Population Differentiation in *Enoplognatha ovata* (Araneae: Theridiidae) and the Detection of Selection

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Summary

1. Samples of *Enoplognatha ovata* from 14 populations in the Stockholm archipeligo, Sweden, were assessed for colour morph frequency and for levels of variation at 4 polymorphic allozyme loci, Amylase (AMY), Glucose-phosphate isomerase (GPI), Lactate dehydrogenase (LDH) and Phosphoglucomutase (PGM).
2. AMY and GPI were polymorphic in all populations studied, PGM was polymorphic in all but population 12. LDH was monomorphic in the majority of populations with a heterozygosity of 0.0298 (direct count). ANOVA showed that there were no significant differences in colour morph frequency between and within islands indicating no geographic pattern.
3. θ averaged over all allozyme loci ($\theta_{Allozyme}$) indicated that genetic variation between populations is low (1.9%) and that most variation (98.1%) is found within populations. θ_{Colour} was found to be significantly different from $\theta_{Allozyme}$ indicating that some form of weak selection is operating in different directions in different populations. This is the first time that selection has been shown to operate within local populations.
4. The direction in which selection has been shown to operate suggests that at intermediate colour morph frequencies local divergent selection operates. One possible explanation for this is that habitat heterogeneity between sites may be an important factor despite previous studies which have found no correlation with habitat variables at other sites.
5. At first it may appear that there is disagreement between this study, whereby selection is shown to operate within local populations, and previous studies, whereby genetic drift was implicated as the primary factor determining colour morph frequencies. However, it is suggested that whether selection or genetic drift determines morph frequencies is a function of effective population size whereby the disturbance history at some sites is responsible for the extraordinarily large role of genetic drift in shaping morph frequency.

KEYWORDS: *Enoplognatha ovata*, population differentiation, allozyme variation, selection, colour polymorphism.

Introduction

The determination of the adaptive significance of visible genetic polymorphisms and the mechanisms by which these are maintained in nature is an issue central to the study of population genetics. The significance of such polymorphisms have been shown in a number of systems, one of the most thoroughly investigated of which is of shell colour and banding in *Cepea nemoralis* and *C.hortensis* which appear to be maintained by a complex interaction of environmental heterogeneity and genetic factors (see Jones, Leith and Rawlings, 1977). Colour polymorphisms have proved ideal for this kind of study because there is a direct link between the phenotype in question and its genetic control. An investigation into the mechanisms by which such visible polymorphisms are maintained in nature can be used as an initial step in determining their adaptive significance.

Whilst knowledge of population differentiation has been used to study the partitioning of genetic variation within and between populations and to calculate rates of gene flow its use as an indirect method to test for the presence of selection, as used in this study, is relatively new. It has been used to infer selection acting between populations (e.g. Prout and Barker, 1993; Mithen, Raybould & Giamoustaris, 1995) and to test for selection acting on morphological characters along a cline in *Drosophila melanogaster* (Long and Singh, 1995). In addition it has recently been used for the first time to test for selection in *Theridion grallator* a species of spider exhibiting a rampant colour polymorphism (Gillespie and Oxford, 1998).

E. ovata, the candy-striped spider, is a widespread annual spider which exhibits a rather simple colour polymorphism under the genetic control of a single autosomal locus (Oxford, 1983). Three colour morphs are apparent: lineata (Y) has a plain yellow opisthosoma, redimita (S) has a pair of dorso-lateral carmine stripes superimposed over the opithsoma whilst ovata (R) has a carmine shield superimposed on the opithosoma. The colour polymorphism exhibits a dominance hierarchy such that ovata is top dominant and lineata bottom recessive. Studies of *E.ovata* date back to the 1930s (for example Bristow, 1931), however very little is known about how the colour polymorphism is maintained in nature. There is circumstantial evidence at the global scale that the polymorphism is being maintained: In every population studied thus far there is a consistent rank order of colour morphs (Y>S>R); additionally the polymorphism has a widespread geographical distribution across Europe and North America. A very similar

polymorphism also exists in a sister species *E.latimana* (Oxford and Reillo, 1993). This suggests that the colour polymorphism originated in a common ancestor and has, thus, survived for a long period of time. Golding (1992) has argued that such polymorphisms, whose origins predate speciation events, can only have survived under strong selection. In addition to this circumstantial evidence at the global scale there is also some evidence that selection may be operating along weak climatic clines in Great Britain (Oxford, 1985a) and also in western Europe (Oxford and Reillo, 1993) although the biological significance of the latter is unclear.

Despite this there is no evidence, either direct or indirect, showing that selection acts within local populations to maintain the polymorphism. Indeed, studies utilising perturbation experiments have been unable to show that selection operates and have implicated genetic drift is the primary factor determining morph frequencies most of the time (Oxford and Shaw, 1986; Reillo and Wise, 1988). In addition, modelling experiments have also shown that a simple stochastic model can give a very close fit to the temporal changes in allele frequency observed in local populations (Oxford and Shaw, 1986).

The aim of this project was to use an indirect method to test for the presence of selection and, if present, for indications of the strength and direction of selection operating. The approach used was to compare the degree of population differentiation at the colour locus with that at putatively neutral allozyme loci. Population differentiation for neutral loci is a function of genetic drift and migration, both of which should affect all loci similarly. Therefore, if the degree of population differentiation at the colour locus is significantly different from that at the allozyme loci then the action of selection on the colour locus can be inferred. The magnitude of difference between the two sets of loci should tell us the strength of selection operating: the larger the difference the stronger the selection. Additionally the direction of the difference should tell us the type of selection which is operating: If population differentiation is greater for the colour locus than for the neutral markers then selection is operating in different populations in different directions. In the opposite case, if population differentiation is greater for the neutral markers than for the colour locus then selection would be acting in a global fashion to keep morph frequencies similar between populations.

Materials and methods

Samples of mature female spiders were collected between 5 and 15 August, 1997 by Dr Geoff Oxford and B. Gunnarson (University of Goteborg, Sweden) from 17 sites on islands of the Stockholm archipelago of Sweden. Three sites, 4, 9 and 14 were not used in this study due to a small number of samples. In some cases sites are on separate islands, however, several sites are on the same island but geographically separated (see Fig. 1). Spiders were collected from roadside patches of broad-leaved vegetation. Collection at this time of year has several advantages: Gravid females are found in rolled leaves, guarding egg-sacks, thus collection is independent of colour morph and contamination from ingested foodstuffs can be discounted as a confounding factor. Additionally, as only females were collected, the complication of an interaction between the colour locus and a putative regulatory locus can also be avoided (Oxford, 1993). Collected spiders were stored under cool conditions and transported to York where they were preserved at -80^{o}C until required.

Two previous studies on the same samples of spiders have assessed the levels of variation at two polymorphic loci, Glucose-phosphate isomerase (GPI) and Lactate dehydrogenase (LDH) (St Pier, 1998; Woodhouse, 1998). Further work by Dr. Geoff Oxford during the summer of 1999 identified a further 2 polymorphic loci, Amylase (AMY) and Phophoglucomutase (PGM). The first objective of this project is to build on these earlier studies by assessing levels of variation at the AMY and PGM loci in the 14 populations, and secondly to identify other polymorphic loci and to assess their levels of variation. All data will then be combined for data analysis.

Allozyme frequencies were determined using cellulose acetate electrophoresis (Richardson, Baverstock & Adams, 1986). Individual spiders were homogenised in 75mM dH_20, centrifuged at 13,000g for 5 minutes and the supernatant applied to Titan III cellulose acetate sheets (Helena laboratories). Running conditions for enzyme systems are given in Appendix 2. Buffer recipies are given in Appendix 3. Stain recipes followed Herbert and Beaton (1993) and Richardson *et al*. (1986). Stains were applied using an agar overlay technique.

Figure 1. Sample sites in the Stockholm archipeligo, Sweden.

STRATEGY

30 allozyme systems (see Appendix I) were initially surveyed for staining activity using samples of *E.ovata* from Yorkshire and control samples of *Drosophila melanogaster*. Of these, 23 showed some level of staining but only 14 stained sufficiently. These 14 systems were tested using differing running buffers, run times and voltages and in some cases differing staining recipes to find suitable conditions. Only 4 systems, encoded by 6 different loci, (amylase [AMY], phosphoglucomutase [PGM], isocitrate dehydrogenase 1 and 2 [IDH-1 and IDH-2], and Glycerol-3 phosphate dehydrogenase 1 and 2 [α-GPDH -1 and α-GPDH - 2]) were found to resolve sufficiently. A second PGM locus, PGM-2, was not scored due to unreliability in staining.

The 14 populations were tested for polymorphism at each of the 6 loci with a sample size of 12 individuals from each population. Two of these loci, AMY and PGM, were found to be polymorphic and thus useful in determining genetic structure. A further 12 individuals from each population were then assayed for variation at these 2 polymorphic loci. To ensure that heterozygotes were correctly identified, heterozygotes from different populations were run together in a calibration gel.

DATA ANALYSIS

The degree of population differentiation as revealed by allozyme variation was investigated using the FSTAT (Goudet, 1995) and TFPGA (Miller, 1997) computer programs. Each locus was tested for accordance to Hardy-Weinberg equilibrium using Haldane's exact test (1954). Wright's F-statistics were estimated using Weir and Cockerham's variance component, θ (1984). Allele frequencies for the colour locus were calculated according to Hedrick (1985). F_{st} for the colour locus was calculated according to Weir (1996). Fisher's RxC test was used to determine if significant differences in allele frequencies exist between populations. Contingency table Chi^2 analyses were used to test for significant associations between loci. One sample t-tests were used to determine whether Nei's (1978) heterozygsity levels differed from mean values across populations. One-way ANOVA was used to determine if there were significant differences in colour morph frequency between and within island groups (see Fig. 1 for groupings). Colour morph frequency was first tested for adherence to ANOVA

assumptions - normally distributed (Kolmogrov-Smirnoff test) and homogeneity of variance (Levene's test).

Results

Allele frequencies for all 4 polymorphic allozyme loci are given in Table 1. Colour morph frequencies are given in Table 2. Three alleles were detected at the PGM locus and their distributions differed: PGM was polymorphic in all but one population with allele 2 predominating and allele 1 occurring at very low frequency with population 12 being monomorphic for allele 2. A third allele was present at low frequency in populations 5, 13, 15 and 16. No geographic trend in the occurrence of allele 3 is apparent. AMY was highly polymorphic for all populations studied with two alleles detected. Table 3 gives results of exact tests for deviation from Hardy-Weinberg proportions at either locus (Haldane's exact probability test). Highly significant differences in allelic distributions across populations were found with probabilities of no differentiation of 0.0000, 0.0000, 0.0389 and 0.0000 for GPI, LDH, AMY and PGM respectively (Fishers RxC test).

Table 3. Exact tests for Hardy-Weinberg Equilibrium (Haldane, 1954).

Locus	P	S.E.
GPI	0.3853	0.0223
LDH	1.0000	0.0000
AMY	0.0663	0.0075
PGM	0.5938	0.0135

One-way ANOVA showed that there were no significant differences in colour morph frequency found between and within island groups (see Table 4.)

Similarly no significant differences in the level of heterozygosity at the 4 allozyme loci (Nei, 1978) were observed between populations (one sample t-test).

No association was found between the following allozyme genotypes and colour morphs: GPI (χ^2 , p=0.68, df = 4), AMY (χ^2, p = 0.79, df = 4), PGM (χ^2, p= 0.51, df = 2). A significant

association was found between the LDH and colour loci (χ^2, p=0.02, df =2). Thus LDH cannot be used as an independent measure of variation in these samples.

Table 4. One-Way ANOVA on colour morph frequencies between and within island groups. See Fig. 1 for groupings.

Colour Morph		Sum of Squares	df	Mean Square	F	Sig.
Y	Between Groups	0.130	8	1.620E-02	0.567	0.774
	Within Groups	0.143	5	2.858E-02		
	Total	0.272	13			
S	Between Groups	0.151	8	1.894E-02	0.946	0.551
	Within Groups	0.100	5	2.001E-02		
	Total	0.252	13			
R	Between Groups	4.457E-02	8	5.571E-03	0.911	0.569
	Within Groups	3.057E-02	5	6.115E-03		
	Total	7.514E-02	13			

Estimates of population differentiation (θ) at the GPI, LDH, AMY and PGM loci are 0.0174, 0.0144, 0.0156 and 0.0379 respectively (See Table 5.) An average θ can be estimated as 0.0190 across all allozyme loci. Bootstrapping over loci gave a 99% confidence interval of 0.0363 - 0.0155. θ for the colour locus was calculated as 0.0460.

Table 5. Wright's F-statistics (1951) for the GPI, LDH, AMY and PGM loci. Estimates were calculated according to Weir and Cockerham (1984). Heterozygosity levels are direct counts.

Locus	F_{is}	F_{st}	F_{it}	Heterozygosity
GPI	0.0157	0.0174	-0.0018	0.4573
LDH	-0.0086	0.0144	-0.0234	0.0298
AMY	0.0969	0.0156	0.0825	0.442
PGM	0.0289	0.0379	-0.0093	0.1275
Overall all loci	**0.0522**	**0.0190**	**0.0339**	0.2642
Bootstrap				
99% C.I. Upper	0.0948	**0.0363**	0.0804	
Lower	0.0118	**0.0155**	-0.0151	

Discussion

The significance of the results gained in this type of study rely heavily upon adherence to the assumptions made. Arguably the most significant of these assumptions is the contention that allozymes are selectively neutral. This issue has been contentious for some time and is far from being resolved sufficiently. The wider issue concerns the significance of the observed variability in the genomes of virtually all organisms studied thus far. The arguments fall into two schools: the first, which has been termed "neoclassical" (Lewontin, 1974) contends that genetic polymorphisms are selectively neutral and exist as transient polymorphisms as a result of genetic drift. The other school contends that many polymorphisms are the consequence of various forms of balancing selection. The allozymes chosen for this study are critical metabolic enzymes. Thus it is possible that the identification of different alleles resulting from amino-acid substitutions would have some effect upon the functioning of the enzyme which may in turn affect the physiology of the organisms and thus for such loci to be under some kind of selective pressure. As a corollary, taking a large number of polymorphic allozymes should approximate a selectively neutral sample.

Studies tend to reach different conclusions with regards to the selective neutrality of allozymes. For example, in a statistical study of the frequency distribution of single locus heterozygosity among protein loci in 95 vertebrate and 34 invertebrate species Fuerst, Chakraborty & Nei (1977) reached the conclusion that the majority of data are consistent with the mutation-drift hypothesis, in other words, that the majority of variation is selectively neutral. However, in a study of the genetic variability of *Euphydras* butterflies McKechnie, Erlich & White (1975) showed that the neutrality assumption was untenable for the allozyme loci studied. Selection was implicated as the major factor responsible.

Thus it is clear that the contentious issues surrounding the use of allozymes as neutral markers is far from being resolved and conclusions reached should bear this in mind.

THE USE OF CELLULOSE-ACETATE GEL ELECTROPHORESIS

Although the use of cellulose acetate electrophoresis for studies of population differentiation is widespread there are some noteworthy problems with this method in a study such as this with tight time and budget restrictions.

The number of variables upon which correct staining and interpretation relies upon is large and often only one of these has to fail before gels either do not stain or become uninterpretable. These problems were found to be significant in this study, which was attributed to the use of old chemicals and the use of old samples which had likely been subjected to many freeze-thaw cycles before being used in this study, presumably affecting the tertiary structure, and thus activity, of enzymes.

- Significant problems were encountered with the reliability of the staining activity of various enzyme systems during the initial enzyme screening. The problem was eventually identified as being due to the age of certain chemicals used in the stain.

- Problems were encountered with high levels of condensation formation on the cellulose of the gels whilst gels were running. The problem was eventually identified as being due to the wide temperature variations in the laboratory between day and night and was solved by installing the electrophoresis apparatus in a refrigerator to equalise temperatures.

- Several enzyme systems were rejected from the initial screening due to the difficulty in interpreting the bands in a simple mendelian fashion. Other systems were rejected due to their unresolvability despite several combinations of buffer systems and running conditions. It is possible that given more time the ideal conditions for more enzyme systems could have been determined. Guidelines for appropriate buffer pH, running times and voltages and stain recipes are available for cellulose acetate electrophoresis (see Herbert and Beaton, 1993 and Richardson *et al.* 1986) but these conditions are dependent upon the system in question and since this series of projects are the first to use cellulose acetate electrophoresis in *E.ovata* these ideal conditions have not yet been determined fully.

- In many cases the amount of homogenate remaining for this study was very low and only sufficient to run one set of plates for both AMY and PGM. Any spurious or subjective results were discounted from further analysis since repeats could not be done.

Despite the problems alluded to above no problems with age-related enzyme modifications (such as wide, smeary bands) were encountered when using freshly homogenised spiders, despite their being stored for over 2 years prior to usage. However, samples which were

homogenised in 1997 and later stored at -80^{o}C did show problems which were attributed to their age. For example, AMY, a very consistently staining enzyme system became difficult to score objectively and some samples had to be repeated. PGM showed a reduced staining activity which was overcame by increasing the quantities of active reactants in the stain recipe and leaving gels to stain for longer periods of time. In some cases PGM activity from old samples produced wide, smeary bands which did not resolve clearly by repeating the samples. These subjective results were not included in the data analysis shown here.

It is surprising that so few enzyme systems were found to stain reliably and at a high enough level for scoring. Enzymes chosen for testing are critical metabolic enzymes which should be present in *E.ovata*. In some cases crude homogenate from *D.melanogaster* also failed to stain which suggested a problem with the stain ingredients or conditions rather than a lack of enzyme activity. Had fresh chemicals been used throughout this initial screening process it is possible that more systems could have been developed sufficiently to assess the levels of allozyme variation in the 14 populations.

The addition of NADP to the grinding medium was tested to see if it had any noticeable effect on enzyme resolution. It had no effect on the systems tested except that its presence altered the enzyme activity of several useable enzyme systems. Another problem with age-related enzyme modification is the presence of sub-banding on some gels. The use of β-mercaptoethanol is recommended to reduce this (Richardson *et al.* 1986), however for the systems under use it was found to be ineffective. The best method for reducing these problems would be to minimise the number of freeze-thaw cycles between homogenisation and application to gels.

VARIATION AT THE COLOUR LOCUS

Every population of *E.ovata* examined was polymorphic for colour and only in population 10 was the top dominant morph missing. There were no difficulties in distinguishing redimata and ovata morphs as reported by Hippa and Oksala (1979). Colour-morph frequencies varied between populations but there were no significant deviations between and within island groups (One-way ANOVA) indicating no geographical trend in colour morph frequency.

Figure 2. shows the distribution of the redimata and ovata colour morphs at each site. Only at site 3 is morph frequency highly skewed. No geographical pattern in colour morph frequencies is apparent.

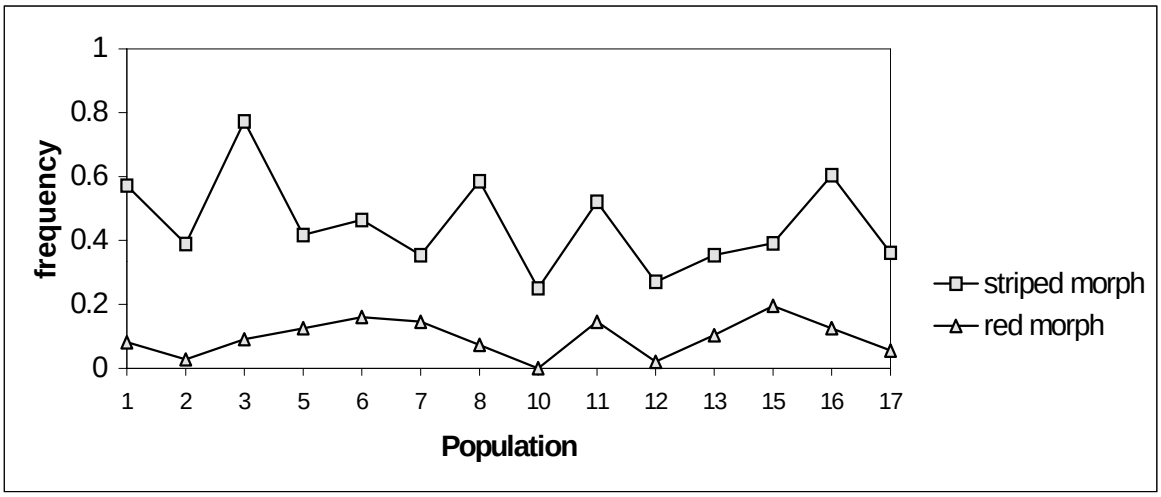

Figure 2. Spatial distribution of colour morph frequencies for the 14 study sites.

ALLOZYME VARIATION

Two loci were found to be polymorphic, AMY and PGM. Thus allele frequencies for a total of 4 loci are available for data analysis. Richardson *et al.* (1986) suggest that 6 polymorphic loci at a 90% confidence level is sufficient. Our results fall short of that required and thus care must be taken when interpreting results.

No population has a level of heterozygosity (averaged over all loci) which is significantly different than the average (t-test, $t = 0.003$, $df = 13$, $p = 0.998$) indicating that there is no further genetic structuring in each population. Only population 10 is fixed for the fast allele at the PGM locus, all others being polymorphic.

Table 5. gives the estimated F-statistics for each allozyme locus. The Fst values for allozymes calculated here are similar to that found in other studies. For example, Douwes and Stille (1988) looked at conspecific populations of Embla butterflies to assess the effect of selective versus stochastic processes in genetic differentiation. They calculated an average F_{st} value of 0.02 for allozyme markers which was quoted as being comparable to that of allozymes in mice from a study by Selander *et al.* (1971). Our results are also similar to those found in *Danaus*

plexipus (Monarch butterfly) and *Pieris rapae* with Fst values of 0.009 and 0.014 respectively (Eanes and Koehn, 1978).

These results indicate that over all allozyme loci only 1.9% of the genetic variation in this set of samples is found between populations, 98.1% being found within populations. At the colour locus a higher degree of genetic variation is found between populations (4.6%). The confidence intervals estimated by bootstrapping indicate that the degree of population differentiation is significantly greater at the colour locus than the allozyme loci. Thus, selection can be inferred as acting above the level of migration and genetic drift to keep differentiation at a higher level for the colour locus. This is a significant result since it is the first time that selection has been shown to operate at the local level.

The magnitude of difference in θ between the two sets of loci should tell us the net effect of selection, i.e. that operating above the level of genetic drift, rather than the absolute strength of selection. Thus, the small difference between the two sets of loci in this case suggests that the net selective forces operating in this system are weak. Thus selection may be acting strongly but is masked, partially in this case, by the strong action of genetic drift. Lawson and King (1996) during a study of garter snake melanism estimated an F_{st} value of 0.031 for allozymes and 0.150 for colour. The difference between the two sets is clearly much greater than in this study and implies that the selection operating in *E.ovata* is weak in comparison.

Recently Gillespie and Oxford (1998) have shown that the degree of population differentiation in the Hawaiian happy-face spider, *Theridion grallator*, based on variation at eight allozyme markers was greater than θ for the colour locus thus suggesting that some form of balancing selection, such as bird mediated predation, is likely operating.

It is at first surprising that in this study we have shown that selection is operating in different directions in different populations to increase variability at the colour locus. It is difficult to see from this result how such selection could be involved in maintaining the colour polymorphism. In contrast, I suggest that the selection operating in this case could be a result of habitat heterogeneity between sites. It is conceivable that there are habitat variables at each site which are important to the differential survival of colour morphs; thus selection could favour different morphs in different populations at intermediate frequencies and it is only when frequencies become highly skewed that some form of balancing selection operates to protect

the polymorphism. Other investigations into the role of selection at the local population level at Nidderdale, UK were unable to find any correlation between differentiation and various habitat variables (Oxford and Shaw, 1986 and see Oxford, 1993) however, it is possible that either the situation in Nidderdale is unusual in some fashion or that there is some habitat variable which is important but which has been overlooked.

All studies of local populations of *E.ovata* have been unable to infer the action of selection. Rather, genetic drift has been implicated as the major force determining colour morph frequencies (Oxford and Shaw, 1986; Oxford, 1991; Reillo and Wise, 1988). Thus the results given here appear to contradict those of previous studies. However, this may not be so. Below I speculate as to why the action of weak selection was detected in this case but other researchers have been unable to identify such selection at other sites:

Most of the work at the local population level has been done at Nidderdale in Great Britain, a site known to have been heavily disturbed in the past century (Oxford and Shaw, 1986). Another study site in Pembrokshire (Oxford, 1991) is also likely to have been disturbed to some unknown extent due to the nature of land-uses changes in Great Britain over the last several centuries. This situation may not be the same for the islands within the Stockholm archipeligo - it is generally accepted that islands have been less heavily disturbed than mainland sites (Oxford, 1989). At these disturbed sites it is likely that the high levels of disturbance have resulted in a patchy, fragmented series of habitats each with a low population size. If this is the case then genetic drift may play an extraordinarily large role in shaping local morph frequencies at these sites thus, overshadowing the weak selection. At the less disturbed sites in Sweden, the habitat may be more continuous leading to larger effective population sizes and thus the relative strength of genetic drift in these populations may be less, thereby allowing the weak effect of selection to be detected. Whilst it is pure speculation that high levels of disturbance have caused habitat fragmentation and its possible consequences, this suggests an avenue for further work.

Thus, whether colour morph frequencies are determined primarily by genetic drift or selection at intermediate morph frequencies may be a function of effective population size.

The results shown here suggest several avenues for further work:

1) An important follow-up would be to test the hypothesis presented above. This could involve a similar approach to that taken here but by selecting sites with large population sizes and continuous habitat and comparing the results to sites with smaller, fragmented habitats - ideally variation at many more allozyme loci would be assessed to give greater confidence in results.

2) An investigation involving the detection of selection at sites where the colour morph frequencies have been manually skewed would be able to detect whether selection acts in a balancing fashion in contrast to that shown here.

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Appendix 1.
Enzyme systems initially tested for staining activity.

Enzyme system	Abbreviation	Staining activity	Variability
Acid Phosphatase	ACP	Weak	
Adenylate Kinase	AK	Glass Slide only	
Alcohol Dehydrogenase	ADH	Weak	
Aldehyde Dehydrogenase	AlDH	Weak, Fuzzy	
Aldehyde Oxidase	AO	Weak, Fuzzy	
alpha-alpha-Trehalase	alpha-alpha-T	Glass Slide only	
Amino aspartate transferase	AAT	Strong	Uncertain interpretation
Amylase	AMY	Strong	Yes
beta-hydroxybutyrate Dehydrogenase	beta-HBDH	No	
Esterase	EST	Weak	
Fumarate	FUM	Weak	
Glucose Dehydrogenase	GLDH	Weak	
Glucose Phosphate Isomerase	GPI	Strong	Yes
Glyceraldehyde-3-Phosphate DH	G3PDH	Strong	No
Glucose-6-Phosphate Dehydrogenase	G6PDH	Strong, Fuzzy	
Glutamate Dehydrogenase	GDH	Weak	
Glycerol-3-phosphate Dehydrogenase	alpha-GPDH	Strong	No
Hexokinase	HEX	Glass Slide only	
Isocitrate Dehydrogenase	IDH	Strong	No
Lactate Dehydrogenase	LDH	Strong	Yes
Leucine Amino Peptidase	LAP	Strong, bad resolution	
Malate Dehydrogenase	MDH	Strong, bad resolution	
Malic Enzyme	ME	Strong	Not easily interpretable
Mannose Phosphate Isomerase	MPI	Glass Slide only	
Phosphoglucomutase	PGM	Strong	Yes
6-Phosphogluconate Dehydrogenase	6PGDH	Weak, Fuzzy	
Sorbitol Dehydrogenase	SDH	Weak, Fuzzy	
Superoxide Dismutase	SOD	No	
Xanthine Dehydrogenase	XDH	Glass Slide only	
Xanthine Oxidase	XO	Glass Slide only	

Appendix 2.
Running conditions for loci used to screen for polymorphisms

Enzyme System	Buffer	Run time (minutes)	Voltage	# of Applications	Stain recipe	Staining time (minutes)
AMY	Tris-Glycine pH 8.5	40	200	2	See Below *	40
PGM	Tris-Glycine pH 8.5**	40	200	2	H & B (1993)	5
IDH	Tris-Maleate pH 7.8	30	200	2	H & B (1993)	20
alpha-GPDH	Phosphate, pH 6.3	20	200	2	H & B (1993)	40-60

* Stain Recipe for AMY:

1. Starch solution: 0.5g agar, 0.12g starch, 30mg NaCl in 50mls Tris/Glycine buffer pH 8.5.
2. Stain gel on agar/starch layer for 40 minutes
3. Add solution of 2.5g Iodine and 1.25g KI in 500ml water.

** Tris-Maleate was used with samples homogenised in 1998 to aid resolution

Appendix 3.
Agar and Buffer recipes

Agar overlay (Herbert and Beaton, 1993):

1.6g Bacterial grade agar
100ml dH_20
Stored at 60^oC.

Tris-Glycine buffer, pH 8.5:

3.03g Tris
14.41g Glycine
Make up to 1 litre with dH_20

Tris-Maleate buffer, pH 7.8:

12.11g Tris
4.64g Maleic acid
Make up to 1 litre with dH_20

Phosphate buffer, pH 6.3:

Solution A: 0.04M $NaH_2PO_4.2H_2O$ (4.68g/7850 ml)
Solution B: 0.04M Na_2HPO_4 (2.13g/375ml)