

# Chapter 12

## The Use of Geometric Morphometrics in Studying Butterfly Wings in an Evolutionary Ecological Context

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### Idea and Aims

In order to quantify shape variation, many powerful, free, easy-to-use and dedicated software packages have been developed that quickly digitize and/or analyse landmark data (e.g. the tps suite by Rohlf, <http://life.bio.sunysb.edu/morph/>). Furthermore, powerful shape analyses can also be carried out in statistical packages such as R (Claude 2008). Unlike 10–20 years ago, these days it is therefore no longer difficult to accurately record the position of landmarks on any biological structure. Furthermore, such landmark configurations can easily be compared within and between species using a variety of analyses that together comprise the field of geometric morphometrics (Zelditch et al. 2004). The theoretical core of geometric morphometrics has been well described and is easy to understand, and as such geometric morphometrics can easily be implemented in a wide variety of research fields, such as evolutionary ecology (Zelditch et al. 2004). Using the speckled wood butterfly *Pararge aegeria* (L.) (Nymphalidae, Satyrinae) as our model species, we will illustrate the variety of uses to which geometric morphometrics can be applied to understand the effects of the environment on possibly adaptive butterfly wing size and shape variation in ecologically relevant contexts.

### Butterfly Wings and Their Function

In Pterygota (i.e. winged insects), fitness is strongly affected by flight performance (Speight et al. 2008). Behavioural activities such as locating oviposition and foraging sites, courtship, and predator avoidance are often highly dependent on an efficient and precise ability to fly. The design of flight morphology in winged insects is therefore expected to be under strong selection for these behavioural

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activities which involve staying aloft, manoeuvrability and thrust production (Betts and Wootton 1988; Dudley 2000). A higher wing loading (total body weight/total wing area) (cf. Betts and Wootton 1988) and wing aspect ratio (mean forewing length<sup>2</sup>/mean forewing area), for example, would indicate the capacity to fly faster and more extensively (Betts and Wootton 1988). This is, of course, a somewhat simplified view as studies on flight performance in butterflies have shown that flight speed and pattern also depend on factors such as thoracic mass (principally flight muscles), wing damage, centre of body mass, behavioral adaptations (e.g. predation avoidance), thermoregulation, wing asymmetry, and the ability to make use of spatial and temporal variation in the prevailing winds (Srygley and Chai 1990; Srygley and Dudley 1993; Srygley et al. 1996; Srygley 1999; Dudley 2000; Srygley 2000; Srygley and Kingsolver 2000; Srygley 2001, 2003; Berwaerts and Van Dyck 2004).

Male and female butterflies have different life-histories and differ in associated behaviour (Wiklund 2003). Males, for example, spend a significant portion of their time in pursuit of obtaining matings, either by patrolling or perching. Females typically focus on the search for suitable foraging and oviposition sites. Therefore, behavioural sexual dimorphism is often associated with differences in flight morphology and design of the flight apparatus (Breuker et al. 2007a).

Butterfly wings are also used for signalling (Breuker et al. 2006a). The development of the wing pattern elements is to some extent integrated in overall wing development (see later in this chapter). This means that butterfly wings experience a multitude of different natural and sexual selection pressures either simultaneously or sequentially, which will select for a particular wing size and shape in response to the environmental and ecological conditions experienced. These conditions are very likely to be heterogeneous across time and space. Often, what is actually not clear is how this spatio-temporal variation in environmental conditions affects the phenotypic variation of flight morphology.

## **The Concept of Developmental Plasticity in Ecology**

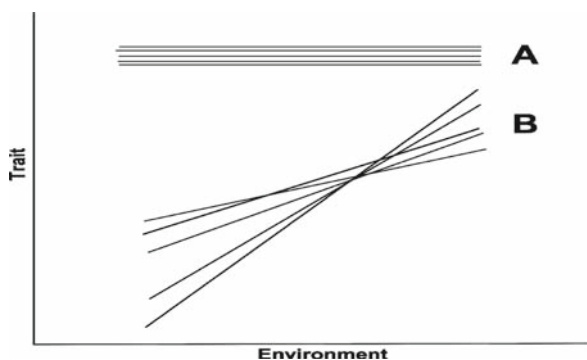
Individuals of the same butterfly species may be able to develop and live in a variety of habitats, or are forced to do so through habitat fragmentation (Hill et al. 2001, 2002; Dover and Settele 2009). Furthermore, the conditions that individuals encounter in these different habitats are very likely to change over time. Habitats may change either suddenly (e.g. a natural catastrophic event), on a regular and more or less predictable basis (e.g. seasonal variation), or progressively (e.g. due to global warming) (Vitousek et al. 1997; Hill et al. 2002). In order to track adaptive peaks, (local) genetic adaptation of flight morphology may occur, which tends to be a relatively slow process as this may take quite a number of generations. In perfectly constant environments, selection is likely to favour just one optimal wing size and shape. Given that most environments, however, are heterogeneous in both space and time, it may be more advantageous to allow for flexibility in wing development, or traits in general, and thus to be able to develop the most optimal flight design in each of the different environments (Bradshaw 1965; Stearns and Koella

1986; Stearns 1989; Schlichting and Pigliucci 1998). Selection may thus operate on the plasticity of traits relevant for survival and reproduction such as flight morphology, thereby ensuring that the development of these traits is flexible enough to allow for a quick response to varying conditions across space and time (Schlichting and Pigliucci 1998; Sultan 2003; West-Eberhard 2003; Sultan 2004). Selection for such developmental flexibility in the case of seasonal variation, for example, is highly likely when selection pressures are different between seasons (Shapiro 1976; Tauber et al. 1986).

This characteristic of development to respond flexibly and adaptively to environmental variability has been labeled developmental plasticity (for an overview of different views on the concept of plasticity, especially concerning its adaptedness, see West-Eberhard 2003), while the resulting phenotypes display a so-called phenotypic plasticity (Bradshaw 1965; Schlichting and Pigliucci 1998; Stearns 2000). Developmental plasticity is thus the modification of development by the environment. This may involve changes in the dynamics of the genetic regulatory network underlying development, thereby resulting in developmental switches (i.e. *developmental conversion* (Smith-Gill 1983)). In general, a set of phenotypes produced by a single genotype across a range of environmental conditions is known as a reaction norm (Woltereck 1909; Pigliucci 2005). A reaction norm with a non-zero slope indicates a plastic response (Fig. 12.1). An example would thus be a range of different wing sizes and shapes across a season or seasons, as for example has been found in damselflies (Bots et al. 2009). These reaction norms may be gradual (i.e. continuous plasticity) or stepwise (i.e. discrete plasticity, as in many examples of seasonal plasticity) (Schlichting and Pigliucci 1998). When individual reaction norms cross, there is genetic variability in the effect environmental heterogeneity has on development (i.e. so-called Genotype by Environment (GxE) effects). GxE effects are commonly observed in life-history traits (Stearns 1989; Via et al. 1995) and are thought to indicate that plasticity can evolve through selection (Schlichting 1989; Moran 1992).

Reaction norms commonly cross, and it is therefore often assumed that most, if not all, observed developmental plasticity is adaptive, and that plasticity is a trait that has been selected for (Scheiner and Lyman 1991). This need not be

**Fig. 12.1** Theoretical example of inter- and intra-population trait value differences. Reaction norms of five population ‘A’ families and five population ‘B’ families. Population A and B differ in both trait mean and variation. Population A displays no plasticity (hence no GxE)



the case, however, as the observed plasticity may simply be a non-adaptive by-product of the physiology and biochemistry of an organism interacting with the variable environment during development (Smith-Gill 1983; Via et al. 1995), with no genetic basis whatsoever (i.e. *developmental modulation* (Smith-Gill 1983)). Developmental conversions are therefore the most likely candidates for adaptive developmental plasticity, as they are presumed to have a genetic basis whereas developmental modulations do not (Smith-Gill 1983). A major complication of GxE studies in a classical quantitative genetic context is the fact that developmental modulation results in, sometimes crossing, reaction norms not unlike those resulting from developmental conversions of the gradual kind. In fact various researchers, for example Smith-Gill (1983), actually lump all *continuous* reaction norms under developmental modulation, and label it non-adaptive. This, however, is considered too restrictive by many (West-Eberhard 2003). There are various ways of testing whether an observed continuous reaction norm is of the developmental conversion or modulation kind, including estimating a selection surface or performing reciprocal transplant experiments. So far, however, the best understood cases of adaptive plasticity remain those where alternative phenotypes fall into discrete classes (e.g. seasonal polyphenism (Shapiro 1976) or wing polyphenism in ants (Abouheif and Wray 2002; Nahmad et al. 2008)).

## **Developmental and Phenotypic Plasticity in *Pararge aegeria* Wings**

Even in the case of seasonal polyphenism, relatively few attempts have been made to explicitly test the hypothesis that the seasonal forms are adapted to the season, and hence the environment individuals live in (Brakefield and Frankino 2009). Butterflies, however, including our model species *P. aegeria*, have been reasonably well-studied for their (adaptive) developmental and phenotypic plasticity of life-history traits and body morphology across space (i.e. different habitats and in relation to habitat fragmentation) and time (mainly seasonal variability in habitat conditions) (Nylin et al. 1989; Van Dyck and Matthysen 1999; Fric and Konvicka 2002; Norberg and Leimar 2002; Van Dyck and Wiklund 2002; Karlsson and Van Dyck 2005; Fric et al. 2006; Breuker et al. 2007a).

Developmental plasticity in *P. aegeria* across landscapes and in relation to habitat fragmentation is still poorly understood. Variation in a number of life-history traits and flight morphology is associated with landscape variability and habitat fragmentation (e.g. Karlsson and Van Dyck 2005), but whether this variation necessarily indicates adaptive plastic responses or merely local adaptation as a result of geographical isolation is unknown as formal tests assessing fitness curves for the plastic traits across landscapes are largely lacking. Merckx and Van Dyck (2006) recently addressed this issue by reciprocally transplanting larvae across different landscapes in a split-brood design. They found that body morphology and flight design showed landscape specific plasticity rather than local adaptation. However, one aspect of

flight design did not appear to be plastic: wing shape, measured as forewing aspect ratio. Nevertheless, this trait does show significant seasonal plasticity (Van Dyck and Wiklund 2002). In another respect this is a somewhat puzzling result as wing shape tends to be very sensitive to environmental variation in insects (Azevedo et al. 1998; Klingenberg et al. 2001; Hoffmann et al. 2002).

It should be noted that none of these *P. aegeria* evolutionary ecological studies used landmarks for the wing shape analyses, which we feel is somewhat of a shortcoming as geometric morphometrics allows one to quantify more subtle wing shape variation. To illustrate how geometric morphometrics can be applied in wing plasticity studies in an evolutionary ecological context, we present an outline of the methods to be used and the results of a recent and unpublished preliminary study.

In this study we tested the hypothesis put forward by Merckx and Van Dyck (2006) that variation in forewing shape in relation to landscape variability is predominantly environmentally determined (i.e. due to developmental plasticity). It has, however, been hypothesised for butterflies that natural selection has favoured changes in flight morphology due to the altered costs and benefits of dispersal in more fragmented landscapes (Hill et al. 1999a, b). Incidentally, studies investigating this hypothesis, for example in *P. aegeria* (Hill et al. 1999a), measured flight morphological traits such as thorax size and relative wing size, but not wing shape (e.g. measured by using a landmark-based approach). We can thus put forward the following hypotheses regarding *P. aegeria* wing shape. If landscapes strongly select for particularly shaped wings, then unrelated populations inhabiting similar landscapes should have similar wing shapes, different from populations from other landscape types, when reared under similar conditions. If, however, wing shape variation is largely due to environmental conditions experienced during development in a particular landscape, then similar rearing conditions are likely to result in a similar wing shape, irrespective of landscape of origin. Rather than performing (large-scale) quantitative genetic experiments in the laboratory to establish the exact role of genetic and environmental variation, and their interaction, on wing morphology (Loh and Bitner-Mathe 2005), we took a somewhat simplified approach in this preliminary study as the experimental design detailed below will make clear.

## Quantifying Wing Shape Variation in *Pararge aegeria*

### *Experimental Design*

Speckled wood butterflies were collected from eight different, unrelated, populations in Belgium. Only males were collected. Two representative populations were used from each of four landscape types: (1) large forested woodland (Meerdaalwoud and Bos Ter Rijst), (2) isolated wood (Averechten and Walsbergen), (3) fragmented woodlots (Boshoek and Glabeek), and (4) agricultural landscape (Hoegaarden and Rillaar). The agricultural landscape is a highly fragmented landscape consisting mainly of intensively used fields and pastures, and to a lesser extent of houses,

**Table 12.1** The eight different Belgian populations (abbreviations between brackets) – two different populations in each of four different types of landscape – where male speckled wood (*P. aegeria*) butterflies were collected. The number of males (N) is indicated as well as the suitability of the landscape type for *P. aegeria* (1 = poor, 4 = very suitable; ranking is based on Merckx and Van Dyck 2006)

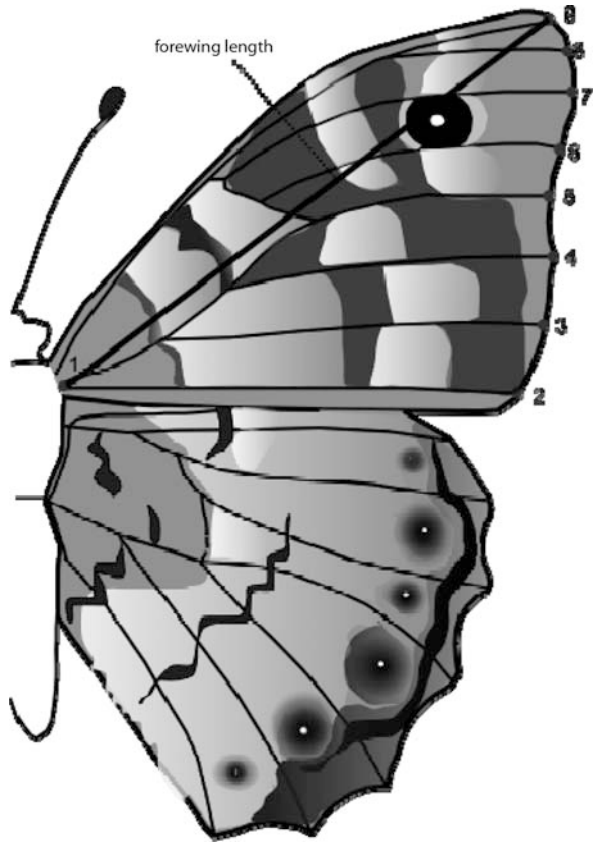
Landscape type	Site	Suitability	N
Large forested woodland	Meerdaalwoud (MDW)	4	17
Large forested woodland	Bos Ter Rijst (BTR)	4	22
Isolated wood	Averechten (AVE)	3	19
Isolated wood	Walsbergen (WAL)	3	18
Fragmented woodlots	Boshoek (BOS)	2	19
Fragmented woodlots	Glabeek (GLA)	2	18
Agricultural landscape	Hoegaarden (HGD)	1	24
Agricultural landscape	Rillaar (RIL)	1	19

farms, orchards and sunken roads with hedgerows. This landscape is largely unsuitable for *P. aegeria* containing no optimal habitat and only an estimated 3% (plus the length of the sunken roads) of suboptimal habitat (Merckx and Van Dyck 2006). Temperatures are highest in this type of landscape compared with the other landscapes in summer (Merckx and Van Dyck 2006; Merckx et al. 2008). For each population 20 individuals were collected, except for Bos Ter Rijst and Hoegaarden, for which 25 individuals were collected. Individuals with excessive wing wear, however, were excluded from the analyses, resulting in the sample size numbers given in Table 12.1.

The males were collected in early spring. This was done deliberately. *Pararge aegeria* butterflies flying in early spring spend the winter in a pupal diapause. Such individuals are very likely to have experienced similar microclimatic environmental conditions during the period that wing development, and thus the position of the wing veins is determined (see below), takes place in the pupa. The direct developing summer generation animals are for example much more likely to experience a range of developmental conditions in the different landscapes. We can thus investigate whether individuals originating from different populations inhabiting different landscapes differ in wing shape under similar rearing conditions in the field. We measured landmarks along the whole of the forewing, at locations where a wing vein meets the edge of the wing (Fig. 12.2), and analysed the data with geometric morphometrics (i.e. Procrustes analyses). For the sake of completion we also investigated forewing length, as this was found to be seasonally plastic in previous studies on *P. aegeria* wing plasticity (Van Dyck and Wiklund 2002).

The forewings were carefully removed from the thorax, placed in between two glass slides and digital images were then taken of the ventral and dorsal wing surface with an Olympus Camedia C-3030 under carefully controlled light conditions (cf. Breuker et al. 2007b). Photographs were randomized with respect to population and landscape type.

**Fig. 12.2** Ventral wing surface of a speckled wood *P. aegeria* butterfly. Numbers on the forewing indicate the landmarks measured (i.e. where a wing vein meets the edge of the wing). Forewing length (in mm) is the distance between landmark 1 and 9



### *Choosing Landmarks*

Given that landmark positions can convey important phylogenetic, developmental and functional information, one should carefully consider which landmarks to digitize and analyze before commencing any morphometrics study (Zelditch et al. 2004). Zelditch et al. (2004) list five criteria to choose landmarks. Landmarks should be (1) homologous, (2) to a degree independent from other landmarks in their location, (3) easy to identify and reliable to measure, (4) lie within the same plane, and (5) provide an adequate coverage of the morphological structure whose shape is to be measured.

In butterflies, and insects in general, the landmarks most often measured are the locations where a wing vein meets the edge of the wing and the wing vein intersections as these landmarks satisfy all five criteria (Dworkin and Gibson 2006; Breuker et al. 2007a, b). Wing vein based landmarks are very easy to identify and measure, and lie in the same plane, as a butterfly wing is a flat structure. In order to fully appreciate how these landmarks satisfy the homology and independence criteria,

and that these landmarks are actually biologically highly relevant when studying wing shape variation, we will briefly explain how insect wings develop.

There are many genes involved in regulating wing size and shape in insects (Mezey and Houle 2005; Mezey et al. 2005), and although many still remain unidentified (De Celis 2003), it has become clear that the basic gene regulatory network underlying wing development is shared among all Pterygota (i.e. winged insects) (Carroll et al. 1994; De Celis 1998; Abouheif and Wray 2002; Carroll et al. 2005). Three key aspects of wing development are integrated: wing vein positioning, cell growth and identity, and intervein cell differentiation (De Celis 1998; Carroll et al. 2005). Decapentaplegic (*dpp*), a signaling molecule of the TGF- $\beta$  superfamily, plays a key role in this developmental integration as it regulates growth, patterning and differentiation of wing compartments (Nussbaumer et al. 2000; De Celis 2003; Crozatier et al. 2004; Martin et al. 2004; Dworkin and Gibson 2006; Schwank et al. 2008; Perez-Garijo et al. 2009). *Dpp* is thus involved in regulating wing size and shape, and the wing venation pattern. Furthermore, the exact identity and position of each individual wing vein is determined progressively in a developing wing disc by an unique combination of up- and down-regulated genes (Nussbaumer et al. 2000; Cook et al. 2004; Hurlbut et al. 2009). In fact, each individual wing vein and intervein region can be regulated independently from other veins and intervein areas (Birdsall et al. 2000; Zimmerman et al. 2000). This independence played a key role in the evolution of species-specific wing venation patterns and wing size and shape (Nussbaumer et al. 2000; De Celis and Diaz-Benjumea 2003). These aspects of wing development satisfy not only the independence criterion, but also the homology criterion. This allows for homologous sets of wing vein based landmarks to be compared within and across species (Breuker et al. 2007b). An example of among species comparisons would be to investigate how the evolution of wing development has created the observed diversity of wing size, shape and wing vein patterning in butterflies.

This means there is one criterion left, that of choosing a set of landmarks to provide adequate coverage of wing shape. In the case of butterflies there are quite a number of landmarks that can be chosen (see the wing venation pattern in Fig. 12.2). Experimental studies that seek to manipulate the transcription and/or translation of genes involved in wing development would need to measure both where a wing vein meets the edge of the wing and wing vein intersections to accurately quantify the effects on the wing venation pattern (Birdsall et al. 2000; Zimmerman et al. 2000; Dworkin and Gibson 2006). In a study such as the one described in this chapter it is sufficient to only measure the locations where a wing vein meets the edge of the wing, and as such capture the ecologically relevant overall “outline” shape of the forewing. We have added landmark 1 (a landmark based on crossing wing veins) to our set of landmarks (Fig. 12.2), as this allowed us to have a better coverage of the overall shape of the wing and have a more reliable measure of wing size (see next section) than would have been obtained with landmarks 2–9 only.

There is one more aspect in choosing landmarks that should be considered here. The fifth criterion speaks of an *adequate* number of landmarks (Zelditch et al. 2004). The lower limit of the number of landmarks is determined by the fact that too few



landmarks would of course not convey enough shape information. The upper limit may to a large extent be determined or constrained by the sample size. Shape is a multivariate trait and in order to have enough degrees of freedom to carry out geometric morphometrics analyses the number of individuals measured should be in excess of twice the number of landmarks minus 4 (Zelditch et al. 2004). Given our sample sizes (Table 12.1), 9 landmarks is definitely the upper limit. Ideally, one should really have larger sample sizes, but for most butterfly species it is difficult to actually catch large numbers of undamaged individuals in a limited period (such as early spring, which is about two weeks) in different sites at the same time. Furthermore, it is not always easy to catch similar numbers of females and males. In this study, for example, insufficient numbers of females were found, as their flight behaviour is much more cryptic than that of males.

### ***Geometric Morphometrics***

The 9 landmarks were digitized on the ventral side of the forewing in ImageJ (freely available on <http://rsb.info.nih.gov/ij/>) (Fig. 12.2). Variation in shape was examined by using geometric morphometrics based on generalized least squares Procrustes superimposition methods (Goodall 1991; Dryden and Mardia 1998; Klingenberg and McIntyre 1998). Procrustes methods analyze shape by superimposing configurations of landmarks of two or more individuals to achieve an overall best fit. It involves four steps, which have been described in mathematical and descriptive detail elsewhere (see details in Klingenberg and McIntyre 1998): (1) reflection of either left or right configurations (i.e. so left and right are now orientated the same way), (2) scaling to unit centroid size (to remove size and shape associations), (3) superimposing the centroids of all configurations, and finally (4) rotation of the configurations around their centroid to obtain the optimal alignment. Step 1 only needs to be performed when wings from both sides are used.

To estimate the amount of measurement error, and thereby to ensure that measurement error due to imaging and digitizing was negligible compared to biological shape and size variation, repeated photos and measurements were taken for a subset of individuals, and a Procrustes ANOVA (Klingenberg and McIntyre 1998) was performed. This will establish how reliable our 9 landmarks were to measure. Measurement error was found to be negligible as the mean squares for individual, side and asymmetry between the sides (the side  $\times$  individual interaction) significantly exceeded the mean squares of the error term ( $P \ll 0.001$ ; Table 12.2).

We used the Procrustes Distance to summarize shape differences between the average shape of two populations (Klingenberg and McIntyre 1998). The square root of the sum of the squared distances between corresponding landmarks of two optimally aligned configurations is an approximation of this Procrustes Distance. Differences in shape between populations were analysed by means of canonical variates analysis (CVA) applying 10,000 permutations, which is more than a reasonable number of permutations for the vast majority of shape comparisons. A permutation test is used here to determine statistical significance as a

**Table 12.2** Analysis of measurement error in digitizing landmarks on a *P. aegeria* forewing. Procrustes analysis of shape variance (Klingenberg and McIntyre 1998) of the amounts of shape variation attributable to different sources, for a random subset of 103 individuals, which were digitized twice. Nine landmarks were measured. The measurement error consists of both the imaging and digitizing error. Sums of squares and mean squares are in units of squared Procrustes Distance. \*\*\*  $P < 0.001$

Source	Sums of squares	Degrees of freedom	Mean squares $\times 10^6$
Individuals	0.3211	1428	224.86***
Sides	0.002142	14	152.97***
Sides $\times$ Individuals	0.03370	1428	23.60***
Error	0.01966	2884	6.82

permutation test does not need to assume any statistical distribution for shape variation. CVA finds the axes (i.e. canonical variates, CVs) that optimize the between population differences relative to the within population variation in landmark configurations. Patterns of shape differentiation that CVs represent can be plotted graphically. Multiplying the original shape variables by the coefficients of the CVs, and summing them, produces a series of vectors of relative landmark displacement that illustrates the shape differentiation represented by the CVs (Zelditch et al. 2004).

The centroid size of all nine landmarks of the forewing was used in this study as a measure of forewing size. Centroid size is the square root of the sum of squared distances from a set of landmarks to their centroid (i.e. mean x and y coordinate of a set of landmarks per individual) (see e.g. Klingenberg and McIntyre 1998). Forewing length is the distance (in mm) between landmark 1 at the base of the wing, and landmark 9 at the wing tip (Fig. 12.2).

The Procrustes ANOVA to determine the measurement error was performed in SAGE, written by E. Marquez (<http://www-personal.umich.edu/~emarquez/morph/>). Wing size and forewing length differences between populations were analysed by means of ANOVA in R 2.8.1 (<http://cran.r-project.org>). Procrustes superimposition and the canonical variates analyses to determine the forewing shape differences between populations and different landscapes were performed in MorphoJ (version 1.00e) (Klingenberg 2008).

## Results

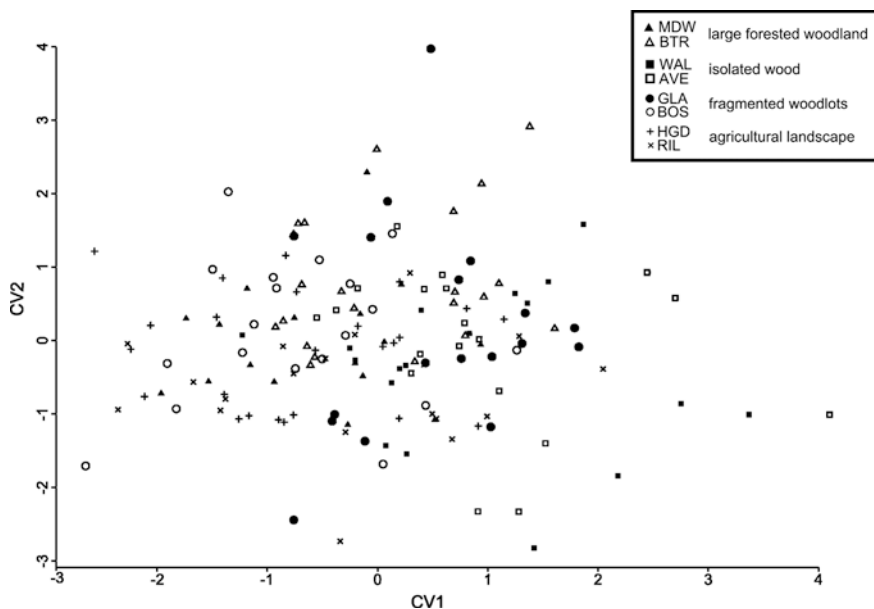
### *Forewing Shape Differences Between Populations and Different Landscapes*

The CVA results in Table 12.3 show that male forewing shape differences between any two populations are non-significant. This means that the shape variation within a landscape type (i.e. between the two populations of a landscape) is similar to the variation among landscapes. A scatter plot with the two CVs (CV1 and CV2) that optimized the between population forewing shape differences relative to within

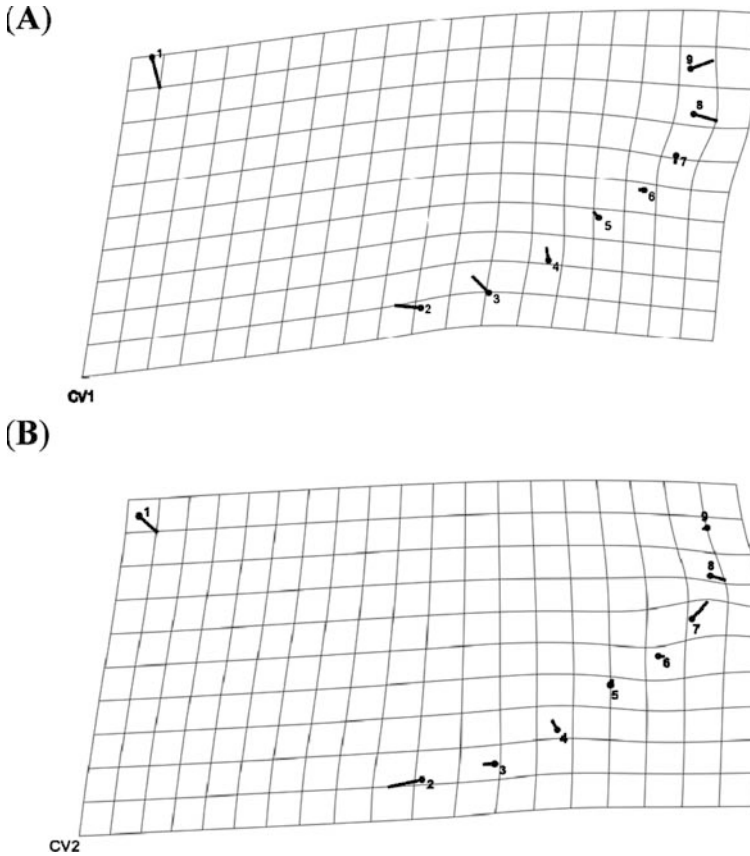
**Table 12.3** Comparison among the eight populations for male *P. aegeria* forewing shape by means of Canonical Variate Analysis (CVA). Shape variation between two populations is estimated as the Procrustes Distance (Klingenberg and Monteiro 2005). P-values were calculated with 10,000 random permutations per test

	AVE	BOS	BTR	GLA	HGD	MDW	RIL
BOS	0.013 <i>P</i> = 0.16						
BTR	0.0076 <i>P</i> = 0.56	0.0098 <i>P</i> = 0.24					
GLA	0.0089 <i>P</i> = 0.51	0.0084 <i>P</i> = 0.48	0.0085 <i>P</i> = 0.39				
HGD	0.0153 <i>P</i> = 0.14	0.0045 <i>P</i> = 0.94	0.0119 <i>P</i> = 0.21	0.0087 <i>P</i> = 0.56			
MDW	0.0089 <i>P</i> = 0.49	0.0065 <i>P</i> = 0.70	0.0071 <i>P</i> = 0.53	0.0056 <i>P</i> = 0.83	0.0079 <i>P</i> = 0.64		
RIL	0.014 <i>P</i> = 0.13	0.005 <i>P</i> = 0.90	0.0115 <i>P</i> = 0.15	0.008 <i>P</i> = 0.57	0.0043 <i>P</i> = 0.96	0.0068 <i>P</i> = 0.69	
WAL	0.0041 <i>P</i> = 0.96	0.010 <i>P</i> = 0.21	0.0062 <i>P</i> = 0.59	0.0064 <i>P</i> = 0.64	0.0121 <i>P</i> = 0.22	0.0065 <i>P</i> = 0.61	0.0108 <i>P</i> = 0.19

population shape variation illustrates this result (Fig. 12.3). Figure 12.4 illustrates the patterns of shape differentiation they represent. None of the CVs, including CV1 and CV2, were effective discriminators between the populations as there are no significant forewing shape differences between the populations.



**Fig. 12.3** Scatterplot (CV1 versus CV2) from Canonical Variate Analysis of male *P. aegeria* forewing shape from eight different, non-overlapping populations inhabiting 4 different landscapes. Abbreviations of the populations, and more information on them, can be found in Table 12.1



**Fig. 12.4** Shape differentiation associated with CV1 (a) and CV2 (b) for the eight populations from which male *P. aegeria* were collected. The lollipops and deformation grid indicate the directions and magnitude of shape variation along the CV

### *Wing Size Differences Between Populations*

Males from the eight populations did not differ significantly in forewing length (one-way ANOVA:  $F_{7,149} = 0.75$ ,  $P = 0.63$ ), nor did they differ in wing size as estimated by centroid size (one-way ANOVA:  $F_{7,149} = 1.28$ ,  $P = 0.26$ ). Pooling the two populations per landscape type showed exactly the same pattern for forewing length (one-way ANOVA:  $F_{3,153} = 0.60$ ,  $P = 0.61$ ) and centroid size (one-way ANOVA:  $F_{3,153} = 1.39$ ,  $P = 0.25$ ).

### **Phenotypic Plasticity?**

The results seem to indicate that populations of *P. aegeria* butterflies inhabiting different landscapes developed very similar wing shapes and sizes. These results were of course based on small sample sizes, and it is therefore clear that this experiment

needs to be repeated, in both the field and the lab (which is currently being done). It seems, however, that there is an indication that developmental plasticity plays a crucial role in wing morphology in *P. aegeria* butterflies.

Although numerous studies have reported the existence of wing and trait plasticity in general (Van Dyck and Wiklund 2002; Merckx and Van Dyck 2006), investigated the quantitative genetics of plasticity (Winterhalter and Mousseau 2007), and even modeled its evolution (Via and Lande 1985; de Jong 2005), the actual developmental and genetic mechanisms underlying plasticity of fitness-related traits such as wing morphology and its adaptive value in different ecological contexts are still very poorly understood (Marden 2006). Knowing these genetic mechanisms is essential as it will make it possible to determine whether a (continuous) reaction norm is the result of a developmental conversion (and therefore possibly adaptive) or merely developmental modulation. More importantly though, it will unlock many of the secrets underlying the flexibility in gene expression patterns that provide both developmental robustness and plasticity, which are of profound evolutionary significance (Abouheif and Wray 2002; Marden 2006; Nahmad et al. 2008).

As indicated in the section on choosing landmarks, changes in the dynamics of the regulatory network underlying wing development, either by means of careful experimental manipulation of gene expression or induced by the environment, can thus have changes on overall wing size and shape, which can be traced to individual wing veins or compartments (Birdsall et al. 2000; Zimmerman et al. 2000). This can be quantified at the phenotypic level by means of geometric morphometrics, which will indicate which veins changed position (Birdsall et al. 2000; Zimmerman et al. 2000; Breuker et al. 2006b; Dworkin and Gibson 2006). Carefully designed experiments would therefore offer the possibility of elucidating the developmental mechanisms underlying wing shape developmental plasticity observed in a variety of ecological contexts. A fully integrated research programme on adaptive plasticity in butterfly wing morphology would thus need to establish spatio-temporal variation due to environmental regulation of development, and demonstrate that this regulation is adaptive. We argue that geometric morphometrics will also play a crucial role in these developmental studies (cf. Klingenberg and McIntyre 1998; Birdsall et al. 2000; Zimmerman et al. 2000; cf. Dworkin and Gibson 2006).

## Summary

In this chapter we have illustrated, using the speckled wood butterfly *P. aegeria* as our model species, the ease with which geometric morphometrics can be applied in evolutionary ecology, using free and easy-to-use morphometric software packages. Great care should be taken in choosing landmarks and multiple criteria should be met when choosing a set of landmarks. Landmarks based on the position of butterfly wing veins do not only meet these criteria, they are also biologically relevant as wing veins play a key role in our understanding of the developmental processes underlying wing shape and its evolution. This makes butterfly wings ideal for the

implementation of geometric morphometrics in a wide variety of studies, not only evolutionary ecological studies, but also developmental studies.

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