

8 OVERVIEW: this thesis and some ideas for future research

The work reported in this thesis explores different aspects of some of the processes which account for the patterns of morphological variation in butterfly wing colour patterns. In particular, we have studied the size of the two eyespots characteristically present on the dorsal surface of the forewings of *Bicyclus anynana* butterflies. The objective of this overview is to provide a very brief summary of each of the different chapters and to connect them all. Details (sometimes interesting!) and references have been left out, but both can be found throughout all other chapters.

This overview is divided into four parts. The first section introduces the subject of this thesis into the context of modern research in evolutionary developmental biology, and provides essential background information about our model system. The second section summarizes the work reported in CHAPTERS 2 to 5 which examined different processes shaping population-level variation in *B. anynana* patterns of eyespot size. These include exploring the potential of standing genetic variation present in a laboratory population to produce independent evolutionary changes in different eyespots (CHAPTERS 2 and 3), a description of the genetic correlations across the whole eyespot pattern (CHAPTER 4), and a test of the relevance of male eyespot size phenotypes to female mating preferences (CHAPTER 5). CHAPTERS 6 and 7 are summarized in the third section which looks at different aspects of the proximate mechanisms underlying pattern formation and variation at the individual level. We examined the ways in which the cellular, and molecular mechanisms of eyespot size specification have been altered to produce phenotypic variation in this trait, and discussed different types of genetic variation which might contribute to the evolutionary diversification of butterfly wing patterns. Finally, the last section lists some of the many questions left unanswered and raised by our work, and discusses possible future avenues of research in butterfly wing pattern evo-devo.

BACKGROUND

The last decade has witnessed increased efforts attempting to integrate the fields of evolutionary and developmental biology, which grew independently for most of the twentieth century. Development is the process that translates genotypes into phenotypes, and thus it is fundamental for any complete understanding of evolutionary change. Natural selection can favour any number of traits but it can act only on those morphological variants produced as a result of ontogeny. The understanding of the generation of phenotypic variation has been identified as one of the major challenges for contemporary evolutionary developmental biology (or evo-devo). The comparison of developmental mechanisms (and resulting “body plans”) across sometimes very distantly related species has generated much research work in evo-devo and contributed evidence of the striking evolutionary

conservation of many developmental processes. However, studies of variation at the intra-specific level have been largely neglected. Since adaptive evolution involves the sorting by natural selection of the phenotypic variants present in populations of organisms, the generation of different phenotypes within a species will be very important to further our knowledge of the evolutionary processes that eventually result in wide differences across species.

The colour patterns on butterfly wings provide ideal material for an integrated study of the reciprocal interactions between evolution and development. There is a bewildering diversity in wing patterns both across and within species of butterflies. These patterns of colour are interesting from an evolutionary perspective because they represent visually compelling products of natural selection, often with a known adaptive value in intra- or inter-specific visual communication. They are also interesting from a developmental perspective because they are structurally simple and have proven amenable to a developmental characterization at different levels. The colour patterns on butterfly wings are produced by the spatially ordered arrangement of pigmented scale cells in a single cell layer, and variation in wing patterns has been described at the molecular, genetic, biochemical, cellular, physiological, and ecological levels. Furthermore, despite the diversity in wing patterns, comparisons across species are made easier by the recognition of a basic wing pattern “groundplan” composed of different series of pattern elements whose more or less profound changes can explain the generation of most extant patterns in butterfly wings.

Much work on wing pattern evolution and the interaction between genetic variation and developmental mechanisms has focused on the eyespots of the tropical butterfly *Bicyclus anynana*. This species has a series of marginal eyespots on different wing surfaces, and much potential to produce phenotypic variation in eyespot patterns. In *B. anynana* there are high levels of additive genetic variance that have enabled a progressive and gradual response to artificial selection on different features of eyespot morphology, including their size, colour composition, shape, and position. Dramatic phenotypic changes in wing pattern also occur due to spontaneous mutations of large effect isolated in laboratory populations. It has been found that both types of genetic variation usually affect all eyespots on the same butterfly in a concerted manner. For example, the mutant allele *Bigeye* produces larger eyespots on all wing surfaces, and selection for changes in the colour-composition of one eyespot produce strong correlated responses across all eyespots. Such correlations are presumably a consequence of the fact that all eyespots share the same developmental basis. Each eyespot is formed through the action of a central group of organizing cells, which is associated with a characteristic pattern of expression of several developmental genes.

PROCESSES SHAPING POPULATIONAL VARIATION IN EYESPOT PATTERNS

In *B. anynana* strong correlated responses to artificial selection have been reported for each of several different features of eyespot morphology, including the size of the two eyespots on the dorsal forewing of these butterflies. Selection targeting the posterior eyespot consistently produced a strong correlated response for the same feature in the anterior eyespot. These genetic correlations raised the prediction that evolutionary

changes of individual eyespots might be constrained. In CHAPTERS 2 and 3 we have used artificial selection to directly test the prediction that the developmental coupling between individual eyespots constrains evolution of wing patterns involving independent change of different eyespots on the same wing surface.

In CHAPTER 2 we compared the response to artificial selection between lines selected for coupled changes in eyespot size (*i.e.* both eyespots selected for larger size or both selected for smaller size) and lines selected for uncoupling changes (*i.e.* one eyespot selected for larger size and the other selected for smaller size). We have shown that, against our prediction, and despite the fact that coupled changes produced more extreme phenotypes, the rate of response to selection (measured relative to the cumulated selection differential in each line) was not consistently lower for the uncoupling directions. High realized heritabilities for individual eyespot sizes led to the production of extreme phenotypes not represented in the starting laboratory population. We have shown that artificial selection based on standing genetic variation present in a single laboratory population of *B. anynana* butterflies was sufficient to produce all phenotypes found within the genus, including one which is not explored in any extant species. We have argued that the developmental properties of eyespot formation are unlikely to constrain any process of evolutionary change in the pattern of relative size of *Bicyclus* butterfly eyespots.

CHAPTER 3 used additional artificial selection lines to ask specifically whether, and to what extent, selection for a larger eyespot depended on the selection imposed on the other eyespot. We have compared the response for increased size of one eyespot in three situations which differed with respect to the selection imposed on the other eyespot: i) concerted selection for increased size, ii) independent stabilizing selection on size and iii) antagonistic selection for decreased size. We observed that the response to selection for the size of one particular eyespot was only to a small extent dependent on the selection regime imposed on the other. As for CHAPTER 2, the results discussed in CHAPTER 3 revealed a great potential for independent evolution of individual eyespots based on the variation present in our laboratory stock of *B. anynana*. These results suggest that evolutionary history might have favoured the developmental independence of different eyespots, which presumably originated as highly integrated serially repeated elements.

Despite the great potential for independent evolution of individual eyespots, the patterns of covariation between them can be important in determining the patterns of the (phenotypic) variation found in butterfly wings. In CHAPTER 3 we discuss how the genetic correlation between individual eyespots might be important when limited time is available for evolution to occur, and in CHAPTER 4 we analyze and discuss correlated responses to selection across different wing surfaces. The evidence that all eyespots in one butterfly are developmentally and genetically coupled had led to suggestions that the whole of the eyespot pattern, rather than individual eyespots, should be considered a developmental and evolutionary unit. In CHAPTER 4 we use lines derived by artificial selection on the two eyespots on the dorsal forewing to dissect the modular organization of the whole eyespot pattern. We show that there are genetic correlations between eyespots on different wings and different wing surfaces, and also that some (generally weaker) correlations exist

between eyespot size and other features of eyespot pattern morphology. The relative ease with which the coupling between eyespots on the same wing surface has been broken (CHAPTERS 2 and 3), suggests that the (weaker) genetic correlations described in CHAPTER 4 are also probably not a major force constraining eyespot pattern evolution.

The great flexibility in wing pattern evolution uncovered in CHAPTERS 2 and 3 shows that the developmental properties of eyespot formation are unlikely to constrain the evolution of the pattern of relative size of *B. anynana* eyespots. Two alternative processes are generally put forward to explain conserved patterns of morphology; the action of constraints *versus* some form of stabilizing selection. We found no evidence for developmental constraints biasing the production of different eyespot patterns, and we have suggested that natural selection, together with population-level properties, rather than the generation of phenotypic variation, were likely to dominate in shaping the evolution of patterns of eyespot size. Since previous results had suggested that the dorsal forewing pattern of eyespot size might play a role in courtship and sexual selection, in CHAPTER 5 we tested for differences in attractiveness to females between males with different combinations of eyespot sizes. We found no evidence that females preferred to mate with males depending on their eyespot size phenotype. We have argued that, in natural conditions, other types of intra- or interspecific interactions or population structure limitations might be involved in maintaining the relative eyespot size pattern of *B. anynana* butterflies. These results make it clear that progress in understanding the patterns of variation in eyespot size in *B. anynana* will need to take into account the ecological interactions involving natural populations of this species.

MECHANISMS GENERATING VARIATION IN EYESPOT PATTERNS

The eyespots of *B. anynana* butterflies provide a system where morphological variation can be studied in a very integrative manner, from its molecular underpinnings to its ecological relevance. In this section I summarize work that has taken advantage of knowledge about the molecular and cellular basis of eyespot formation, and of our selected lines with different eyespot sizes (CHAPTER 2), to characterize the mechanistic basis of variation in this trait.

In recent years, the study of gene expression patterns has identified some of the genetic pathways involved in eyespot formation. The genes whose expression in pre-adult wing primordia is associated to the eyespot field provide candidate genes which might contribute to variation in eyespot patterns. CHAPTER 6 reports on the study of the contribution of the gene *Distal-less* (*Dll*) to quantitative variation in eyespot size. Several characteristics of the patterns of *Dll* expression in *B. anynana*, suggested that this gene might harbour molecular variation underlying phenotypic variation in eyespot patterns. We found quantitative differences in *Dll* expression between phenotypically divergent lines with different eyespot sizes, and evidence for the co-segregation of DNA polymorphisms in *Dll* with eyespot size variation in crosses between those lines. These data suggested that molecular variants at *Dll* itself contributed to response to selection on eyespot size, and might also contribute to evolution of butterfly wing patterns across species.

Other candidate genes which might have been involved in the response to selection include several genes of the *hedgehog* genetic pathway. Preliminary work aimed at testing the contribution of *engrailed* and *hedgehog* to variation in *B. anynana* eyespot size is described in CHAPTER 7. We show that there are quantitative differences in *engrailed* expression in association with differences in adult eyespot size, and report on the cloning of a *B. anynana* homologue of the *hedgehog* gene, a potential regulator of *engrailed* expression in the presumptive eyespot field. In this chapter we also discuss (very!) preliminary results of manipulative experiments examining how the properties of the cellular interactions underlying eyespot formation can be changed in a localized manner to produce changes on different individual eyespots on the same wing surface. We show that different components of such interactions have contributed differently to changes in eyespot size across selection lines. This might be the result of different spatial- or temporal patterns of action of those components; some might be more readily changed locally in different wing regions, or might be the basis for evolutionary response at different stages of the response to selection. These experiments gave very exciting preliminary results, but also revealed a series of problems with data collection and analysis which will need to be solved in future experiments. Finally, CHAPTER 7 also reports on the phenotypic characterization of a number of mutant alleles of large effect on the size of the anterior and posterior dorsal forewing eyespots. Such type of genes might have played an important role in promoting developmental and evolutionary independence of individual eyespots. In CHAPTER 7 we discuss future experiments aiming at confirming and advancing the preliminary results presented, and knowledge on butterfly wing patterning in general.

CONCLUDING REMARKS

The distinction between variation at the population level and the proximate mechanisms of generating inter-individual variation which is used to organize this overview is, of course, not a clear distinction in biological terms. The proximate mechanisms which generate individual phenotypes contribute to the variation found at the population level! For example, the developmental constraints which potentially bias the production of adult phenotypes (as discussed in relation to population-level variation) are processes that act at the level of the individual, whilst the different types of genetic variation underlying phenotypic variation (discussed in relation to the proximate mechanisms of generation of different eyespot size phenotypes) apply at the population level. Nonetheless, I think that such distinction was useful to attempt a clear and organized summary of the results of this thesis.

Throughout this thesis, we have identified several old questions that our experiments left unanswered, and also new questions generated by our results. At different stages, we have proposed new experiments or directions of research which could be pursued to provide the required answers. Here we briefly discuss some of the issues which, in our opinion, are important avenues for future research on butterfly wing pattern evo-devo.

We have found no evidence for a strong involvement of developmental constraints in shaping variation in *B. anynana* dorsal forewing eyespot size, but also

no evidence for differences in fitness between selected lines with divergent phenotypes. However, we have argued that despite the fact that there are no obvious fitness differences in laboratory conditions these might still exist in nature where intra- and inter-specific interactions are much stronger. Not only is sexual selection probably stronger in the field, but also interactions with other species might be playing a role. The many examples of colour pattern mediated interactions described in butterflies include intra-specific recognition and predator avoidance. Natural populations might also be exposed to other specific conditions which can potentially regulate variation in wing patterns. Even though we found no evidence for the operation of developmental/ genetic constraints, other types of constraints involving population-level properties may play a role in natural conditions. These considerations illustrate how important more information of different aspects of the ecology of *B. anynana* will be to understand the patterns of phenotypic variation in natural populations.

This thesis has characterized different types of genetic variation present in our laboratory population of *B. anynana*. Standing quantitative genetic variation has enabled the gradual and progressive response to artificial selection on dorsal forewing eyespot size which lead to the production of divergent phenotypes not represented in the base population. On the other hand, a number of mutant alleles with extreme effects on eyespot size, have also been isolated from laboratory stocks with no (direct) selection on this trait. Thus both types of genetic variation are readily available in *B. anynana*, at least in laboratory populations. Are the two types of genetic variation based on different alleles at the same loci? What is their relative importance in natural populations? The first question can be addressed using gene mapping techniques, whilst the second requires a better understanding of natural populations of *B. anynana*. Both will be further discussed below.

Different types of linkage-based analysis can be used to identify the molecular nature of different types of genetic variation. Such techniques, including candidate gene approaches and more unbiased genome-wide searches for genetic determinants, can be used to map both the quantitative trait loci (QTLs) that contributed to our response to selection on eyespot size, and the mutants of large effect on this trait. Different types of QTL mapping approaches will be used to identify the genes contributing to variation in eyespot size and other features of eyespot morphology (*e.g.* their colour-composition), and also to estimate the magnitude of their effects and of interactions with other genes. Even though the molecular characterization of genetic variation is a greater challenge for non-model organisms such as *B. anynana*, for which developmental genetic tools are not (yet) fully developed, many recent technological and conceptual advances make it readily feasible and guarantee the success of gene mapping experiments. The importance of these new ventures is increasingly being recognized. Non-model organisms might not have the luxury of a sequenced genome and a long history yielding a very diverse body of data but the importance of these new ventures is increasingly being recognized. Study of non-model organisms provides invaluable information that is essential for a deeper understanding of biological patterns and processes. Very importantly, only such studies can show how general the findings obtained for the

small number of model organisms are. Furthermore, because of different biological properties, these new systems allow for the tackling of different questions or for different approaches to old (many unsolved) problems. [For example, *Drosophila* wings do not have spectacularly diverse wings colour patterns, nor plasticity in wing pattern with great ecological relevance, nor easily manipulable pupae!]

One of the strengths of the *B. anynana* system for studies of evolution and development is that variation in eyespot patterns can be described at different levels, from its molecular underpinnings to its ecological relevance. To make full use of this strength, the different aspects of the mechanisms underlying the production of phenotypic variation in *B. anynana* eyespot size should eventually be brought together in a fully integrated manner. How are the physiological, cellular, biochemical, genetic, and molecular aspects of eyespot formation related to each other? Eventually, the integration of the knowledge on the proximate mechanistic basis of variation in eyespot size, can be extended to other traits of eyespot morphology (*e.g.* eyespot number, position and colour-composition) and other aspects of wing pattern formation (*e.g.* physiological basis of seasonal polyphenism). Particularly interesting would be trying to use the information on the mechanistic basis of eyespot size specification for the dorsal forewing eyespots to understand ventral eyespot formation, and its interaction with development temperature, insect hormones, and ecological interactions in the field.

To what extent is the variation at the different levels discussed in the previous paragraphs relevant to evolution in natural populations of this butterfly? In the end, the evolutionary relevance of the mechanisms of generation of wing pattern variants as uncovered in *B. anynana* laboratory populations need to be analyzed in natural populations. A complete understanding of the mechanisms underlying variation in *B. anynana* wing patterns has to be combined with an analysis of their ecological significance and of the evolution of butterfly colour patterns across species. Which phenotypic and genetic variants are the basis of differences in morphology observed across species? Such questions will be fundamental to understand the evolutionary diversification that has produced the spectacular diversity of butterfly wing colour patterns which has long been fascinating biologists and laymen alike. Hopefully, another 4 years of research on *B. anynana* wing patterns will obtain answers for some of these questions.