Ann. Zool. Fennici 47: 278–286 ISSN 0003-455X (print), ISSN 1797-2450 (online)
Helsinki 30 August 2010

Genotype shifts along one generation of the blue butterfly *Polyommatus coridon* without changes in allele frequencies

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Received 1 Sep. 2009, revised version received 2 Dec. 2009, accepted 15 Dec. 2009

Kühne, G. & Schmitt, T. 2010: Genotype shifts along one generation of the blue butterfly *Polyommatus coridon* without changes in allele frequencies. — *Ann. Zool. Fennici* 47: 278–286.

Evolutionary and ecological effects of DNA and protein polymorphisms are controversially discussed, since the raising of molecular approaches. Functional differences among different genotypes of allozymes might strongly influence evolutionary processes and spatial distributions. Therefore, we analysed nine polymorphic allozyme loci for 127 individuals sampled from one population of the chalk-hill blue *Polyommatus coridon* during one flight season. We observed fluctuations in the genotype frequencies during this flight season, but only two enzyme loci showed significant changes. The percentage of observed heterozygosity was decreasing in more than three quarters of all loci over time (e.g. *Mdh2* decreasing from 72.7% to 36.4%). These changes might be due to different adaptations of heterozygotic individuals if compared with homozygotic ones. In contrast to genotypes, no changes in allele frequencies were observed (e.g. low F_{ST} value for *Mdh2*). Therefore, allozyme data represent a suitable tool for studying ecological adaptation and biogeographical pattern.

Introduction

Analyses of DNA polymorphisms in a wide range of different animal and plant species have gained increasing importance in the fields of taxonomy, ecology and population genetics (e.g. Ayala 1976, Oxford & Rollinson 1983, Loxdale & den Hollander 1989, Watt 1994). Nevertheless, the analysis of protein polymorphisms is still a powerful tool in this field (e.g. Schmitt 2007, 2009). However, the application of genetic marker systems in most cases assumes neutrality of the different alleles (Avise 2000).

Although this neutrality hypothesis often might be true, numerous studies using allozyme polymorphisms in extant populations have revealed positive as well as negative impacts on selection processes. These impacts are often due to different physiological characteristics of various allozyme alleles that, therefore, perform differently in changing environments (Eanes 1999). As is well known, different genotypes of some allozyme loci show remarkable differences in their kinetic parameters (e.g. k_m and V_{max}) and thermostability (Watt 1977, 1983, Watt *et al.* 1983, 1996, 2003). Although high frequencies of genetic polymorphisms have been demonstrated for large numbers of different allozyme loci (cf.

Johannesen *et al*. 1996, 1997, Vandewoesteijne *et al*. 1999, Schmitt & Hewitt 2004a, Habel *et al*. 2005, 2009, Schmitt *et al*. 2005a, Schmitt & Haubrich 2008), most of the analyses addressing the functional aspects of allozyme polymophisms of native populations and their fitness consequences were performed with one single locus, the phosphoglucose isomerase (*Pgi*).

In *Colias* butterflies, genetic differences in *Pgi* have remarkable effects on the performance of individuals: (i) The proportion between *Pgi* homozygotic and heterozygotic individuals changes during the flight period starting in Hardy Weinberg equilibrium and ending up with a strong heterozygote excess (Watt 1977). (ii) Also the flight performance during a single day depends on the *Pgi* genotype with the heterozygotic individuals performing better than the homozygotic ones as regards the time of flight initiation, breadth of flight time and overall densities throughout the day (Watt *et al*. 1983). (iii) The likelihood of a male *Colias meadii* mating a female is higher for *Pgi* and *Pgm* heterozygotic individuals than for their homozygotic conspecifics most probably due to heterosis effects (Watt *et al*. 1996). (iv) Finally, different adaptation advantages result in genotype distributions along ecological gradients of temperature and altitude (Watt *et al*. 2003).

Comparing different species of *Colias* butterflies, similar electromorph genotypes were detected for the *Pgi* locus (Watt *et al*. 1996). However, these similar electromorphs do not represent identical alleles but only similar electromorphic characteristics. The resulting tradeoff situation (heat adapted but less kinetically active *versus* temperature sensitive but kinetically more active) clearly explains the *Pgi* adaptation and selection to the respective habitat conditions. A similar phenomenon was described by Karl *et al*. (2008) for the copper butterfly *Lycaena tityrus* with the *Pgi* genotype 2-2 being one of several genotypes in lowland populations but being mostly fixed in Alpine populations. This genotype allows a quicker recovery from chill coma, thus maybe representing a better adaptation to alpine environments with their extreme climatic conditions. However, the analysis of a set of 18 allozyme loci showed only a strong geographic differentiation in *Pgi* (Karl *et al*. 2009). Also studies of the glanville fritillary *Melitaea cinxia* revealed longer moving distances under suboptimal conditions in *Pgi* heterozygotic individuals (Niitepold *et al*. 2009) and dependence on lifespan and egg hatching rate (Klemme & Hanski 2009).

Furthermore, analyses of the *Pgi* locus in the butterfly *Maniola jurtina* (Goulson 1993) and the moth *Epiphyas postvittana* (Gu 1991) revealed a strong influence of the different genotypes on the flight capacity of the respective individuals. Thus, *M. jurtina* showed an increase of the frequency of the best suited genotype for flight in higher (and thus windier) locations. These and also other findings demonstrate that functional differences among different genotypes of allozymes might strongly influence long-lasting evolutionary processes and hence spatial distributions.

These analyses show that *Pgi* and, although less documented, *Pgm* are strongly influenced by selection. However, such phenomena are much less studied in other loci for which high frequencies of polymorphisms are well known. Furthermore, it is widely unknown to what extent kinetic differences among genotypes of these and other frequently polymorphic enzyme loci of the glycolysis (cf. Habel *et al*. 2005, 2009, Schmitt *et al*. 2005a) have a significant influence on the population genetic structure along a single generation. Thus, some alleles or genotypes might enable an enhanced larval development resulting in temporal genotype or even allele shifts within a single generation.

As an example, we selected the chalk-hill blue *Polyommatus coridon*, a species known for its high genetic diversity at various allozyme loci (Schmitt & Seitz 2001, 2002a, 2002b, Schmitt *et al*. 2002, 2005a). The long flight season (mid-July to mid-September, males mostly have vanished by the beginning of September) and the close ecological linkage to semi-natural calcareous grasslands in central Europe (Ebert & Rennwald 1991, Schmitt 1998) make this species particularly suitable for this type of study. We, therefore, sampled 127 male individuals of one *P. coridon* population along its entire flight season of the year 2005 from mid-July to the end of August and analysed nine polymorphic allozyme loci.

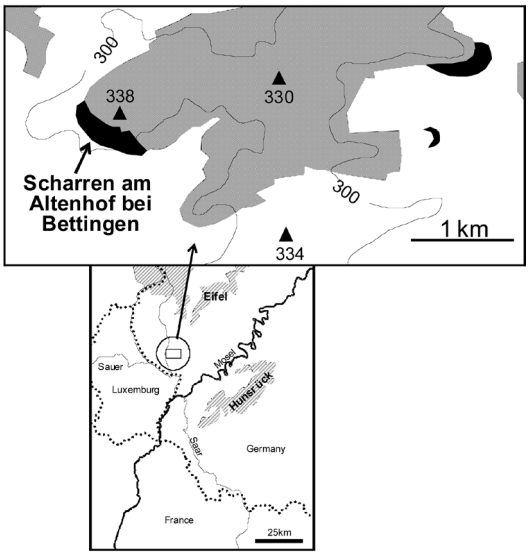

Fig. 1. Sampling locations of the four temporal samples of *Polyommatus coridon* in the nature reserve Scharren am Altenhof bei Bettingen. Semi-natural calcareous grasslands are given in black, forests are grey, hay meadows and fields are white.

By this analysis we want to answer the following questions:

i. Do genetic shifts in allele and genotype frequencies take place along one single generation of the calcareous grassland butterfly *P. coridon*?
ii. If so, do only the genotype frequencies change over time, or do also the allele frequencies vary along one generation?
iii. What are the consequences for the evolution of the genetic make-up of allozyme polymorphisms?
iv. What are the implications of these results for the analyses of allozyme polymorphisms in ecology and biogeography?

Material and methods

Study species

The chalk-hill blue *Polyommatus coridon* (Poda 1761) is a common lycaenid butterfly species of semi-natural calcareous grasslands (Weidemann 1986, Ebert & Rennwald 1991, Tolman & Lewington 1998, Asher *et al*. 2001) and is widely distributed in southern and central Europe (Kudrna 2002). As these habitat types are strongly fragmented in the otherwise intensively used agricultural matrix of central and western Europe, populations are mostly isolated from each other (Weidemann 1986, Ebert & Rennwald 1991, Asher *et al*. 2001). The butterflies are sedentary and only rarely disperse between different habitat patches; so far only some cases of long distance dispersal are known (Ebert & Rennwald 1991, Asher *et al*. 2001, Schmitt *et al*. 2006a). In western Germany, the larvae exclusively feed on *Hippocrepis comosa* (Ebert & Rennwald 1991), but more eastern populations frequently feed on *Coronilla varia* (Settele *et al*. 1999). As well known for many lycaenid butterflies, *P. coridon* is mutualistically associated with several ant species; the larvae secrete sugars and amino acids, which are harvested by the ants; in return, the larvae are defended by the ants (Fiedler *et al*. 1992, Jordano & Thomas 1992).

Sampling

A total of 127 male individuals were hand-netted on 18 (33 individuals) and 27 July (35) 2005 as well as on 18 (36) and 23 August (23) 2005. Sampling was carried out between 11:00 and 15:00 mostly under constant weather conditions (sunny, no or little wind, about 25 °C). The sampling design of one sample per week was hindered by constantly rainy weather conditions during the first half of August. The study area is the nature reserve Scharren am Altenhof bei Bettingen (Fig. 1) southwest of Bitburg (western Rhineland-Palatinate). This keuper hill (338 m a.s.l.) has a well preserved semi-natural calcareous grassland area of 6.7 ha (Jungbluth *et al*. 1995). The individuals were collected at a homogeneous grassland without ecological and climatic gradients in the south-western part of the hill.

Electrophoresis

Half of the abdomen of each individual was homogenised in *Pgm* buffer (Harris & Hop-

kinson 1978) by ultrasound and centrifuged at 17 000 g for 5 min. Electrophoresis was run on cellulose acetate plates (Hebert & Beaton 1993). The electrophoresis conditions are given in Schmitt and Seitz (2001). The alleles were classified according to their relative mobility (allele 1 being the slowest). A total of six common enzyme systems representing nine polymorphic loci were analysed. All of them were involved in the metabolic pathways of the glycolysis and gluconeogenesis (Table 1). Therefore, all of them have major impacts on the energy supply of animals.

Statistics

The obtained data set was analysed with the programs Arlequin 2.000 (Schneider *et al*. 2000) for *F*-statistics (Weir & Cockerham 1984), AMOVA, hierarchical *F*-statistics and Hardy-Weinberg equilibrium (Louis & Dempster 1987) and G-Stat (Siegismund 1993) for allele frequencies, Mantel tests (Mantel 1967) and Nei's standard genetic distances (Nei 1978). The *U* test, Cochran *Q* test, Wilcoxon matched-pairs test, Kruskal-Wallis ANOVA and Person correlation were performed with STATISTICA (StatSoft Inc.). All means are given with their standard deviations.

Results

The average number of alleles detected per locus per capture day was 3.0 ± 0.3 (mean ± SD), ranging from 2.9 to 3.4. The percentage of polymorphic loci was 91.7% ± 5.6%, ranging from

Table 1. The six analysed enzyme sytems, their abbreviations and EC numbers, number of loci (isoenyms) and their function in metabolic pathways.

Enzyme system	EC numbers	Isoenzym	Function
Aspartate-aminotransferase (Aat)	2.6.1.1	Aat1 (cytosol), Aat2 (mitochondria)	Urea cycle, malate-aspartate shuttle: *reversible conversion of aspartate and alpha-ketoglutarate to oxaloacetate and glutamate*
Isocitrate-dehydrogenase (Idh)	1.1.1.41	Idh1 (mitochondria)	citric acid cycle (NAD-dependend): *reversible conversion of isocitrate to ketuglutarate*
	1.1.1.42	Idh2 (cytosol, mitochondria)	NADPH supply (NADP-dependend): *reversible conversion of isocitrate to ketuglutarate*
Malate-dehydrogenase (Mdh)	1.1.1.37	Mdh1 (mitochondria)	citric acid cycle, aspartic acid cycle, malate-aspartate shuttle (NAD* –> NADH + H*): *reversible conversion of malate into oxaloacetate*
		Mdh2 (cytosol)	citric acid cycle (after arginine biosynthesis), aspartic acid cycle, malate-aspartate shuttle (NADH + H* –> NAD*): *reversible conversion of malate into oxaloacetate*
6-phosphogluconate -dehydrogenase (6Pgdh)	1.1.1.44		Pentose phosphate pathway (cytosol): *conversion 6-phosphogluconate into ribulose-5-phosphate*
Phosphoglucose-isomerase (Pgi)	5.3.1.9		metabolic pathways of glycolysis and gluconeogenesis (cytosol): *reversible conversion of glucose-6-phosphate into fructose-6-phosphate*
Phosphoglucomutase (Pgm)	5.4.2.2		metabolic pathways of glycolysis and gluconeogenesis (cytosol): *reversible conversion of glucose-1-phosphate into glucose-6-phosphate*

88.9% to 100%. Applying a 95% criterion, the mean number of polymorphic loci was 66.7% ± 15.7% and range 55.6%–88.9%. The high percentage of polymorphic loci coincided with high observed heterozygosities (25.6% ± 3.0%, range = 21.8%–29.2%). Expected heterozygosity was 25.9% ± 3.1% (range = 21.6%–28.8%). All parameters of genetic diversity excluding the mean number of alleles per locus were decreasing from the beginning to the end of the flight period (Table 2). A constant decrease from the first to the last sampling day was observed for the observed heterozygosity of the loci *Mdh2*, *6Pgdh*, *Pgi*, *Pgm* and *Aat1* (Table 3), but only *Aat1* and *6Pgdh* were significantly correlated with time (Person correlation: $p = 0.031$ and 0.022, respectively).

F_{IS} including all individuals was not significant (0.022, $p = 0.22$). However, the values of two single loci were significant (*6Pgdh*: $F_{IS} = 0.478$, $p < 0.01$; *Aat2*: $F_{IS} = 0.199$, $p = 0.01$). Treating the daily samples as units, the difference among them was significant ($F_{ST} = 0.018$, $p = 0.025$) whereas the difference among the individuals within daily samples was not ($F_{IS} = 0.008$, $p = 0.35$). The highest pairwise F_{ST} value (0.0325, $p = 0.0088$) was between the first and the last sampling day; the second significant value was between the second and the third day with a rainy period in between (0.0224, $p = 0.0303$).

The most important contribution among the daily samples are delivered by the loci *6Pgdh* ($F_{ST} = 0.068$, $p = 0.001$) and *Mdh2* ($F_{ST} = 0.037$, $p = 0.021$). The overall F_{IS} values were not significant for each of the daily samples (all $p > 0.05$). However, three significant cases were obtained for the loci *6Pgdh* and *Aat2* (Table 4). The genetic distances (Nei 1978) among the four daily samples ranged from 0.024 to 0.039 (Table 5). No correlation between "days elapsed between two sampling events" and "genetic distances" (Nei 1978) was observed (Mantel test: $p > 0.4$).

We also tested the two samples at the beginning of the flight season in July before the rainy period (in the first half of August) against the two samples from the second half of August. The genetic difference between these two periods ($F_{ST} = 0.015$, $p = 0.017$) was a bit smaller than

Table 2. Average number of alleles per locus (*A*), expected (H_e), observed heterozygosity (H_o), proportion of polymorphic loci [total (P_{tot}) and at the 95% level (P_{95})] and number of individuals of each daily sample of *Polyommatus coridon* from the Scharren am Altenhof bei Bettingen.

	18.VII.2005	27.VII.2005	18.VIII.2005	23.VIII.2005	Mean	SD
A	2.9	3.4	2.8	2.8	3.0	0.3
H_e (%)	28.8	26.4	26.7	21.6	25.9	3.1
H_o (%)	29.2	25.3	26.1	21.8	25.6	3.0
P_{tot} (%)	100.0	88.9	88.9	88.9	91.7	5.6
P_{95} (%)	88.9	66.7	55.6	55.6	66.7	15.7
N	33	35	36	23	31.8	

Table 3. Observed heterozygosity (H_o, %) of nine polymorphic loci of *Polyommatus coridon* at the Scharren am Altenhof bei Bettingen on four sampling days of the species flight period in 2005.

Locus	18.VII.2005	27.VII.2005	18.VIII.2005	23.VIII.2005	Mean	SD
6Pgdh	9.1	8.6	0.0	0.0	4.4	5.1
Idh1	21.2	14.7	47.2	18.2	25.3	14.8
Idh2	9.1	11.4	3.0	4.5	7.0	3.9
Mdh1	3.0	0.0	8.3	4.5	4.0	3.5
Mdh2	72.7	50.0	52.8	36.4	53.0	15.0
Aat1	12.1	8.6	5.9	5.0	7.9	3.2
Aat2	17.2	31.4	16.7	40.9	26.6	11.7
Pgi	72.7	57.1	55.6	59.1	61.1	7.8
Pgm	45.5	45.7	45.5	27.3	41.0	9.1

Table 4. F_{IS} values of each daily sample and all individuals pooled. The F_{IS} values are given for all analysed allozyme loci of a population of *Polyommatus coridon* at the "Scharren am Altenhof bei Bettingen".

Locus	18.VII.2005	27.VII.2005	18.VIII.2005	23.VIII.2005	Total F_{IS}
6Pgdh	0.624*	–0.030	0	0	0.478**
Idh1	–0.106	–0.080	–0.075	0.147	–0.042
Idh2	–0.021	–0.034	0.000	0.000	–0.025
Mdh1	0	0	–0.019	0.000	–0.013
Mdh2	–0.363	0.048	0.040	0.054	–0.007
Aat1	–0.036	–0.020	0	0	–0.025
Aat2	0.323*	0.297*	0.170	–0.025	0.199**
Pgi	–0.255	–0.076	–0.017	–0.056	–0.136
Pgm	0.153	–0.073	0.093	0.042	0.084
Total F_{IS}	–0.014	0.021	0.025	–0.006	0.022

* $p < 0.05$; ** $p < 0.01$.

in the case of the four daily samples; F_{IS} was not significant (0.014, $p = 0.296$). Four parameters of genetic diversity showed more loci with decrease than increase, and only P_{tot} showed a balance; however, only the case of H_o was significant (Table 6).

Discussion

Our allozyme data give some evidence of changes in the genetic composition during one single flight season of one *P. coridon* population: More than three quarters of all loci showed decreasing amounts of heterozygotic individuals from the two July samples to the two August samples representing an overall significant decline rate. *Mdh2* was the most extreme case decreasing from 73% (18 July) to 36% (23 August). Furthermore, we observed a negative significant correlation between sampling date and percentage of heterozygotic individuals for two loci (*6Pgdh*, *Aat1*). Hence, heterozygotic individuals are the ones appearing with higher percentages than expected by Hardy-Weinberg equilibrium at the beginning of the flight season and at lower rates at the end of the respective generation.

If we compare the parameters of genetic diversity of the individuals sampled before and after the cool and rainy period in the first half of August, all parameters (except P_{tot} not showing a trend) support a decrease of genetic diversity; however, only the decrease in the observed heterozygosity was significant. Therefore, H_o might be the only factor of genetic diversity really affected. This coincides with the observation that the changes of the allele frequencies along the flight season were relatively weak as reflected in low F_{ST} values among the two and also four temporal samples. As an example, the percentage of heterozygotic individuals for *Mdh2* dropped by 50% along the flight season, but the allele frequencies did not change as reflected in a low F_{ST} value for this locus.

Table 5. Genetic distances (Nei 1978) of nine polymorphic loci of *Polyommatus coridon* at the Scharren am Altenhof bei Bettingen on four sampling days of the species flight period in 2005.

	27.VII.2005	18.VIII.2005	23.VIII.2005
18.VII.2005	0.0257	0.0243	0.0388
27.VII.2005		0.0339	0.0391
18.VIII.2005			0.032

Table 6. Number of loci showing decrease, increase or no change for different parameters of genetic diversity of the *Polyommatus coridon* individuals sampled in July 2005 at the Scharren am Altenhof bei Bettingen compared with the ones sampled in August 2005. Significance of differences between July and August samples was tested using a Wilcoxon matched-pairs test for H_e, H_o and A, and using a Cochran Q test for P_{tot} and P_{95}.

	Decrease	Increase	No change	p
H_e	7	2	0	0.139
H_o	7	2	0	0.038
A	4	1	4	0.178
P_{tot}	1	1	7	> 0.999
P_{95}	3	0	6	0.083

The decrease in the percentage of heterozygosity during the flight season might be due to an advantage of heterozygotic individuals in contrast to homozygotic ones. As the heterozygotic individuals comprise two (monomeric enzymes e.g. *Pgm*), three (dimers e.g. *Pgi*, *Mdh*, *6Pgdh*, *Idh*) or five (tetramers, no case studied in this survey) genetic types of the same enzyme, it is possible that the heterozygotic individuals are adapted to a greater variety of environmental and thermal conditions. Therefore, larval and pupal development times might be shorter in heterozygotic than in homozygotic individuals resulting in a somewhat earlier hatching of the former, and a prevailing of the latter at the end of the flight season. A further possibility is that the hatching is not dependent from being hetero- or homozygotic, but that this feature allows adult activities over a larger range of environmental conditions. This might result on average in an earlier death of heterozygotic individuals leaving homozygotic ones alive at the end of the flight season. A clear disentangling of both possibilities has to be analysed by further studies. The possibility of changes in allele frequencies and heterozygosities by dispersing individuals can mostly be ruled out as the amount of dispersing individuals is a rather small fraction of all individuals (Schmitt *et al*. 2006a).

Although selection seems to act with respect to homozygotic and heterozygotic individuals, there should not be a remarkable selection in favour of one of the main alleles because no major allele shift was observed in any of the loci analysed. This also should be expected as otherwise advantageous alleles would rapidly become fixed and disadvantageous ones would quickly disappear from the gene pool of a population. However, some weak effects of gene frequency shifts are detectable at the very end of the flight season when stochastic effects due to rapid senescence and death of many individuals might somehow impact the allele frequencies themselves. Furthermore, the long cool and rainy period during the first half of August might have similarly affected the population genetic structure, as a considerable amount of individuals of this thermophilic butterfly species might not have survived this period of disadvantageous conditions. Both of these shifts are mirrored in the pairwise F_{ST} values.

Although the genotypes might be under active selection and even might shift along one single generation, the allele frequencies themselves remain largely constant. This phenomenon was observed (i) along one single generation with only some minor changes most probably caused by the death of a larger proportion of the individuals (this work), (ii) over a series of generations (Raijmann & Menken 2000), or (iii) along climatic gradients (Besold *et al*. 2008).

Suitability of allozyme analysis for ecophysiological experiments is especially due to the fact that different genotypes and alleles show differences in their kinetics and stability thus making them subjected to selection and evolutionary processes; however, these effects have mostly been demonstrated for the *Pgi* locus (e.g. Gu, 1991, Goulson 1993, Watt 1994, Watt *et al*. 1996, 2003, Karl *et al*. 2008). As most of these effects are only relevant for a minority of the loci frequently studied, these effects might be stabilised over time. Thus, selection within the time of one generation might only affect genotype frequencies and not allele frequencies. While these allele frequencies are more important for those calculations most analyses in phylogeography are based on, allozymes also should represent a powerful tool to reveal the history of populations and taxa (e.g. Schmitt & Seitz 2001, 2004, Schmitt & Hewitt 2004b, Schmitt *et al*. 2005b, 2006b, 2006c, 2007, Schmitt & Haubrich 2008).

Acknowledgements

We acknowledge the grant of the Deutsche Forschungsgemeinschaft (grant SCHM 1659/2-1) enabling the allozyme electrophoresis. We are grateful to the authorities in Koblenz for the sampling permit and Karola Haubrich collecting the last *P. coridon* sample.

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