slugs. In homogenates of whole individuals this enzyme is present in *A. a. ater* as a single band which develops on the

Table 3. *The number of alleles of esterase and tetrazolium oxidase in the slug* Arion ater rufus *sampled from different localities*

(No specimens from Kloten were scored for tetrazolium oxidase.)

	Esterase					Tetrazolium oxidase	
	F	I^B	I^N	*S*	*O*	*F*	*S*
England							
Yorkshire, Sheffield	1	1272	—	104	3	1096	530
Derbyshire, Castleton	179	409	—	—	—	270	94
Netherlands							
Groningen, Groningen	89	—	29	—	—	87	13
Friesland, Leeuwarden	201	—	17	—	—	105	105
Overijsel, Zwolle	192	—	12	—	—	126	78
Zuid Holland, Gouda	195	—	5	—	—	195	5
Limburg, Valkenburg	125	—	15	—	—	136	22
Switzerland							
Zurich, Kloten	22	—	6	—	—	—	—

Table 4. *The number of alleles of esterase and tetrazolium oxidase in the slug* Arion ater ater *sampled from different localities*

	Esterase					Tetrazolium oxidase	
	F	I^B	I^N	*S*	*O*	*F*	*S*
Scotland							
Isle of Arran	—	56	—	—	—	56	—
Rosshire, Achiltibuie	—	50	—	—	—	50	—
England							
Northumberland, Bamburgh	—	100	—	—	—	100	—
Lancashire, Cartmel	—	10	—	—	—	10	—
Yorkshire							
York	—	20	—	—	—	20	—
Thorne	—	80	—	—	—	80	—
Sheffield	—	400	—	—	—	400	—
Sheffield 2	—	140	—	—	—	140	—
Derbyshire, Cave Dale	23	255	—	—	—	198	2
Ulster							
Londonderry, Magilligan	—	32	—	—	—	32	—
Eire							
W. Cork, Skibbereen	—	30	—	—	—	30	—

zymogram well before any of the other esterase bands. Populations of *A. a. rufus*, as shown by the data summarized in Table 3, are polymorphic for four different anodally migrating electrophoretic variants of this enzyme: fast *F*, intermediate (Netherlands) I^N, intermediate (Britain) I^B and slow *S*. A null allele giving no detectable enzyme activity also occurs. The R_F values using the Poulik buffer system were *F* 0·73, I^N 0·69, I^B 0·68, *S* 0·63. In addition to the small difference in electrophoretic mobility between I^N and I^B using Poulik and also Markert buffer

Table 5. *Number of individuals of each genotype, and the corresponding allele frequencies, for esterase and tetrazolium oxidase in overlapping populations of* Arion ater ater *and* A. a. rufus *in adjacent environments at Castleton*

	Esterase					
	I^BI^B	I^BF	FF	p(I^B)	q(F)	χ^2_1
A. a. ater, dale,	118	19	2	0·917	0·083	51·9**
A. a. rufus, village	137	135	22	0·696	0·304	
	Tetrazolium oxidase					
	FF	FS	SS	p(F)	q(S)	
A. a. ater, dale	98	2	0	0·990	0·010	54·6**
A. a. rufus, village	99	72	11	0·742	0·258	

(χ^2_1 tests the difference in allele frequencies between the respective populations; ** denotes $P < 0{\cdot}01$.)

systems (see Materials and Methods), these isozymes differ in that I^N shows no detectable activity in the Syner & Goodman buffer system whereas the activity of I^B is quite normal.

Populations of *rufus* from the Netherlands and Switzerland are polymorphic for the F and I^N isoenzymes. Three phenotypes are found in these populations: individuals with a single band in the F position (FF homozygotes), or in the I^N position (I^NI^N homozygotes), and individuals with one F and one I^N band each with about half the activity, as indicated by density of staining, as individuals with a single band (FI^N heterozygotes). The enzyme is apparently a monomer specified by a single structural gene.

The F allele present in the *rufus* population at Castleton appears to be identical to that in the Netherlands populations. It occurs at very low frequency in the Sheffield population where a single FI^B heterozygote was found. Both populations are polymorphic, but for different combinations of alleles. The Sheffield population contains the S and a low frequency of null alleles. At both Sheffield and Castleton I^B is the most common allele. In I^BS heterozygotes there are two bands corresponding to the I^B and S positions, respectively, each with approximately half the activity of the single band in the corresponding homozygotes.

Populations of *A. a. ater* from each of the localities sampled in the British Isles proved to be monomorphic for I^B, with the exception of the Cave Dale population at Castleton which is polymorphic for F and I^B (Table 4). This population is immediately adjacent to the Castleton village population of *rufus*, which is also polymorphic for these two alleles.

Tetrazolium oxidase activity is recognized by the presence of achromatic regions against the blue background which forms on starch gels incubated with nitroblue tetrazolium and phenazine methosulphate – reactants normally used for the detection of dehydrogenases (Brewer, 1967). Three separate and tissue-specific tetrazolium oxidases can be recognized in *Arion*. The results to be described here refer to the enzyme (tetrazolium oxidase-1) present in the salivary gland tissue.

Table 6. *Number of individuals of each genotype, and the corresponding allele frequencies, for three enzymes in* Arion ater *and* A. a. rufus *sampled from overlapping populations in the two adjacent environments at Sheffield*

	Esterase				
	I^BI^B	I^BS	SS	p(I^B)	q(S)
A. a. ater, meadow	200	0	0	1·000	0
A. a. rufus, gardens	101	20	3	0·895	0·105
	Tetrazolium oxidase				
	FF	FS	SS	p(F)	q(S)
A. a. ater, meadow	200	0	0	1·000	0
A. a. rufus, gardens	98	85	23	0·682	0·318
	Leucine aminopeptidase				
	FF	FS	SS	p(F)	q(S)
A. a. ater, meadow	0	0	150	0	1·000
A. a. rufus, gardens	2	24	130	0·089	0·910

Populations of *A. a. rufus* from the British Isles and the Netherlands are each polymorphic for anodally migrating electrophoretic variants of this enzyme (Table 3). Three phenotypes are present in these populations: (i) individuals with a single fast-migrating band (FF homozygote), (ii) with a single slowly migrating band (SS homozygote), and (iii) with three bands – one at the F position and one at the S position, together with a hybrid band of intermediate mobility (FS heterozygote). Using the Poulik buffer system the R_F values for the F, S and hybrid bands are 0·39, 0·14 and 0·26, respectively. This enzyme appears to be a dimer of two polypeptide chains specified by a single structural gene for which two alleles are segregating in populations of *A. a. rufus*.

Populations of *A. a. ater* are monomorphic for the F form of tetrazolium oxidase, with the exception of the Cave Dale population in which the S allele occurs at low frequency (Table 4).

(iii) *Gene-flow between adjacent populations of* ater *and* rufus

At Castleton *rufus* and *ater* form two overlapping populations in adjacent environments. The *rufus* population is present in village gardens, and *ater* is present on the sheep-grazing in Cave Dale. Table 5 shows the number of individuals of each genotype together with the allele frequencies for esterase and tetrazolium oxidase from collecting areas within the two respective populations. These collecting areas were some 150 yards apart, avoiding the zone of overlap between the two populations. The *rufus* population is polymorphic for both enzymes. Clear evidence for gene-flow between the two subspecies is given by the presence of the F allele for esterase and the S allele for tetrazolium oxidase in the Cave Dale population of *A. a. ater*. The differences in allele frequency between the two populations are highly significant ($P < 0·01$) for both enzymes. The 21 individuals carrying the esterase F allele, and two individuals heterozygous for the S allele of tetrazolium

Table 7. *Annual variation in allele frequencies for esterase and tetrazolium oxidase in the garden population of* Arion ater rufus *at Sheffield*

(No observations on tetrazolium oxidase were made in 1968.)

	Esterase			Tetrazolium oxidase		
Year	n	I^B	S	n	F	S
1968	248	0·895	0·105	—	—	—
1969	416	0·947	0·046	416	0·682	0·318
1970	310	0·916	0·084	622	0·697	0·302
1971	406	0·916	0·081	592	0·644	0·356

oxidase, were typically *A. a. ater* in appearance, having black body colour, grey/black foot fringe, together with a grey foot sole. Genital morphology was not examined in these animals.

At Sheffield there is a rather sharp transition zone between the *rufus* garden population and the meadow population of *ater*. Animals of the two populations are recognizable even in the overlap zone not only by the differences in colour, but also by a difference in body size which is apparent during the summer months from about May onwards. Specimens of *rufus* are larger on average than *ater*. Table 6 gives data for three enzymes in samples taken from both populations in the same year (1969), including leucine aminopeptidase for which three genotypes can be recognized: FF homozygotes with a single anodally migrating fast band, SS homozygotes with a single slowly migrating band, and FS heterozygotes with two bands – each with about half the intensity of staining of the single band of either of the respective homozygotes. The garden *rufus* population is polymorphic for esterase, tetrazolium oxidase and leucine aminopeptidase, whereas the adjacent meadow population of *ater* appears to be monomorphic for each of these three enzymes. In contrast to the situation at Castleton there is no evidence for gene flow from the *rufus* into the *ater* population. The possibility that gene flow has taken place in the reverse direction in both populations is discussed below.

(iv) *Stability of the polymorphism for esterase and tetrazolium oxidase*

Allele frequencies for esterase and tetrazolium oxidase in the garden population of *A. a. rufus* in consecutive years are shown in Table 7. For esterase $\chi^2 = 11{\cdot}4$, $P < 0{\cdot}02$ for heterogeneity over the 4-year period for which data are available. The greater part of the heterogeneity here is due to the reduction in frequency of the S allele in 1969, the frequencies remaining stable in the ensuing 2 years at just below the level of 1968. The allele frequencies for tetrazolium oxidase remained stable over the 3 years 1969–71 (χ^2 4·2, $P > 0{\cdot}20$). There is no evidence for any directional change in allele frequency for either of these enzymes during the period of observation, and consequently there appears to be a balanced polymorphism for esterase and tetrazolium oxidase in this population.

Table 8. *Number of individuals of each genotype, and the corresponding allele frequencies, for esterase and tetrazolium oxidase in* Arion ater rufus *sampled from different localities in the Netherlands*

	FF	FI^N	I^NI^N	p(F)	q(I^N)	χ_1^2
Groningen	39	111	0	0·890	0·110	0·05
Leeuwarden	93	15	1	0·922	0·078	0·20
Zwolle	90	12	0	0·941	0·059	0·40
Gouda	95	5	0	0·975	0·025	0·06
Valkenburg	57	11	2	0·893	0·107	0·90
			Tetrazolium oxidase			
	FF	FS	SS	p(F)	q(S)	χ_1^2
Groningen	40	7	3	0·870	0·130	4·57**
Leeuwarden	26	53	26	0·500	0·500	0·01
Zwolle	45	36	21	0·618	0·382	6·52**
Gouda	95	5	0	0·975	0·025	0·06
Valkenburg	62	12	5	0·861	0·139	8·10**

(χ_1^2 tests the agreement between the observed and the expected Hardy–Weinberg distribution of genotype frequencies; ** denotes differences significant at the 1 percent level of probability.)

(v) *Genotype frequencies in populations of* Arion ater rufus

In three of the populations for which data are given in Table 8 there are significant departures from the expected Hardy–Weinberg distribution of genotypes for tetrazolium oxidase. These samples were collected in early summer (June) of the same year. Comparable data for English populations of *rufus* are given in Table 5 (χ_1^2 0·18, $P > 0·9$ for Hardy–Weinberg agreement in the village populations at Castleton). Table 9 shows that the goodness of fit for the genotype distributions in the garden population at Sheffield between spring and mid-summer is also excellent in two successive years. Combined data for May–July 1970 for this population is plotted on a de Finnetti diagram, together with that for the Castleton and the Netherlands populations, in Fig. 1. Populations from Leeuwarden and Gouda are in good agreement with expectation, whereas those from Groningen, Zwolle and Valkenburg each show a significant shortage of heterozygotes (Table 8).

The slugs are hermaphrodite, functioning as fertile males and females with reciprocal cross-fertilization. Williamson (1959) reports that in *A. a. ater* self-fertilization frequently occurs among specimens kept in captivity. Broods of offspring resulting from cross-fertilization usually also include selfed offspring as well. If selfing commonly occurs in the natural state this might be expected to reduce the frequency of heterozygous genotypes depending on the actual level of inbreeding in a given population. The sample size for each of the populations in question is small. Consequently, the level of inbreeding would have to be high to be detectable in such small samples. Inbreeding coefficients of 0·38 (Groningen), 0·25 (Zwolle) and 0·37 (Valkenburg) would be required to give rise to the observed deficiency in the frequency of heterozygotes in these populations. Such high levels

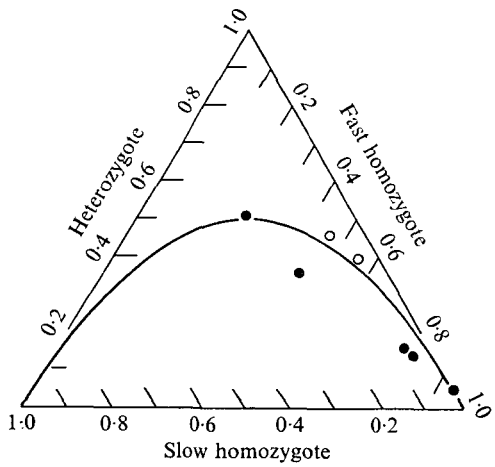

Fig. 1. Genotype frequencies for tetrazolium oxidase in different populations of *Arion ater rufus* plotted on a de Finetti diagram. Populations from the Netherlands are shown using solid circles and English populations with hollow circles. Populations in exact agreement with the Hardy–Weinberg distribution are expected to lie on the parabola, those with a shortage of heterozygotes fall below it (see Li, 1955).

of inbreeding would imply a considerable incidence of selfing. Notwithstanding the possibility that selfing may occur in *A. a. rufus*, and that the relative immobility of these animals is likely to increase the probability of sib-mating, it seems unlikely that the observed deficit of heterozygotes is due to inbreeding. Genotype frequencies for esterase, scored in the same individuals as were scored for tetrazolium oxidase, are at each locality in good agreement with the Hardy–Weinberg expectation. The high inbreeding coefficients necessary to explain the deficiencies of heterozygotes for tetrazolium oxidase would be expected to lead to a corresponding attenuation of heterozygosis for esterase in the same population. There is no evidence for this in any of the samples.

Negative-heterosis implying reduced fitness of heterozygotes, relative to the corresponding homozygotes, seems unlikely. It could be local in occurrence, but would be difficult to reconcile with the evidence for positive heterosis discussed below.

A shortage of heterozygotes could arise if the samples contain a mixture of individuals from two populations differing widely in their gene frequencies. The data in Table 7 show that the frequencies of esterase allele I^N tend to be rather similar in different localities, whereas allele frequencies for tetrazolium oxidase are much more variable. The collecting sites from which these samples were taken were strips of grass or rough herbage between a road and drainage channel bordering grazing or cultivated land. These margins were usually some 3–5 yards wide and approximately 100–200 yards long. Although apparently uniform, these habitats would impose upon the slug population something approaching linear continuity. Under these conditions there is the possibility that a given sample includes individuals from two different subpopulations or semi-isolates with similar

Table 9. *Seasonal variation in allele frequencies for tetrazolium oxidase in the garden population of* Arion ater rufus *in two successive years*

	FF	*FS*	*SS*	p(*F*)	q(*S*)	χ_1^2
1970						
April/May	73	53	11	0·726	0·274	1·00
June/July	48	43	13	0·668	0·332	0·68
August/September	28	40	2	0·686	0·314	7·43**
1971						
June/July	45	45	11	0·668	0·332	0·00
August/September	70	106	19	0·631	0·369	5·44**

(χ_1^2 tests the agreement between the observed and expected Hardy–Weinberg distribution of genotype frequencies; ** denotes differences significant at the 1 percent level of probability.)

allele frequencies for esterase, but with widely differing allele frequencies for tetrazolium oxidase. This could give rise to an apparent deficiency of heterozygotes for that enzyme.

More detailed information is required in order to decide with certainty between these possibilities, which are not mutually exclusive, but the data again serve to illustrate the difficulties inherent in attempting to make inferences about the breeding structure of populations in the absence of a secure understanding of how the sampling method was related to their spatial dispersion (Burnet, 1961).

Grouping of the data to test for seasonal variation is possible for the years 1970 and 1971. Although the sample sizes are small, analysis shows that in both years there is a significant excess of heterozygotes for tetrazolium oxidase towards the end of the summer. No comparable effect was found for esterase. Table 9 shows allele frequencies in samples collected in early, middle and late summer. In August/September 1970 there is a significant departure ($\chi_1^2 = 7{\cdot}4$, $P < 0{\cdot}01$) from the Hardy–Weinberg distribution of genotypes caused by an excess of heterozygotes. No comparable excess is present in the samples collected during the earlier part of the year. In 1971 there is again evidence for an excess of heterozygotes in samples collected in the later part of the summer ($\chi_1^2 = 5{\cdot}4$, $P = 0{\cdot}02$), but the effect is not perhaps as marked as in the preceding year. Since the breeding season extends well into the late summer these data suggest that in this population heterosis could be an important factor in the maintenance of balanced polymorphism for tetrazolium oxidase.

4. DISCUSSION

Populations of *Arion ater rufus* at different localities in the Netherlands are polymorphic for different electrophoretic variants of digestive gland esterase and salivary gland tetrazolium oxidase. The English populations of *A. a. rufus* resemble their continental European counterpart with respect to body colour and the presence of the brightly coloured orange foot fringe. Present in both of these populations, which are also polymorphic for these enzymes are alleles (*Esterase-F* and *Tet. ox-S*), which are present in all the Netherlands populations. In contrast, populations of *A. a. ater* sampled at different localities in the British Isles are similar to one another in their colour characteristics and different from *rufus*

Table 10. *Variant alleles for nine separate enzymes in* Arion ater rufus *distinguished by starch-gel electrophoresis*

(Esterase-1 and tetrazolium oxidase-1 are the enzymes for which data are given in the preceding tables.)

Enzyme	Number of alleles	Hybrid bands
Esterase		
1	5	No
2	2	No
3	3	No
4	1	—
Tetrazolium oxidase		
1	2	Yes
2	2	No
3	3	Yes
Leucine aminopeptidase	2	No
Malate dehydrogenase	2	Yes

populations. They are monomorphic for both enzymes. These facts lend support to the suggestion (Cain & Williamson, 1958) that *A. a. rufus* is an accidental import of continental European origin.

The diffusion of alleles (*Est.-F* and *Tet. ox-S*) from the *rufus* into the adjacent *ater* population at Castleton provides evidence for gene flow. It proves that hybridization occurs on contact between the two forms, and validates the subspecific status presently accorded to them (Quick, 1960). The presence in both of the English *rufus* populations of the alleles *Est.-I^B* characteristic of the endemic *ater* populations provides evidence for gene flow in the reverse direction – from *ater* into *rufus*. On the assumption that the immigrant founder populations of *rufus* had an array of gene frequencies resembling those of the Netherlands populations (i.e. high frequency of *F* and low frequency of I^N), there has evidently been an inflow of I^B alleles into the *rufus* populations. In the population at Sheffield, *F* has been almost totally replaced by I^B: only one *F* allele was present in a sample of 1380 as a clue to its presence. Development of a new balanced polymorphism for *S* and I^B must have followed replacement of *F* in this population. On this interpretation the Castleton *rufus* population is of more recent introduction than that at Sheffield and may not yet have reached a stable equilibrium.

Although the Netherlands is a likely place of origin for the immigrant *rufus* in view of the longstanding inflow of horticultural products from that country, there is, of course, no evidence that the two English *rufus* populations originated there rather than in some other part of Europe. Neither do we know how far the genetic structure of *rufus* in the Netherlands is typical of continental European populations in general. However, the fact that the typical esterase alleles (*F* and I^N) of *rufus* in the Netherlands are also present in the sample from Switzerland suggests that their range of occurrence is rather extensive.

The allele frequencies for tetrazolium oxidase in the two English *rufus* popula-

tions fit well within the range of variation found in the Netherlands, and the evidence for an excess of heterozygotes in the Sheffield *rufus* population raises the interesting possibility that heterosis is an important factor in the maintenance of balanced polymorphism. Whether hybrid vigour for this enzyme is general in the subspecies or, on the contrary, a localized phenomenon cannot be answered at present, but studies on genotype frequencies in the Netherlands in late summer and autumn may well answer the point and also indicate whether the apparent shortages of heterozygotes in certain instances, described here, are real, or an artifact of the sampling method used.

Populations of *A. a. rufus* encountered in the present study are markedly polymorphic for colour and for the three enzymes for which data are given here. There appears to be an appreciable level of heterozygosity for other gene loci as shown by the results summarized in Table 10. The subspecies is polymorphic for eight out of nine loci controlling enzyme proteins which have been studied so far. Although relatively small samples have been used, no comparable variation at these loci has so far been detected in *A. a. ater*. Since *A. a. ater* is polymorphic for colour (Williamson, 1959), the subspecies probably differ only in average levels of heterozygosity. The extent to which the apparently lower average probability of heterozygosity in *A. a. ater* may be a consequence of inbreeding, particularly due to selfing is an interesting problem worthy of further investigation.

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