

Landscape structure and phenotypic plasticity in flight morphology in the butterfly *Pararge aegeria*

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In evolutionary time, varying environments may lead to different morphs as a result of genetic adaptation and divergence or phenotypic plasticity. Landscapes that differ in the extent of habitat fragmentation may provide different selection regimes for dispersal, but also for other ecological functions. Several studies on flying insects have shown differences in flight morphology between landscapes, but whether such differences result from plastic responses have rarely been tested. We did a reciprocal transplant experiment with offspring of speckled wood butterfly females (*Pararge aegeria*) from three types of landscape differing in fragmentation: woodland landscape, landscape with woodland fragments and agricultural landscape with only hedgerows. Young caterpillars were allowed to grow individually on potted host grasses in small enclosures under the three landscape conditions (split-brood design). Mortality in caterpillars was much higher in agricultural landscape compared to the other landscapes. Additive to the effect of landscape of development, landscape of origin also affected mortality rate in a similar way. Flight morphology of the adults resulting from the experiment differed significantly with landscape. Independent of the landscape of origin, males and females that developed in agricultural landscape were the heaviest and had the greatest wing loadings. Females that developed in agricultural landscape had higher relative thorax mass (i.e. greater flight muscle allocation) in line with adaptive predictions on altered dispersal behaviour with type of landscape. In males, relative thorax mass did not respond significantly relative to landscape of development, but males originating from landscape with woodland fragments allocated more into their thorax compared to males from the other types. We found significant $G \times E$ interactions for total dry mass and wing loading. Our results suggest the existence of phenotypic plasticity in butterfly flight morphology associated with landscape structure.

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In evolutionary time, organisms have in principle two alternatives to deal with varying environments. They can either follow the pathway of genetic adaptation and divergence, or of phenotypic plasticity. For instance, seasonal morphological variation can result from differential success of different genetic forms (e.g. *Cepaea nemoralis* – Cain and Sheppard 1954), or, of plastic

responses of the genotype based on environmental cues producing different phenotypic forms (e.g. seasonal polyphenism – Shapiro 1976). The reaction norm or the degree to which a genotype responds in a plastic way to environmental variation can be under genetic control, and hence, be subject to selection (Price et al. 2003).

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Habitat fragmentation is a widely recognised phenomenon that alters the environment in multiple ways for several organisms (Meffe and Carroll 1997, Hanski 1999, Fahrig 2003) providing different selection regimes. In this vein, several studies using flying insects as study models have shown differences in flight morphology between landscapes (Dempster 1991, Taylor and Merriam 1995, Thomas et al. 1998, Hill et al. 1999b, Norberg and Leimar 2002). Morphological patterns have typically been interpreted in terms of assumed changes in dispersal rates with habitat fragmentation, such as selection against mobility with increased isolation in line with Dempster et al. (1976).

Several of those flight-morphological traits (including total body mass, relative thorax mass, wing loading and forewing aspect ratio) have a heritable basis (Hill et al. 1999a, Berwaerts 2004), which is a prerequisite for evolutionary change. In seasonal environments, however, there is evidence for adaptive phenotypic plasticity in the same morphological traits in multivoltine butterflies (Fric and Konvicka 2002, Van Dyck and Wiklund 2002). Reaction norms for morphological and life history traits may differ – ultimately in adaptive terms – between geographic regions (Nylin et al. 1995) or even between populations at close proximity (Sibly et al. 1997). We are not aware of studies testing adaptive morphological plasticity in relation to landscape structure in a context of habitat fragmentation. Moreover, plasticity studies have only rarely been done under field conditions (Lorenzon et al. 2001). In the laboratory, environmental regimes with changed means without changes in variance appear to be the rule (but see Miner and Vonesh 2004). Besides practical constraints, the obvious reason to avoid field experiments relates to the fact that field conditions are typically too complex and variable. However, to test associations between phenotypic plasticity and landscape structure, reciprocal transplant experiments provide an interesting scope. Such experiments are of course limited because they do not allow insight in the environmental cues and causal factors that are operating.

In this paper, we evaluate to what extent a particular genotype produces different phenotypes in different landscapes using the speckled wood butterfly (*Pararge aegeria* L.) as a model. Following a split-brood approach, offspring of individual females was distributed evenly and individually between three landscapes. Furthermore, we monitored mortality during development and explored survival rate relative to landscape of origin and landscape of development. We test for adaptive differences in flight morphology assuming that dispersal rate has changed with landscape structure. Flight morphology includes relative thorax mass (i.e. allocation to flight muscles), wing loading and forewing aspect ratio (i.e. wing shape). For a detailed discussion of the functional significance see Dudley (2000),

Berwaerts et al. (2002) and Van Dyck (2003). Butterfly flight is multifunctional (Shreeve 1992, Watt 2003) and is not exclusively related to dispersal ability, which may confound interpretations (Van Dyck and Matthysen 1999). *Pararge aegeria* males and females differ, however, considerably in their flight behaviour relating to their different ecological roles (Van Dyck 2003). Males spend most of their active time in mate-location. They either adopt an aggressive territorial sit-and-wait strategy on a sunlit patch ('perching') or a search strategy in which a male explores a wider area looking for receptive females ('patrolling') (Wickman and Wiklund 1983, Shreeve 1987). In either case, mate-location mainly covers local movements. On the other hand, there is evidence that females are the more dispersive sex. At considerable distance outside woodland habitat, Baker (1984) only observed *P. aegeria* females traversing the landscape. This is anecdotal evidence, but stronger support comes from comparative work on flight morphology between recently colonised sites (at the edge of the distribution) versus permanently populated sites (at the core area of the distribution). Hill et al. (1999a) found significant differences in *P. aegeria* females only; females from newly established populations were larger, had larger thoraces and had lower forewing aspect ratios. In our earlier work testing variation in dispersal propensity in experimental cages, we found only clear responses in *P. aegeria* females (Merckx et al. 2003).

As a result of altered costs and benefits of dispersal, fragmented landscapes are expected to select for higher mobility since resources are no longer concentrated but scattered over different fragments (Olivieri and Gouyon 1997, Van Dyck and Matthysen 1999). Based on the literature of functional flight-morphology in butterflies (Dudley 2000, Van Dyck 2003) and as applied by Hill et al. (1999a), we predict females to have a larger size (larger total body mass), to have higher wing loading, to allocate more mass to flight muscles (higher relative thorax mass), and to possess lower aspect ratio with increasing degree of fragmentation. Elsewhere we have shown that mate-location is more frequently of an intermediate type between perching and patrolling in agricultural landscape compared to woodland landscape (Merckx and Van Dyck 2005), but this may have little impact when average values of flight morphology are tested among landscapes. So, we predict no differences in male flight morphology.

We can also test a prediction on size that follows from a constraint hypothesis and contrasts with the earlier predictions. We know that fragmented, agricultural landscape is on average warmer than woodland landscape (T. Merckx, et al., unpubl.). Higher ambient temperature makes larvae growing faster which leads to smaller adults (Sibly et al. 1997, Nylin and Gotthard 1998). Hence, from this constraint hypothesis, we predict smaller individuals in more fragmented landscape in

males and females, which is in contrast with our earlier predictions.

Finally, we also use the experimental data on flight morphology to test for significant genotype \times environment interactions as families (or genotypes) may differ in their plastic responses. Such variability is a prerequisite for phenotypic plasticity to respond to natural selection and hence to be adaptive (West-Eberhard 2003).

Methods

Study species

The speckled wood (*Pararge aegeria* L.) is primarily a woodland butterfly (Higgins and Riley 1993), but it also occurs in fragmented, agricultural landscape with only small and scattered pieces of 'woodland' habitat like woodlots and hedgerows (Dover and Sparks 2000, Merckx et al. 2003). This butterfly can use three developmental pathways corresponding to different seasonal cohorts: (i) development with a pupal winter diapause resulting in early-spring adults, (ii) development with a larval winter diapause resulting in late-spring adults and (iii) direct development resulting in summer generation adults (Wiklund et al. 1983, Nylin et al. 1989). Honeydew is the main adult feeding resource and several grasses can be used as larval host plant (Shreeve 1986). Eggs are deposited on isolated grass plants that grow under shaded, rather wet conditions surrounded by some bare ground (Wiklund and Persson 1983). Our recent work has pointed to several differences between speckled woods living in woodland landscape versus fragmented agricultural landscape, including flight morphology (Berwaerts et al. 1998), dispersal propensity (Merckx et al. 2003), temperature-related female fecundity (Karlsson and Van Dyck 2005), mate-location (Merckx and Van Dyck 2005) and habitat finding ability (T. Merckx, and H. Van Dyck, unpubl.).

Reciprocal transplant experiment

In the summer of 2001, we caught females in three different landscapes in central Belgium. Those were: (i) a landscape dominated by deciduous oak woodland (Meerdaalwoud, 1255 ha), (ii) a set of small oak wood-

land fragments (Boshoek, 1.3 to 19.0 ha) with relatively small interpatch distances (85 to 865 m), and (iii) a highly fragmented, agricultural landscape composed of intensively used fields and pastures (the large majority of the area) and to a lesser extent houses and farms, orchards, tiny woodlots and several sunken roads with hedgerows (Hoegaarden). We refer to the three as woodland landscape, landscape with woodland fragments, and agricultural landscape, respectively. The agricultural landscape consisted of 33 sunken roads with hedgerows that made up a total distance of 8.9 km. Only 3% of the study area was covered by six small (0.4 to 11.2 ha) and mainly poplar woodlots (Table 1). The caught females were transported to the laboratory and allowed to lay eggs on potted tufts of grass (*Poa trivialis*) in individual cages. Offspring of two females of each landscape was used in the experimental set-up: 30 second instar larvae of each female were placed in individual enclosures (nylon netting cover, size: 11 \times 11 \times 35 cm) containing a fresh tuft of potted *P. trivialis* each. Grasses had been reared in the laboratory under standardised conditions for light, ambient temperature, nutrition, water and soil substrate (Wiklund et al. 1983). The enclosures (n=180) were equally spread among the three types of landscape. They were placed at sites in each landscape where *P. aegeria* larvae were known to occur naturally. Cages were inspected daily and we recorded when larvae or pupae were dead or when adults were not fully eclosed. Freshly eclosed adults were collected, killed and stored by freezing (-20°C) for morphological measurements. The experiment covered a period from 16 May to 26 July. Next, we repeated the experiment from 24 July to 25 September (n=180). Given summertime conditions, all individuals followed a direct development to the adult stage (Wiklund et al. 1983).

Morphological measurements

Morphological measurements were according to the methods of Van Dyck and Wiklund (2002). Prior to measurement, specimens were dried in an incubator until constant mass during 24 h at 60°C . Total dry mass was measured with a microbalance (Mettler Toledo 5). Next, specimens were dissected by separating head, thorax, abdomen, legs and wings. Thorax mass was also

Table 1. Overview of percentages optimal and suboptimal habitat surface area for given study areas within three landscapes: (i) woodland landscape, (ii) landscape with woodland fragments, and (iii) agricultural landscape. Optimal habitat is oak woodland; Suboptimal habitat is all other habitat (deciduous woodland, structure rich conifer woodland, orchards, hedgerows, sunken rural roads). Based upon a biological validation map of Flanders (version 1.0–2.0–2.1, Institute of Nature Conservation, Brussels).

Landscape type	Study area	Optimal habitat	Suboptimal habitat
Woodland	138 ha	ca 85%	ca 15%
Woodland fragments	757 ha	9%	2%
Agricultural	555 ha	0%	3%+8.9 km sunken roads

weighed. Forewings were photographed (Olympus Camedia C-3030) to measure forewing length and forewing area using an image analyser system (Optimas 1999). Morphological characteristics were grouped into three measures: (1) size: total dry mass, (2) wing shape: aspect ratio ($4 \times \text{forewing length}^2 / \text{forewing area}$) and (3) relative allocation: relative thorax mass and wing loading (total dry mass/forewing area). Both forewing length and forewing area was correlated with total dry mass (Pearson correlation coefficients: $P < 0.0001$). Repeated measurements for mass and wing traits on 20 individuals showed high repeatabilities (between 0.99 and 1.00). Individuals with substantial wing wear were excluded from the analyses.

Analyses and statistics

Counts of dead larvae and pupae and counts of not fully eclosed adults were summed and contrasted with counts of full-grown individuals. Differential survival with landscape of development and landscape of origin was tested by means of χ^2 -tests. Flight-morphological traits were analysed in function of landscape of origin and landscape of development. After removing outliers of two males, original data were normally distributed. Main effects were tested using linear regression models that allow random as well as fixed regression coefficients (Proc Mixed, SAS 2001; Verbeke and Molenberghs 2000). Appropriate degrees of freedom were calculated using the Satterthwaite option (Littell et al. 1996). In line with the predictions, models were run separately for males and females. With the purpose of testing for $G \times E$ interactions, models also included family (i.e. genotype) (nested within landscape of origin) and session as random effects. For each trait it was tested by log-likelihood ratio whether the interaction of the family \times landscape of origin random parameter with landscape of development made a significant contribution to the model. If so, it meant that a significant $G \times E$ interaction was present. Final statistical models were obtained from the full models by the backward stepwise selection procedure eliminating non-significant factors. The non-significant factor with highest P-value was first removed; factors remained in the model as long as they also contributed in significant interaction effects. Residuals of the final models were normally distributed (Shapiro-Wilks statistics $W > 0.95$).

Results

The proportion of individuals that survived to the adult stage was significantly smaller in agricultural landscape (58%) compared to woodland landscape (74%) and landscape with woodland fragments (76%) ($n = 346$,

$\chi^2_2 = 10.59$, $P = 0.005$). Independent of landscape of development, there was also a significant effect of landscape of origin on survival rate ($n = 346$, $\chi^2_2 = 13.42$, $P = 0.001$). Fewer individuals that originated from the agricultural landscape survived to the adult stage (57%) than did individuals that originated from landscape with woodland fragments (72%) ($n = 229$, $\chi^2_2 = 5.44$, $P = 0.020$). Survival of individuals that originated from woodland landscape (79%) did not differ significantly to that of individuals from landscapes with woodland fragments. Even when restricting the analysis to butterflies that developed in the agricultural landscape, the butterflies that originated from this type of landscape still had higher mortality than the two other groups ($\chi^2_2 = 7.77$, $P = 0.02$). Lowest levels of mortality were found in woodland butterflies that developed in either woodland landscape or landscape with woodland fragments (21.6–10.0% mortality). As we worked with a small number of families, it is important to notice that those differences do not depend on a deviation in survival in just one female, but was consistent among offspring of different females. Independent of their landscape of origin, males and females that developed as larvae in the agricultural landscape were heavier and had higher wing loadings than individuals that developed in woodland landscape and landscape with woodland fragments (example female total mass: Fig. 1). Females that developed in the agricultural landscape had higher relative thorax mass than females that developed in the other two types of landscape. In males, relative thorax mass did not respond significantly in relation to landscape of development, but a landscape of origin effect was present. Males originating from the landscape with woodland fragments allocated more mass to the thorax than individuals from woodland landscape and agricultural landscape. Forewing aspect ratio did not differ with landscape of development, or with landscape

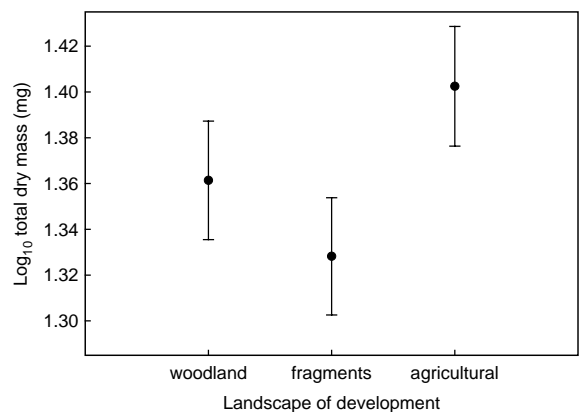


Fig. 1. Total dry mass of female *P. aegeria* offspring from different landscape origin that developed under woodland landscape, landscape with woodland fragments and agricultural landscape conditions in a reciprocal transplant enclosure experiment. We used means \pm SE.

Table 2. Summary of the analyses of different flight-morphological traits of *P. aegeria* offspring from a reciprocal transplant enclosure experiment in relation to landscape of origin (woodland landscape, landscape with woodland fragments or agricultural landscape) and landscape of development (idem). Mixed procedures (SAS) were run for both sexes separately. Full models included the factors landscape of origin, landscape of development and their interaction. Final models were obtained from these full models by the backward stepwise selection procedure eliminating non-significant factors. As models for thorax mass included total body mass as covariate, they represent variation in relative thorax mass.

	Variable	Effect		F	P
Males	total dry mass	landscape of development	F _{2,18.6}	11	0.001
	thorax mass	landscape of origin	F _{2,10.1}	7.9	0.009
		total dry mass	F _{1,105}	772	<0.0001
	wing loading	landscape of development	F _{2,21.8}	6.2	0.008
Females	total dry mass	landscape of development	F _{2,22.3}	6.6	0.006
	thorax mass	landscape of development	F _{2,96.6}	11	<0.0001
		landscape of origin	F _{2,7.66}	3.8	0.071
		total dry mass	F _{1,93.1}	580	<0.0001
	wing loading	landscape of development	F _{2,17.3}	4.2	0.034

of origin (Table 2). Hence, we observed landscape-related phenotypic plasticity for total dry mass, wing loading and relative thorax mass. For the latter trait, however, plasticity was only present in females.

In both sexes, individuals from different families had different responses to the same environmental conditions for total dry mass and wing loading. These differences in the observed phenotypic plasticity among genotypes imply that $G \times E$ interactions were present for these traits. With regard to relative thorax mass, a $G \times E$ interaction was only present in males. No $G \times E$ interaction could be detected for aspect ratio.

Discussion

Our reciprocal transplant experiment in different landscapes, following a split-brood approach, indicated that adult offspring of *P. aegeria* females differed significantly in adult flight morphology between landscapes. This suggests the existence of phenotypic plasticity associated with landscape structure as the same genotype produced a different morphology in, for instance, a woodland landscape versus a highly fragmented agricultural landscape. Interestingly, the observed phenotypic plasticity for body size contrasts with the constraint hypothesis, which predicts smaller individuals with fragmentation in both males and females due to higher growth rates with higher ambient temperatures (Sibly et al. 1997, Nylin and Gotthard 1998). Since our results demonstrated the opposite, they rather point to adaptive differences.

Although our experimental set-up principally aimed to evaluate plastic responses in flight morphology, it also revealed significant differences in juvenile mortality among types of landscape. A much higher mortality in agricultural landscape for caterpillars of this woodland butterfly points to a novel aspect of habitat fragmentation that requires further research. Besides the direct effect of higher mortality under agricultural landscape

conditions, the additive significant effect of landscape of origin suggests some divergence among the populations and types of landscape with respect to the capacity of larvae to deal with (or tolerate) different microclimatic conditions. It shows that evolutionary ecological studies in a context of habitat fragmentation should not be limited to adult ecology alone. Moreover, it needs to be evaluated to what extent an increased mortality biases the variation in phenotypic expression in adults between the landscapes. Increased developmental mortality in fragmented landscapes may be of more general importance in contexts of conservation and environmental change, but to our best knowledge there is still little evidence for such effects. In the leaf miner *Phytomyza ilic*, prevalence was higher and survivorship to adulthood lower at the woodland edge compared to interior parts of the woodland (McGeoch and Gaston 2000). Experimental and manipulative studies are vital if the subtle interaction of factors responsible for high and variable juvenile mortality is to be understood in Lepidoptera and other insects (Zalucki et al. 2002).

In line with the assumption of changed dispersal rate with landscape structure, we showed that individuals that developed in the agricultural landscape were heavier and had higher wing loading than those that developed in woodland landscape and landscape with woodland fragments, irrespective of their landscape of origin. Although these effects were similar among males and females, phenotypic plasticity for relative thorax mass was only present in females. This is in line with the prediction that an adaptive response of variation in flight morphology is easier to detect in females. As predicted according to the adaptive hypothesis, females that developed in the agricultural landscape allocated more mass to flight muscles than did females that developed in woodland landscape and landscape with woodland fragments. However, effects were not consistent for all flight-morphological traits: in both sexes a significant difference among landscapes was absent for aspect ratio. Relative thorax mass was the only trait for which landscape of origin played a role, but only

in males. Males originating from the landscape with woodland fragments allocated more mass to the thorax than individuals from the woodland and agricultural landscape. Clearly this needs further investigation, as it cannot be explained in terms of an adaptive response to changes in dispersal capacity with landscape structure as in females.

Finally, our experimental data indicated that individuals from different families responded in a different way to the same landscape structure. In other words, we showed that different genotypes possessed different reaction norms. Significant $G \times E$ interactions were present for total mass, wing loading, and male relative thorax mass. Such interactions indicate that the observed landscape-related phenotypic plasticity is adaptive (West-Eberhard 2003) and contributes to evolutionary changes in dispersal – and probably other flight-related behaviours that need further research – with changed landscape structure.

Which environmental cue(s) induce plastic responses in flight morphology among different landscapes? Our field experiment does not provide direct answers. Ambient temperature is a candidate because we know that sites in agricultural landscape where *P. aegeria* thrives are on average warmer, and show more variability in temperature than corresponding sites in woodland (T. Merckx et al., unpubl.). Organisms are not only able to induce different morphologies as a response to an environmental parameter with different means. They can also respond morphologically to the amount of environmental variability (Brakefield and Mazzotta 1995, Miner and Vonesh 2004). Hence, both average temperature as well as temperature variability could be cues within directly developing butterflies in order to predict future landscape structure conditions. Seasonal plasticity, on the other hand, operates among pathways. Van Dyck and Wiklund (2002) compared the three seasonal cohorts for the same flight morphological traits, and found evidence that photoperiod functions as the prime cue for this kind of plasticity in *P. aegeria*, although temperature can play a role as well (Stevens 2004).

It may not be a coincidence that we find landscape-associated plasticity in a species that also shows seasonal plasticity. Once a species has developed plastic responses to seasonal environmental conditions, it may have the advantage of using the same mechanisms to respond to other (micro) climate-related variation like variation between landscapes. This leads to the hypothesis that species with high levels of plasticity in morphology and life-history have a greater capacity to deal with changing and highly variable landscapes than other species. Contrary to several other butterfly species that show clear to dramatic declines (Maes and Van Dyck 2001), *P. aegeria* is indeed one of the few species that increased highly significant in distribution and abundance, at least

in NW-Europe (e.g. UK: Asher et al. 2001; the Netherlands: van Swaay and Groenendijk 2004).

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