Dev Genes Evol (2011) 221:297–308
DOI 10.1007/s00427-011-0380-6

ORIGINAL ARTICLE

Convergent, modular expression of *ebony* and *tan* in the mimetic wing patterns of *Heliconius* butterflies

Laura C. Ferguson · Luana Maroja · Chris D. Jiggins

Received: 22 June 2011 / Accepted: 26 October 2011 / Published online: 3 December 2011

Abstract The evolution of pigmentation in vertebrates and flies has involved repeated divergence at a small number of genes related to melanin synthesis. Here, we study insect melanin synthesis genes in *Heliconius* butterflies, a group characterised by its diversity of wing patterns consisting of black (melanin), and yellow and red (ommochrome) pigmented scales. Consistent with their respective biochemical roles in *Drosophila melanogaster*, *ebony* is upregulated in non-melanic wing regions destined to be pigmented red whilst *tan* is upregulated in melanic regions. Wing regions destined to be pigmented yellow, however, are downregulated for both genes. This pattern is conserved across multiple divergent and convergent phenotypes within the Heliconii, suggesting a conserved mechanism for the development of black, red and yellow pattern elements across the genus. Linkage mapping of five melanin biosynthesis genes showed that, in contrast to other organisms, these genes do not control pattern polymorphism. Thus, the pigmentation genes themselves are not the locus of evolutionary change but lie downstream of a wing pattern regulatory factor. The results suggest a modular system in which particular combinations of genes are switched on whenever red, yellow or black pattern elements are favoured by natural selection for diverse and mimetic wing patterns.

Keywords Pigmentation · *Heliconius* · *Ebony* · *Tan* · Mimicry

Communicated by S. Roth

Electronic supplementary material The online version of this article (doi:10.1007/s00427-011-0380-6) contains supplementary material, which is available to authorized users.

L. C. Ferguson · C. D. Jiggins (✉)
Department of Zoology, University of Cambridge,
Downing Street,
Cambridge, UK CB2 3EJ
e-mail: c.jiggins@zoo.cam.ac.uk

L. C. Ferguson
Department of Zoology, University of Oxford,
South Parks Road,
Oxford, UK OX1 3PS

L. Maroja
Biology Department, Thompson Biology Laboratory,
Williams College,
59 Lab Campus Drive,
Williamstown, MA 01267, USA

Introduction

The genetics of pigmentation is an excellent system in which to address questions of constraint and divergence during evolution. The history of evolutionary biology is closely tied to the genetics of pigmentation, and indeed, some of the earliest empirical tests of mammalian Mendelian inheritance, mutation rates, genetic linkage, epistasis and pleiotropy involved studies of melanism in rodents (Hoekstra 2006). In recent years, insect pigmentation has come to the forefront of evolutionary biology as one of the few examples of a trait in which the link has been made between genetic variation and morphological adaptation (Hoekstra 2006).

In mammals and birds alterations in the coding sequence of the melanocortin-1 receptor *MC1R* and its antagonist *Agouti* have repeatedly been linked to changes in melanic phenotype, including convergent mutations responsible for phenotypic shifts in distantly related species such as birds and mice (Mundy 2005; Kingsley et al. 2009). In parallel,

studies on *Drosophila* have demonstrated that adult pigmentation is a two-step process, with well-known developmental loci (e.g. *bric-a-brac* and *optomotor-blind*) 'patterning' the distribution of melanic pigments in time and space, and downstream 'effectors' biochemically producing the pigments (Wittkopp and Beldade 2009; Wittkopp et al. 2003a, b). Whilst in some cases patterning loci have been implicated in melanic shifts (Gompel and Carroll 2003; Kopp et al. 2003; Brisson et al 2004; Kopp et al. 2000; Williams et al. 2008), there is a growing body of evidence that mutation in the *cis*-regulatory regions of pigment genes (the effectors) can also drive phenotypic evolution on the wings, thorax and abdomen, with *yellow*, *tan* and *ebony* recurrent targets of selection both within and between species (Wittkopp et al. 2009, 2002a, 2003a, b; Jeong et al. 2008; Gompel et al. 2005; Prud'homme et al. 2006; Rebeiz et al. 2009; Takahashi et al. 2007; Pool and Aquadro 2007). Thus, despite gross differences in the mode of melanin synthesis, in both mammals and flies there is a clear precedent for genes involved in pigment production accumulating evolutionarily relevant mutations; and pigment genes themselves being prime candidates for adaptive, melanic shifts during evolution.

Pigmentation has also been an attractive model for evolutionary genetics because there is a clear understanding of the adaptive value of many pigmentation phenotypes, notably industrial melanism in the peppered moth (Van't Hof et al. 2011), mate choice and wing pigmentation in *Drosophila* (Prud'homme et al. 2006; Gompel et al. 2005) and coat colour polymorphism in rock pocket mice (Nachman 2005; Steiner et al 2007). Here, we examine another case in which the adaptive value of pigmentation has been clearly established—the warning colours of *Heliconius* butterflies.

The *Heliconius* (Lepidoptera, Nymphalidae, Heliconiinae, *Heliconius*) are found throughout the lowland rainforests of the neotropics, where they display high diversity of wing colour pattern and have radiated into 40 species with over 320 named colour pattern races (Darwin-Initiative 2007). *Heliconius* races have monomorphic wing patterns within a population but are divergent between geographic locations. Most *Heliconius* species are Müllerian mimics, the most notable being the co-mimics *Heliconius melpomene* and *Heliconius erato*, with about 30 races each. Mimetic *Heliconius* species share the cost of educating bird predators of their toxicity, leading to lower mortality for all species involved in a mimicry ring (Kapan 2001). Both within *H. melpomene* and between closely related species, individuals mate assortatively based on wing pattern, demonstrating that wing patterning in *Heliconius* contributes to speciation (Jiggins et al. 2001). Thus, *Heliconius* wing pigmentation has strong adaptive value in mate choice, mimicry and aposematism.

The wing patterns of *Heliconius* are striking, simple, two-dimensional arrays of colour. Three types of pigment have been identified from the wings of *Heliconius*: ommochromes (reds and orange), melanins (black, brown and tan) and 3-OHK (yellow), itself an intermediate of the ommochrome pathway (Nijhout 1991). Thus, genetic analysis of the ommochrome and melanin biosynthesis pathways should provide a basis for a comprehensive understanding of pigmentation across the wing. Previously, we have found a clear correlation between wing pattern and expression level for two ommochrome pathway genes, *cinnabar* (kynurenine-3-monooxygenase) and *scarlet* (Ferguson and Jiggins 2009). Here, we examine the melanin biosynthesis pathway for the first time in *Heliconius*.

Materials and methods

Identification of melanin pathway genes

Reference sequences for six melanin pathway genes (*tyrosine hydroxylase* (*TH*; *pale*), *Dopadecarboxylase* (*Ddc*), *yellow*, *yellow-f*, *NBAD synthetase* (*ebony*) and *NBAD hydrolyase* (*tan*)—Fig. 1) were used to search NCBI dbEST databases and 454 transcriptome sequences for both *H. melpomene* and *H. erato* (Ferguson et al. 2010). Where the resultant contigs were not full-length transcripts, primers were designed from *H. melpomene* or *H. erato* sequence to span contig gaps. The same *Heliconius melpomene malleti* normalised cDNA used to construct the transcriptome library (Ferguson et al. 2010) was used as a template for PCR amplification.

In order to confirm *H. melpomene* gene homology, sequence was also recovered from GenBank for *Bombyx mori*, *Apis mellifera* and *Tribolium castaneum*. Inferred protein translations were aligned in ClustalW (Chenna et al. 2003). Analysis in Treepuzzle (V5.2) (Schmidt et al. 2002) indicated that for *ebony*, *A. mellifera* had an amino acid composition significantly ($p<0.01$) different to the other insects, and the short sequence available for *H. erato* also led to a high proportion of uninformative sites. Trees were therefore inferred for all genes except *ebony* using maximum likelihood, implemented in Phylip 3.67 (Joseph Felsenstein, University of Washington, Seattle, USA) with a JTT model and *A. mellifera* specified as the outgroup (Savard et al. 2006). For *ebony*, a neighbour-joining algorithm was implemented in MEGA 4.1 (Tamura et al. 2007). Tree topology was tested for all genes with 1,000 bootstrap replicates. Tree images were generated using Phylodendron (iubio.bio.indiana.edu/treeapp/treeprint-form.html by D.G. Gilbert). In order to further characterise sequence homology, the percentage of identical amino acids between species was taken from the ClustalW scores table

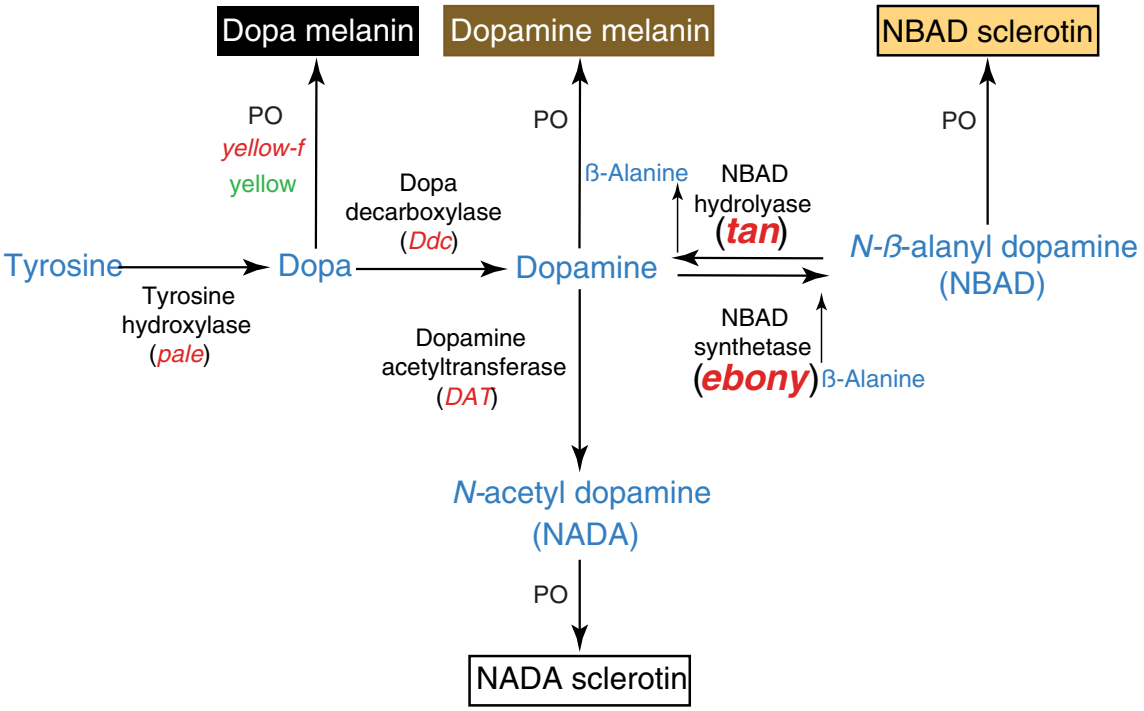

Fig. 1 Model of melanogenesis and scale cell sclerotization. Format modified from a model of *D. melanogaster* pigment metabolism (Wittkopp et al. 2003a, b). The precursor tyrosine and pathway intermediates are shown in *blue*, enzymes in *black* and the loci that encode them in *red*. *PO* phenol oxidase, which may cross-link the precursors to cuticle proteins (True 2003). In *Drosophila*, both of the products dopa and dopa melanin may be black or brown, NADA sclerotin is clear, and NBAD sclerotin yellow or pale tan. The biochemical function of the *yellow* gene has not yet been determined, although it may act upstream of *yellow-f* (Han et al. 2002)

and the percentage of similar amino acids calculated as the number which were identical, or had one or two dots (which indicate degree of biochemical similarity) divided by the shared number of positions shared by both sequences, as determined from sequence alignment (True et al. 2005). Due to recurrent duplications within the *yellow* gene family, *H. melpomene* sequences were defined with respect to the 14 identified *D. melanogaster yellow* genes which gave good support for the identification of *Hm yellow* and *yellow-f*.

Linkage mapping

Due to a lack of crossing over in female Lepidoptera (Turner and Sheppard 1975), maternal alleles from a linkage group are always inherited in complete association and can be used to assign markers to linkage groups. Crossing over occurs in males and can be used to infer recombination distance. Linkage mapping was carried out using individuals from the *H. melpomene* F2 mapping family 'Brood 33' as described previously (Jiggins et al. 2005). Briefly, sequence data were first obtained from the brood parents. Allelic variation diagnostic for the maternally inherited allele was then scored among brood offspring, using either diagnostic restriction enzyme sites or by sequencing. A diagnostic panel of 16 individuals were typed and compared to 'chromosome prints' from previous mapping work (Jiggins et al. 2005), in order to assign genes to chromosomal linkage groups. Due to the lack of crossing over, a perfect correspondence in segregation patterns is expected for linked markers.

qRT-PCR from a developing wing series

Gene expression was surveyed between wing regions and throughout late larval and pupal development using the same cDNA panel assayed previously for ommochrome pathway genes (Ferguson and Jiggins 2009). Tissue was dissected from six stages of pupal developmental (early pupa (EP), pre-ommochrome (PO), ommochrome only (OO), early melanin (EM), mid-melanin (MM) and late melanin (LM) and three wing regions for each stage, representing the proximal (P), red band (R) and distal (D) portions of the wing. Three replicate individuals were used for each sample, and cDNA was normalised to the same concentration (15 ng/μl) and then pooled for the replicated samples, such that there was a single pooled sample for each wing region at each stage. In addition, whole forewings from a single individual were dissected from four earlier stages: two late fifth instar larval and two very early pupal. During the late fifth instar, the larva turns a characteristic dark purple colour and locates a

suitable place for pupation ('crawler' stage), and then becomes suspended by silk with the integument turning a transparent cream colour (pre-pupa (PP)). During pupation the new wings are exposed, and a new pupal case is rapidly formed to cover them, the new pupa (NP) stage is defined as the moment the last larval cuticle falls off the pupa. A single individual was also dissected at 48-h post-pupation

Quantitative reverse transcriptase PCR (qRT-PCR) was carried out as described previously (Ferguson and Jiggins 2009), with three technical replicates for each sample at each stage. Primers were designed from transcripts obtained above and expression was normalised relative to control *elongation factor 1- α* (*ef1-α*) expression level and the highest expression for each gene across all samples. The relationship of gene expression with wing region, developmental stage and the interaction between region and stage was determined for each gene from the normalised expression values of the three technical replicates in an ANOVA using Minitab V.15. Primers are given in Table 1.

qRT-PCR from *Heliconius* races and species

Heliconius individuals were collected from the following sites: *Heliconius ismenius*, *Heliconius hecale melicerta*, *Heliconius erato petiverana* and *Heliconius melpomene rosina*—Gamboa, Panama and *Heliconius melpomene amaryllis* and *Heliconius melpomene aglaope*—near Tarapoto, Peru. All species were subsequently bred at the Smithsonian Tropical Research Institute, Gamboa, Panama. Additional *H. erato* individuals from Surinam were obtained through London Pupal Supplies (London, UK).

We attempted in situ hybridisation and antibody staining for *ebony* and *tan* but encountered significant problems with imaging due to interference of both wing pigments and scale cell cuticle. Instead, we carefully dissected wing patterns from all species into their constituent parts to separate each pigmented region for qRT-PCR (see Supplementary Fig. 2 for examples). Where the pattern consisted of several regions of the same pigment type (e.g. the yellow spots of *H. hecale*), the regions were combined for each wing.

Tissue was stored in RNAlater (Ambion). RNA was extracted using the Qiagen RNAeasy kit and tissue lyzer. cDNA was generated using Bioscript (Bioline) with 1 μg of RNA and random hexamers. qRT-PCR was carried out as before for *ebony*, *tan* and the two control genes *ef1-α* and *Rps3a*, which had been identified as a suitable control from an *H. melpomene* wing developmental microarray (Nadeau, in preparation). The number of biological replicates for each taxon varied (see 'Results'), and three technical replicates were obtained for each sample. Note that biological replicates were not pooled as above but analysed as separate samples. The experimental data were normalised to the mean of the two control genes and the highest expression value for the forewing or hindwing of each species/race. As above an ANOVA was carried out using Minitab V.15

Results

Identification of *Heliconius* melanin pathway genes

We used both existing *H. melpomene* and *H. erato* EST databases and de novo PCR amplification to generate full-length reference transcripts for *TH* (*pale*), *Ddc*, *yellow*, *yellow-f* and *tan*. Alignment against *H. erato* and *Papilio xuthus* transcripts indicates that our *H. melpomene ebony* sequence is lacking four amino acids at the N terminus and 17 amino acids at the C terminus, respectively. All genes examined were found to be expressed in wings from both species, except *yellow-f*, which was not recovered from *H. erato*. Homology of all genes was inferred by phylogeny reconstruction and recapitulation of known insect relationships from gene trees (Fig. 2, trees). The extent of protein sequence conservation varied considerably, with *pale* the most conserved and the yellow family genes the most rapidly evolving (Fig. 2, tables) (Ferguson et al. 2011).

Patterns of expression throughout development

Here, we take advantage of the fact that *Heliconius* wing patterns are predominantly simple, two-dimensional blocks of colour, in order to dissect the wings by pattern element

Table 1 Primers used for quantitative reverse transcriptase PCR

Gene	Forward primer	Reverse primer	T_m	Product size (bp)
Ddc	GGAATGAGTCCATTACGGATGT	GACCGCGCTGGTCTCTTA	60	122
Pale	CGAGCTTCGCTCAGTTCTCT	AAAGTAGAGCAGCGCCGTAA	60	156
Ebony	GTTCCGAAACTTCCCGTTCT	CGATCTGATAATCCGCCAAA	60	185
Yellow	GCTCTTGACGAAGGCATTTC	CTCCCATTGGTGAAGCTGAT	57	219
Yellow-f	GGCTTTGGATGGTTGACACT	TTAAACCTCCCGGAGTCCTT	60	161
Tan	GGTCACACCGAAGATGCTTT	CGTGGAGTGTTTCCAGGTTT	60	229

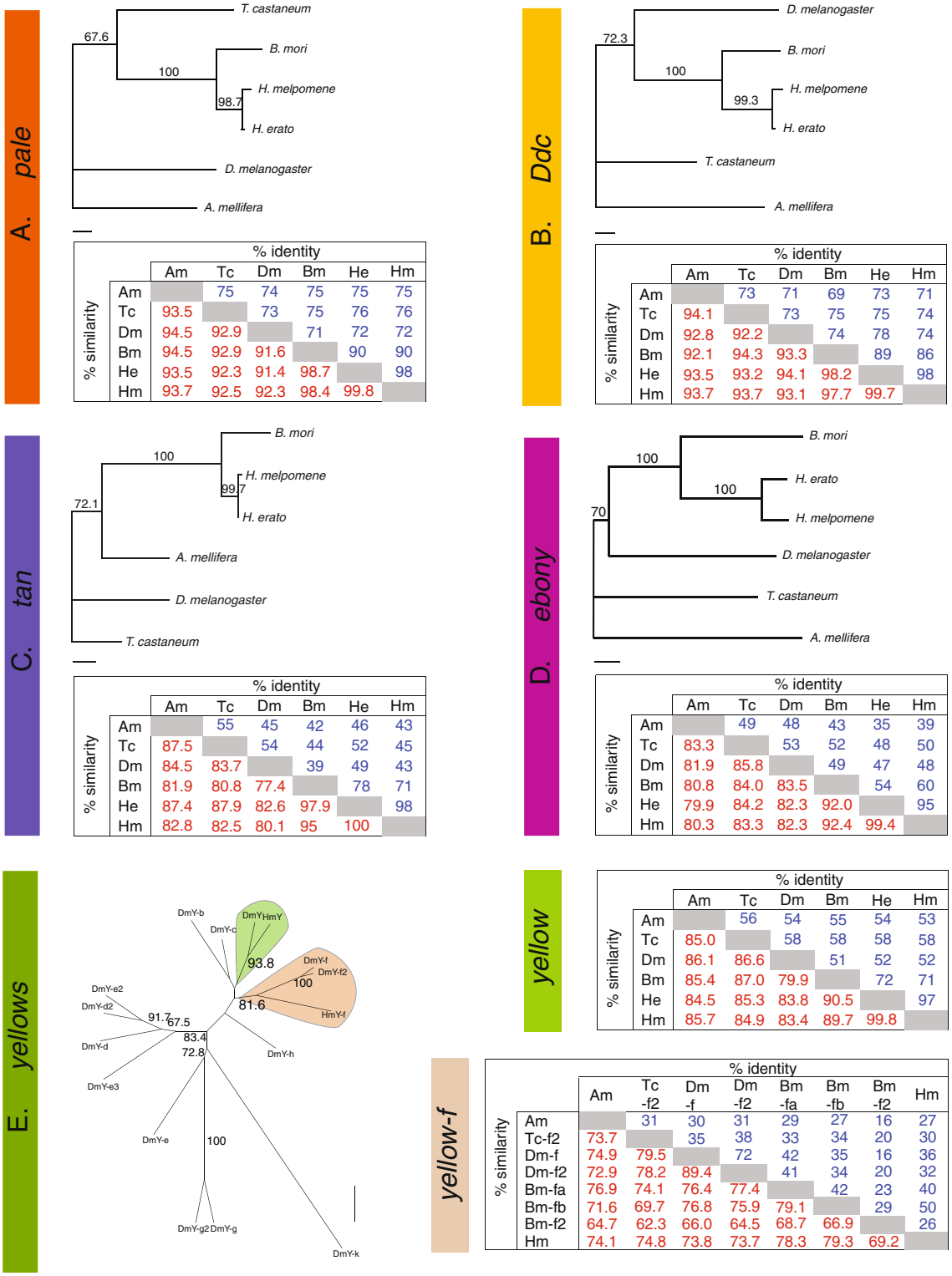

Fig. 2 Support for homology of *Heliconius* melanin pathway genes *Heliconius* sequences were compared to reference sequences for *B. mori*, *A. mellifera* and *T. castaneum* obtained from GenBank. Gene trees were constructed using maximum likelihood for all genes except *ebony*. *HmY H. melpomene yellow* (*green area*), *HmY-f H. melpomene yellow-f* (*pink area*). The percentage of identical (shown in *blue*) and similar (*red*) amino acids calculated between each species. Although all *Heliconius* sequences had high similarity to reference sequences from other insect species, actual levels of sequence conservation varied considerably across the genes (*table inserts a–e*), with highest levels of conservation for *TH*, and lowest levels for *yellow-f*

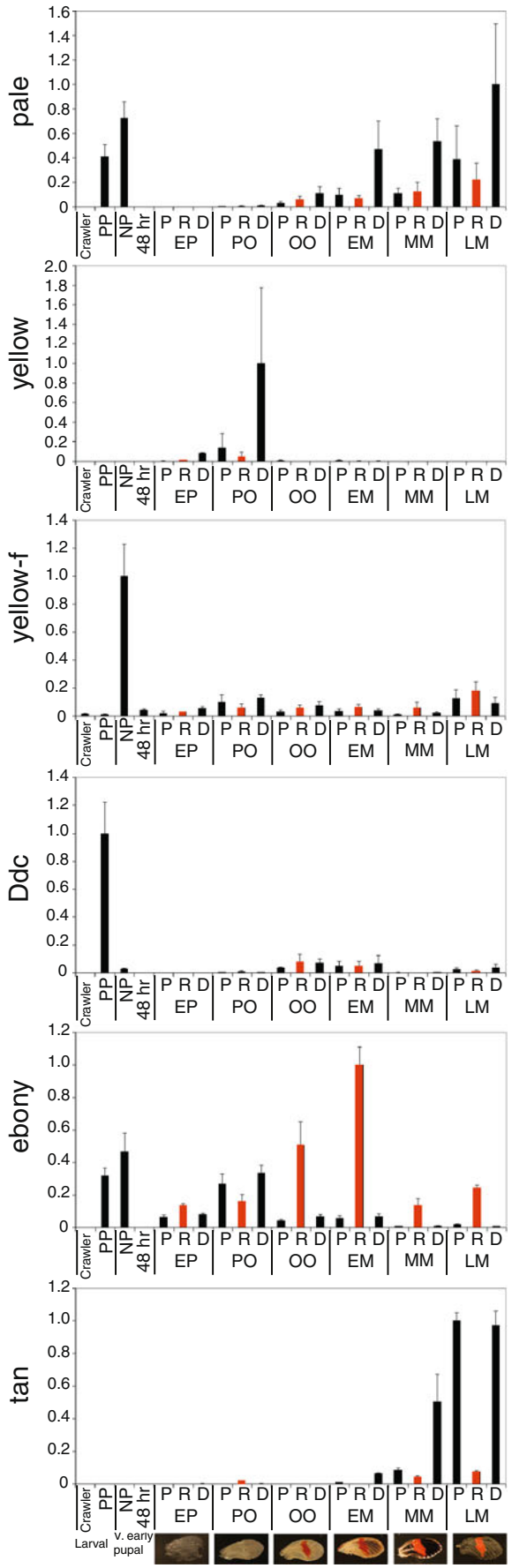

Fig. 3 Expression of genes from the melanin pathway over late larval and pupal wing development in *H. melpomene*. Two late fifth instar larval stages (crawler and almost pupa) and eight pupal developmental stages (new pupa, 48-h pupa, early pupa, pre-ommochrome, early melanin, mid-melanin and late melanin) were sampled, and for the last six pupal stages wings were dissected into three regions—proximal (*P*), red band (*R*, coloured *red*) and distal (*D*). Earlier stage samples were whole forewings. Expression was normalised to expression of a control, elongation factor 1α (ef1α), and is shown relative to the highest experimental reading for each gene. *Error bars* are standard deviations

for gene expression analysis. In the *Heliconius* wing ommochrome pigments are laid down during pupation followed by melanins (see Ferguson and Jiggins 2009 for staging). All genes except *yellow* and *tan* were found to have a spike of expression around pupation, presumably associated with the formation of pupal cuticle, followed by a drop in expression at 48-h post-pupation (Fig. 3). For all genes, the developmental stage or wing region assayed had a significant effect on gene expression level; and there was a significant interaction between wing region and stage demonstrating that gene expression levels change dynamically over both the surface of the wing and the pupal time series (ANOVA $p \leq 0.05$ in all cases).

All genes showed expression across the wing, and for *DDC* and *yellow-f* there was no clear relationship between expression level and pigmentation. Melanisation occurs in a distal to proximal direction on the wing (Supplementary Fig 1), and *pale* was upregulated in the distal region from the stage prior to melanisation (OO) onwards. The strongest correlations with spatial localisation of pigment however were *ebony*, *tan* and *yellow*. First, *ebony* was unregulated in the red band relative to both the proximal and distal melanic wing regions from the OO stage onwards. In contrast, the expression of *tan* was inverted relative to *ebony*, with strongly upregulated expression levels in the melanic regions but downregulation in the forewing band. For both genes, differential expression was most evident during later stages of melanisation. Expression of *yellow* was again upregulated in the future melanic tissue, and specifically the distal region; but in this instance at least 48 h prior to visible pigmentation of the wing at the Pre-Ommochrome stage. Overall, the most striking result was clear inverse expression of *ebony* and *tan* in a pattern correlated with the presence of melanin pigments in the adult. The pattern is consistent with the known roles of these genes in *Drosophila melanogaster*, in which *ebony* suppresses and *tan* promotes melanisation (Wittkopp et al. 2003a, b).

Expression levels of the *ebony* and *tan* genes are associated with mimetic *Heliconius* wing patterning

We captured and bred a variety of divergent and convergent phenotypes from wild *Heliconius* populations in order to

further investigate the relationship between *ebony* and *tan* expression and the *Heliconius* wing pattern radiation. Our sampling of *H. melpomene* included two geographically isolated subspecies, *H. melpomene rosina* (Panama) and *H. melpomene amaryllis* (Peru), that share a 'postman' pattern, and one 'rayed' form, *H. melpomene aglaope* (Peru) (Fig. 4). In the co-mimic species, *H. erato*, we sampled the postman race *H. erato petiverana* (Panama, Fig. 4) and a hybrid population of *H. erato* from Surinam that was obtained through a supplier. Finally, we also studied two co-mimic species, *H. ismenius* and *H. hecale*, from the phenotypically divergent 'tiger' mimicry ring in Panama. Phylogenetic reconstruction of ancestral phenotypes in a character as labile as the wing patterns of *Heliconius* is problematic and as yet the ancestral *Heliconius* wing pattern is not known, but our sampling covers multiple cases of both divergence and convergence across the genus (Beltran et al. 2007).

Prior to expression analysis we increased the resolution of developmental staging around melanisation to reduce error associated with developmental variability (Supplementary Figs. 1 and 2). We found strikingly predictable expression patterns across both divergent and convergent *Heliconius* wing patterns both within and between species (Fig. 4, the number of biological replicates for each taxon is shown below the name). Convergent domains of melanin pigment gene expression therefore underlie convergent wing phenotypes in *Heliconius* butterflies.

For red and black wing regions, the results largely confirmed expectations based on preliminary experiments from *Heliconius melpomene cythera* (Ecuador, Fig. 3). First, *ebony* was significantly upregulated in red pattern elements in virtually all cases, and second, *tan* was upregulated in melanic regions in a similar pattern (Fig. 4). The only exception was the rayed hindwing of *H. erato* from Surinam, in which *ebony* was upregulated in the red rays, as expected, but *tan* expression did not differ significantly between melanic and non-melanic regions (Fig. 4). However, these *H. erato* individuals were taken from a population segregating for the hindwing ray phenotype so may be heterozygote genotypes at the locus controlling the rayed pattern. Further sampling of rayed *H. erato* species from non-hybrid populations will be required to confirm whether this divergent expression pattern for *tan* is a feature of rayed *H. erato* wings in general.

Our expectation was that non-melanic yellow wing regions would show similar patterns of expression to the red wing regions as both are devoid of melanic pigments. Surprisingly however, this was not the case. Although *tan* was significantly downregulated in yellow wing regions relative to melanic tissue, in all cases *ebony* expression did not differ significantly between yellow and melanic regions (Fig. 4). Thus, both genes are expressed at low levels in yellow wing regions in all wing patterns surveyed. It is not clear whether downregulation of *tan* is sufficient to prevent formation of melanin in yellow regions, or whether an additional gene is responsible for suppressing melanisation in place of *ebony*. Either way, these results suggest that despite both being 'non-melanic', suppression of melanisation in red and yellow wing regions shows different patterns of genetic control.

Melanin pathway genes do not map to major wing patterning loci

In order to determine whether the melanin pathway genes might be the locus of evolutionary change in *Heliconius* as in some studies from *Drosophila*, we mapped these genes with respect to known loci controlling wing pattern polymorphism. Within *Heliconius*, the presence/absence of the large wing pattern elements are determined by pattern switching alleles at four major loci, located on four of the 21 *Heliconius* chromosomes (linkage groups 1, 10, 15 and 18) (Joron et al. 2006b; Sheppard et al. 1985). Recent characterisation of two of these loci from *H. melpomene* (Baxter et al. 2010; Ferguson et al. 2010; Salazar et al. 2010) failed to identify coding sequence for pigmentation genes within the mapped intervals. To determine whether the remaining two loci might involve candidate melanin pathway genes, or whether these genes might lie just outside the regions already sequenced, we assigned all the genes identified above in *H. melpomene* to chromosomal linkage groups, except *tan* due to a lack of segregating variation. For each locus, 16 individuals were genotyped and a perfect correlation observed between segregation of alleles derived from the maternal genome and one of the existing 'chromosome prints' for this mapping family. All of the mapped genes were located on different linkage groups and none are tightly linked to major wing pattern loci. *pale* mapped to the Z chromosome, *ebony* to LG 19, *yellow* to LG 17 and *yellow-f* to LG 6. *Ddc* has been previously mapped to LG 1, which contains the *K* patterning locus, but the two are not tightly linked (Jiggins et al. 2005). Thus, neither the coding nor *cis*-regulatory regions of melanin pathway genes control shifts in melanic patterning in *Heliconius* butterflies.

Discussion

We have isolated and characterised *H. melpomene* melanin pathway genes, studied the expression of these genes over a time course of pupal wing pattern development, mapped the genes relative to known wing pattern loci and assayed the expression of two candidates, *ebony* and *tan* in a range of *Heliconius* representing both inter- and intra-specific diversity.

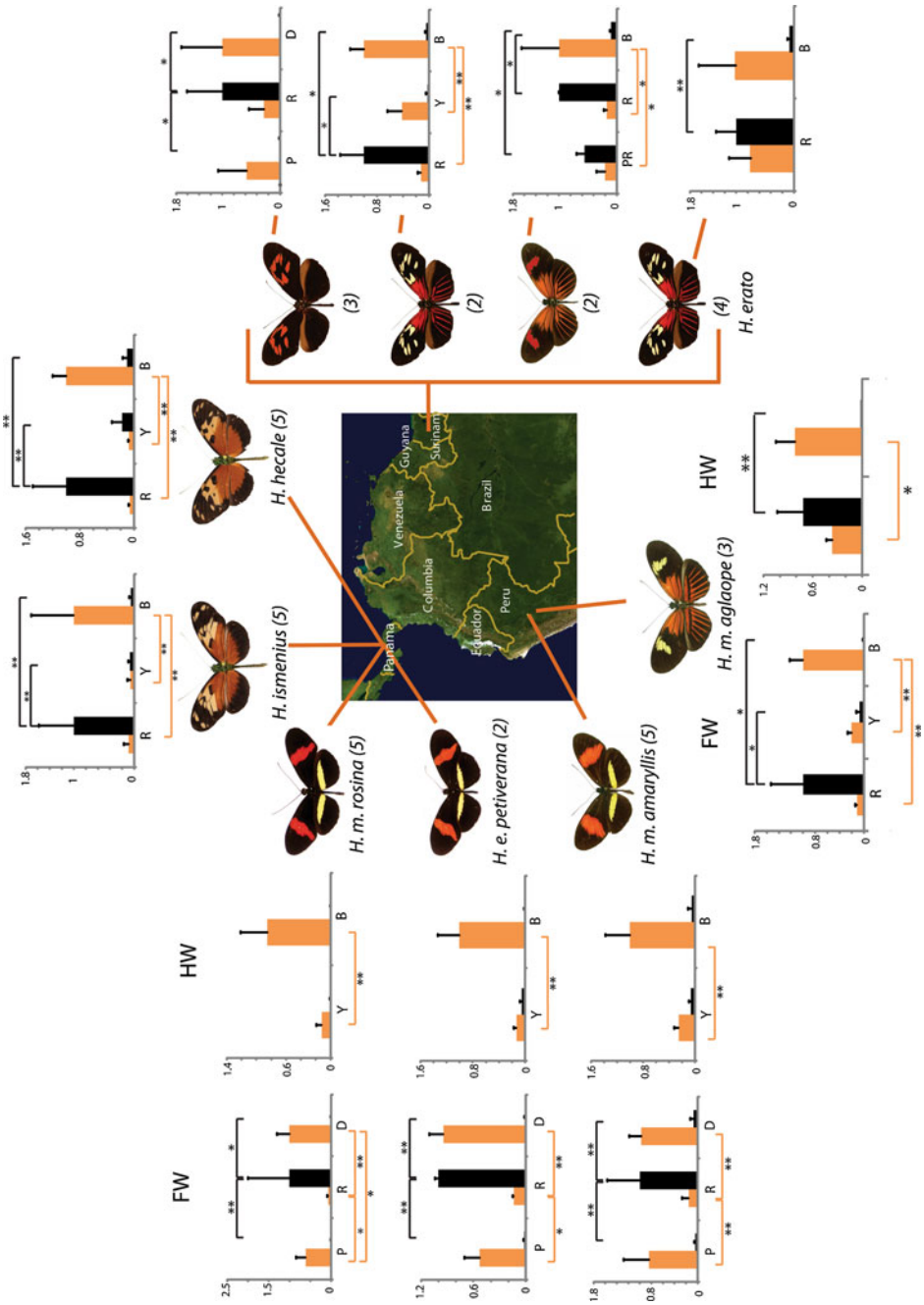

Fig. 4 Expression of the *ebony* and *tan* genes is associated with mimetic patterning in *Heliconius* butterflies. Four species of *Heliconius* were sampled from across South and Central America, including three races of *H. melpomene* and two races of *H. erato*. *H. erato* from Surinam are hybrids showing polymorphism in colour and shape of the forewing band and the presence of hindwing rays (absence of rays was not assayed). In the absence of adult specimens, wing colours of Surinam specimens were altered using Photoshop to illustrate the phenotypic variation sampled. The rayed hindwing of Surinam *H. erato* was assayed from individuals with various forewing phenotypes, including that illustrated. The wings of staged individuals were dissected into proximal (*P*), distal (*D*), red (*R*), yellow (*Y*) and black (*B*) regions (*PR* proximal red). The results show that in all instances except the *H. erato* hindwing rays *ebony* transcripts were localised to red pattern elements (*black bars*) and *tan* to black (melanic) elements (*tan bars*; *p* values of *≤0.05, **≤0.01, one-tailed *t* test; *error bars* are standard deviation). Yellow elements unexpectedly had very low levels of both *tan* and *ebony* expression. The number of biological replicates sampled is shown *in brackets* for each phenotype

The major findings from this study are (1) there is a highly conserved mechanism for generating regions of red, yellow and black pigment across distantly related *Heliconius* species, (2) there is a different developmental basis for supressing melanisation in the red and yellow wing regions, (3) that there may be a conserved module for melanic patterning between lepidopteran larval epidermis and pupal wing tissue and (4) in contrast to several studies from *Drosophila,* genetic variation in genes of the melanin pathway does not control wing pattern polymorphism in the *Heliconius*.

The *Heliconius* have long been famed for their wing pattern diversity and convergence across the tropics (Bates 1862). The molecular genetic basis of this convergence is only now starting to be uncovered, both in respect to pattern (Baxter et al. 2010; Counterman et al. 2010; Joron et al. 2006a; Ferguson et al. 2010), and pigmentation (Reed et al 2008; Ferguson and Jiggins 2009). Previously, convergent gene expression has been shown for the ommochrome pathway gene *cinnabar* between *H. melpomene* and *H. erato*, whilst expression of the *vermillion* gene was found to differ (Reed et al 2008; Ferguson and Jiggins 2009). We have shown that with the possible exception of *H. erato* hindwing rays, regulation of *tan* and *ebony* is strikingly conserved across the genus both within and between species.

Transcript levels in wing regions pigmented with melanins and red ommochromes (dihydroxykynurenine) follow our expectations, with inverse upregulation of *ebony* in red regions and *tan* in melanic regions; whereas wing scales pigmented with yellow ommochromes (3-hydroxykynurenine) are downregulated for both genes. Whilst this result is surprising, red, black and yellow/white (non-pigmented) scale cells are developmentally distinct in *Heliconius* and form three distinct morphological classes that mature from their epidermal precursors and sclerotize at different stages of pupal development (Gilbert 1988). One explanation for our findings could be that that the dual role of dopamine as a precursor for melanisation and sclerotization (Fig. 1) results in divergent *ebony* expression in red and yellow scales as they sclerotize at different stages. However, we do not see any upregulation of *ebony* in yellow scales during melanogenesis (Fig. 4), whereas *ebony* is upregulated in future red scales throughout pupation (Fig. 3), suggesting that *ebony* does not induce stage-specific sclerotization on *Heliconius* wings.

Alternatively, a model of wing pattern development has been suggested in which the developmental status of the wing cell determines its 'competency' to respond to pigmentation cues (Ffrench-Constant and Koch 2003; Koch et al. 2000a). Under this model, pigmentation cues could be present across the wing but only in regions where the cells are developmentally competent would pigment gene expression result in pigmented scales. This model is not supported by our data, as both ommochrome and melanin pathway genes have highly localised gene expression corresponding to wing pattern. Therefore, although scale cell development and melanin gene expression are correlated in *Heliconius*, differential pigmentation of the wing is unlikely to be driven only by the physical properties of scale cell maturation. Instead, the localised expression of a limited number of pigment biosynthesis genes from both pigmentation pathways suggests that their enzyme products play a direct functional role in determining the pigment boundaries. Indeed, in this study we note that *ebony*, *tan* and also *yellow-f* showed much higher levels of expression relative to the control than *Ddc*, *pale* and *yellow* (Supplementary Table 1). This may suggest that a subset of melanin pathway genes play a disproportionate role in production or distribution of the melanic pigment.

Finally, divergent *ebony* expression between red and yellow scale types could indicate that the exclusion of melanic pigment has independent evolutionary origins in *Heliconius.* This hypothesis is mirrored in *D. melanogaster* where *ebony* expression is correlated with melanic patterns on the thorax, but not the wing or abdomen, again leading to the suggestion that the genetic control of melanisation has different molecular mechanisms in different body regions (Wittkopp et al. 2002b; True et al. 1999). The fact that *ebony*/*tan* expression is conserved for all *Heliconius* species and patterns (with the possible exception of the *H. erato* hindwing rays) clearly suggests that the association of pigment gene expression and scale cell type predated the radiation of the *Heliconius*.

We suggest the presence of pigment-scale cell 'modules' in the *Heliconius* ancestor, which involve loci for both pigment production and differential scale cell maturation. A 'red patch' module, for example, might involve upregulation of *ebony*, downregulation of *tan*, upregulation of the ommochrome genes *cinnabar* and *vermilion* and expression of an uncharacterised factor for the maturation of red-scale type morphology. The loci within such modules could be co-ordinately regulated in response to activity from a patterning locus; and be recruited to novel wing patterning networks in response to selection for convergent mimetic phenotypes; providing a mechanism for the rapid radiation of *Heliconius* wing patterns. The convergence in wing patterning would then be underpinned by convergence in co-option of these modules as new patterning networks evolve to generate a mimetic phenotype. Because the nymphalid eyespot butterfly *Bicyclus anynana* also has a conserved relationship between pigmentation and scale cell morphology (Janssen et al. 2001), it could be that these pigmentation-scale cell modules predated the divergence of the nymphalids. If so, we would predict that the patterns of pigment gene expression in the *Heliconius* would also hold for *Bicyclus* and other divergently patterned nymphalid butterflies.

The extent to which genes implicated in *Drosophila* morphogenesis underlie phenotypic adaptation in other

lineages is an outstanding issue in evolutionary developmental biology (Wittkopp and Beldade 2009). It is now clear that whilst both patterning and effector genes have been implicated in *Drosophila* phenotypic divergence, a limited number of pigment genes are recurrent targets of selection in different insect lineages. Work on the swallowtail butterfly *P. xuthus* and silkmoth *B. mori* has led to the suggestion that there may be a lepidopteran 'melanisation module' (Futahashi et al. 2010). In *Papilio, yellow*, *ebony*, *DDC*, *TH* (*pale*) and *tan* expression was correlated with the presence of black markings on the larval epidermis, and *tan* with the *Bombyx* larval epidermis (Futahashi et al. 2010). Here, we find a correlation with wing markings for all these genes except *pale* (Fig. 3), suggesting that there may be a conserved set of pigmentation genes regulating melanic patterning between lepidopteran larval epidermis and pupal wing pattern.

It has also been proposed that larval *Papilio* melanisation occurs in two phases. In the first phase agents responsible for oxidising dopamine to dopa-melanin (*yellow/Laccase2* (not studied here)) are generated, and in the second dopamine itself is formed *via* the activity of *pale/Ddc/tan*. The 'pre-patterning' of *yellow* expression thus ensures that the protein product is available for the deposition/formation of dopamine-melanin in the cuticle during dopamine synthesis (Futahashi et al. 2010). In this study, the strong upregulation of *yellow* expression at least 48 h prior to upregulation of *tan* and visible melanisation of the wing strongly supports this model, and again suggests that there is conservation of melanisation mechanisms between *Papilio* larval epidermis and *Heliconius* pupal wings.

A number of factors might have suggested that the genes assayed here are prime candidates for controlling melanic phenotypic shifts in the *Heliconius*. Not only do we find tight spatial regulation of gene expression corresponding to wing pattern, but extensive crosses between *Heliconius* races have demonstrated that wing pattern loci shift the boundaries of melanisation across the wing surface to cover or reveal 'windows' of underlying pattern elements, such as the forewing band (Gilbert 2003). This model is reminiscent of the situation in *Drosophila* in which mutations in the independent *cis*-regulatory elements of *tan*, *yellow* and *ebony* determine the distribution of melanic pigment on the thorax, abdomen and wings (Jeong et al. 2008; Rebeiz et al. 2009; Wittkopp et al. 2009; Prud'homme et al. 2006). Expression of *ebony* has also been linked to melanic shifts in the wings and body of *Papilio* butterflies (Futahashi and Fujiwara 2005; Koch et al. 2000b), and recent work from *Bombyx* in which the *chocolate* and *sooty* and *rouge* larval laboratory mutants were mapped to the *yellow*, *ebony* and *tan* genes, respectively (Futahashi et al. 2008; Futahashi et al. 2010) seem to indicate that the melanisation genes themselves can be a predictable target for melanic shifts in the Lepidoptera as in *Drosophila*.

Nonetheless, characterisation of two patterning loci from *H. melpomene* has failed to find coding sequence of pigmentation genes within mapped intervals (Baxter et al. 2010; Ferguson et al. 2010; Salazar et al. 2010). Genetic mapping in this study further rules out any linkage between pigmentation loci and the loci of phenotypic adaptation in *Heliconius*. Recent work on the moth *Biston betularia*, famed for shifting its melanic phenotype in response to industrial pollution, has also failed to associate any melanic pigmentation gene with wing pattern polymorphism (van't Hof and Saccheri 2010).

Recent work has demonstrated that instead, red patterns are controlled by the *optix* transcription factor (Reed et al. 2011). In contrast, yellow patterns are determined by an unlinked locus that also shows homology to a region controlling *Bicyclus* eyespot variation, and melanic shifts in *Biston* (Van't Hof et al. 2011; Beldade et al. 2009; Ferguson et al. 2010), suggesting that a wing patterning locus may be an ancestral feature of the Lepidoptera. Either way, these patterning loci therefore likely underlie the extensive diversity of the lepidopteran wing relative to other insects, and lie upstream of the pigmentation genes studied here.

The gene network linking wing patterning and pigmentation in the butterflies is currently unknown, but the relationship we find between scale cell type and pigment gene activity suggests that butterfly wing pattern development may involve an enhanced 'effector' module consisting of both genes for pigment biosynthesis and wing scale cell differentiation. Unravelling the genetic network that links wing pattern switches with insect hormones, pigment genes and the well-characterised developmental genes involved in butterfly eyespot elaboration (Martin and Reed 2010; for review see Beldade and Brakefield 2002) is a major challenge for lepidopteran genetics in the future.

Acknowledgements We thank Nick Mundy for the use of his qRT-PCR machine, and Simon Baxter and Claire Webster for contributing to linkage mapping. Breeding was carried out with assistance from R. Merrill, and Moises Abanto. Ana Portugal and Grace Wu assisted with dissections. This work was funded by a NERC Ph.D. Studentship to L. Ferguson, BBSRC support of L. Maroja and Leverhulme Trust Research Leadership grant and a Royal Society Fellowship to C. Jiggins.

References

Bates HW (1862) Contributions to an insect faunaof the Amazon valley. Lepidoptera. Heliconidae. Trans Linn Soc Lond 23:495–566

Baxter SW, Nadeau NJ, Maroja LS, Wilkinson P, Counterman BA, Dawson A, Beltran M et al (2010) Genomic hotspots for adaptation: the population genetics of Müllerian mimicry in the *Heliconius melpomene* Clade. PLoS Genet 6(2):e1000794

Beldade P, Brakefield PM (2002) The genetics and evo-devo of butterfly wing patterns. Nat Rev Genet 3(6):442–452

Beldade P, Saenko SV, Pul N, Long AD (2009) A gene-based linkage map for *Bicyclus anynana* butterflies allows for a comprehensive analysis of synteny with the lepidopteran reference genome. PLoS Genet 5(2):e1000366

Beltran M, Jiggins CD, Brower A, Bermingham E, Mallet J (2007) Do pollen feeding, pupal-mating and larval gregariousness have a single origin in *Heliconius* butterflies? Inferences from multilocus DNA sequence data. Biol J Linn Soc 92(2):221–239

Brisson JA, Templeton AR, Duncan I (2004) Population genetics of the developmental gene optomotor-blind (omb) in *Drosophila polymorpha*: evidence for a role in abdominal pigmentation variation. Genetics 168(4):1999–2010

Chenna R, Sugawara H, Koike T, Lopez R, Gibson TJ, Higgins DG, Thompson JD (2003) Multiple sequence alignment with the Clustal series of programs. Nucleic Acids Res 31(13):3497–3500

Counterman BA, Araujo-Perez F, Hines HM, Baxter SW, Morrison CM, Lindstrom DP, Papa R et al (2010) Genomic hotspots for adaptation: the population genetics of Mullerian mimicry in *Heliconius erato*. PLoS Genet 6(2):p.e1000796

Darwin-Initiative (2007) Tropical andean butterfly diversity project—databases. Available at http://www.andeanbutterflies.org/database.html. Accessed 23 May 2011

Ferguson LC, Jiggins CD (2009) Shared and divergent expression domains on mimetic *Heliconius* wings. Evol Dev 11(5):498–512

Ferguson LC, Lee SF, Chamberlain N, Nadeau N, Joron M, Baxter SW, Wilkinson P et al (2010) Characterization of a hotspot for mimicry: assembly of a butterfly wing transcriptome to genomic sequence at the HmYb/Sb locus. Mol Ecol 19(Suppl 1):240–254

Ferguson LC, Green J, Surridge A, Jiggins CD (2011) Evolution of the insect yellow gene family. Mol Biol Evol 28(1):257–272

Ffrench-Constant RH, Koch PB (2003) Mimicry and melanism in swollowtail butterflies: toward a molecular understanding. In: Boggs CL, Watt WB, Ehrlich PB (eds) Butterflies: ecology and evolution taking flight. University of Chicago Press, Chicago

Futahashi R, Fujiwara H (2005) Melanin-synthesis enzymes coregulate stage-specific larval cuticular markings in the swallowtail butterfly, *Papilio xuthus*. Dev Genes Evol 215(10):519–529

Futahashi R, Sato J, Meng Y, Okamoto S, Daimon T, Yamamoto K, Suetsugu Y et al (2008) Yellow and ebony are the responsible genes for the larval color mutants of the silkworm *Bombyx mori*. Genetics 180(4):1995–2005

Futahashi R, Banno Y, Fujiwara H (2010) Caterpillar color patterns are determined by a two-phase melanin gene prepatterning process: new evidence from tan and laccase2. Evol Dev 12(2):157–167

Gilbert LE (1988) Correlations of ultrastructure and pigmentation suggest how genes control development of the wing scales of *Heliconius* butterflies. J Res Lepidoptera 26:141–160

Gilbert LE (2003) Adaptive novelty through introgression in *Heliconius* wing patterns: evidence for a shared genetic "tool box" from synthetic hybrid zones and a theory of diversification. In: Boggs CL, Watt WB, Ehrlich PB (eds) Butterflies: ecology and evolution taking flight. University of Chicago Press, Chicago

Gompel N, Carroll SB (2003) Genetic mechanisms and constraints governing the evolution of correlated traits in drosophilid flies. Nature 424(6951):931–935

Gompel N, Prud'homme B, Wittkopp PJ, Kassner VA, Carroll SB (2005) Chance caught on the wing: *cis*-regulatory evolution and the origin of pigment patterns in *Drosophila*. Nature 433 (7025):481–487

Han Q, Fang J, Ding H, Johnson JK, Christensen BM, Li J (2002) Identification of *Drosophila melanogaster* yellow-f and yellow-f2 proteins as dopachrome-conversion enzymes. Biochem J 368 (Pt 1):333–340

Hoekstra HE (2006) Genetics, development and evolution of adaptive pigmentation in vertebrates. Heredity 97(3):222–234

Janssen JM, Monteiro A, Brakefield PM (2001) Correlations between scale structure and pigmentation in butterfly wings. Evol Dev 3 (6):415–423

Jeong S, Rebeiz M, Andolfatto P, Werner T, True JR, Carroll SB (2008) The evolution of gene regulation underlies a morphological difference between two *Drosophila* sister species. Cell 132 (5):783–793

Jiggins CD, Naisbit RE, Coe RL, Mallet J (2001) Reproductive isolation caused by colour pattern mimicry. Nature 411 (6835):302–305

Jiggins CD, Mavarez J, Beltrán M, McMillan WO, Johnston JS, Bermingham E (2005) A genetic linkage map of the mimetic butterfly *Heliconius melpomene*. Genetics 171(2):557–570

Joron M, Jiggins CD, Papanicolaou A, McMillan WO (2006a) *Heliconius* wing patterns: an evo-devo model for understanding phenotypic diversity. Heredity 97(3):157–167

Joron M, Papa R, Beltrán M, Chamberlain N, Mavárez J, Baxter SW, Abanto M et al (2006b) A conserved supergene locus controls colour pattern diversity in *Heliconius* butterflies. PLoS Biol 4 (10):p.e303

Kapan DD (2001) Three-butterfly system provides a field test of mullerian mimicry. Nature 409(6818):338–340

Kingsley EP, Manceau M, Wiley CD, Hoekstra HE (2009) Melanism in peromyscus is caused by independent mutations in *Agouti*. PloS One 4(7):e6435

Koch PB, Behnecke B, Ffrench-Constant RH (2000a) The molecular basis of melanism and mimicry in a swallowtail butterfly. Curr Biol 10(10):591–594

Koch PB, Lorenz U, Brakefield PM, Ffrench-Constant RH (2000b) Butterfly wing pattern mutants: developmental heterochrony and co-ordinately regulated phenotypes. Dev Genes Evol 210 (11):536–544

Kopp A, Duncan I, Godt D, Carroll SB (2000) Genetic control and evolution of sexually dimorphic characters in *Drosophila*. Nature 408(6812):553–559

Kopp A, Graze RM, Xu S, Carroll SB, Nuzhdin SV (2003) Quantitative trait loci responsible for variation in sexually dimorphic traits in *Drosophila melanogaster*. Genetics 163 (2):771–787

Martin A, Reed RD (2010) Wingless and aristaless2 define a developmental ground plan for moth and butterfly wing pattern evolution. Mol Biol Evol 27(12):2864–2878

Mundy NI (2005) A window on the genetics of evolution: MC1R and plumage colouration in birds. Proc R Soc B Biol Sci 272 (1573):1633–1640

Nachman MW (2005) The genetic basis of adaptation: lessons from concealing coloration in pocket mice. Genetica 123(1–2):125–136

Nijhout HF (1991) The development and evolution of butterfly wing patterns. Smithsonian Institution Press, Washington

Pool JE, Aquadro CF (2007) The genetic basis of adaptive pigmentation variation in *Drosophila melanogaster*. Mol Ecol 16(14):2844–2851

Prud'homme B, Gompel N, Rokas A, Kassner VA, Williams TM, Yeh S-D, True JR et al (2006) Repeated morphological evolution through *cis*-regulatory changes in a pleiotropic gene. Nature 440 (7087):1050–1053

Rebeiz M, Pool JE, Kassner VA, Aquadro CF, Carroll SB (2009) Stepwise modification of a modular enhancer underlies adaptation in a *Drosophila* population. Science 326(5960):1663–1667

Reed RD, McMillan WO, Nagy LM (2008) Gene expression underlying adaptive variation in *Heliconius* wing patterns: non-modular regulation of overlapping cinnabar and vermilion prepatterns. Proc R Soc B Biol Sci 275(1630):37–46

Reed RD, Papa R, Martin A, Hines, Heather M, Counterman BA, Pardo-Diaz C, Jiggins CD et al (2011) Optix drives the repeated convergent evolution of butterfly wing pattern mimicry. Sci N Y 333(6046):1137–1141

Salazar C, Baxter SW, Pardo-Diaz C, Wu G, Surridge A, Linares M, Bermingham E et al (2010) Genetic evidence for hybrid trait speciation in *Heliconius* butterflies. PLoS Genet 6(4):p.e1000930

Savard J, Tautz D, Richards S, Weinstock GM, Gibbs RA, Werren JH, Tettelin H et al (2006) Phylogenomic analysis reveals bees and wasps (Hymenoptera) at the base of the radiation of holometabolous insects. Genome Res 16(11):1334–1338

Schmidt HA, Strimmer K, Vingron M, von Haeseler A (2002) TREE-PUZZLE: maximum likelihood phylogenetic analysis using quartets and parallel computing. Bioinformatics 18(3):502–504

Sheppard PM, Turner JRG, Brown KS, Benson WW, Singer MC (1985) Genetics and the evolution of muellerian mimicry in *Heliconius* butterflies. Philos Trans R Soc B-Biol Sci 308(1137):433–610

Steiner CC, Weber JN, Hoekstra HE (2007) Adaptive variation in beach mice produced by two interacting pigmentation genes. PLoS Biol 5(9):p.e219

Takahashi A, Takahashi K, Ueda R, Takano-Shimizu T (2007) Natural variation of ebony gene controlling thoracic pigmentation in *Drosophila melanogaster*. Genetics 177(2):1233–1237

Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol Biol Evol 24(8):1596–1599

True JR (2003) Insect melanism: the molecules matter. Trends Ecol Evol 18(12):640–647

True JR, Edwards KA, Yamamoto D, Carroll SB (1999) *Drosophila* wing melanin patterns form by vein-dependent elaboration of enzymatic prepatterns. Curr Biol 9(23):1382–1391

True JR, Yeh S-D, Hovemann BT, Kemme T, Meinertzhagen IA, Edwards TN, Liou S-R et al (2005) *Drosophila* tan encodes a novel hydrolase required in pigmentation and vision. PLoS Genet 1(5):p.e63

Turner JRG, Sheppard PM (1975) Absence of crossing-over in female butterflies (*Heliconius*). Heredity 34(2):265–269

van't Hof AE, Saccheri IJ (2010) Industrial melanism in the peppered moth is not associated with genetic variation in canonical melanisation gene candidates. PLoS ONE 5(5): e10889

Van't Hof AE, Edmonds N, Dalíková M, Marec F, Saccheri IJ (2011) Industrial melanism in British peppered moths has a singular and recent mutational origin. Science 332(6032):958

Williams TM, Selegue JE, Werner T, Gompel N, Kopp A, Carroll SB (2008) The regulation and evolution of a genetic switch controlling sexually dimorphic traits in *Drosophila*. Cell 134(4):610–623

Wittkopp PJ, Beldade P (2009) Development and evolution of insect pigmentation: genetic mechanisms and the potential consequences of pleiotropy. Semin Cell Biol Dev Biol 20(1):65–71

Wittkopp PJ, True JR, Carroll SB (2002a) Reciprocal functions of the *Drosophila* yellow and ebony proteins in the development and evolution of pigment patterns. Development 129(8):1849–1858

Wittkopp PJ, Vaccaro K, Carroll SB (2002b) Evolution of yellow gene regulation and pigmentation in *Drosophila*. Curr Biol 12 (18):1547–1556

Wittkopp PJ, Carroll SB, Kopp A (2003a) Evolution in black and white: genetic control of pigment patterns in *Drosophila*. Trends Genet 19(9):495–504

Wittkopp PJ, Williams BL, Selegue JE, Carroll SB (2003b) Drosophila pigmentation evolution: divergent genotypes underlying convergent phenotypes. Proc Natl Acad Sci U S A 100(4):1808–1813

Wittkopp PJ, Stewart EE, Arnold LL, Neidert AH, Haerum BK, Thompson EM, Akhras S et al (2009) Intraspecific polymorphism to interspecific divergence: genetics of pigmentation in *Drosophila*. Science 326(5952):540–544