



# CRISPR/Cas9 as the Key to Unlocking the Secrets of Butterfly Wing Pattern Development and Its Evolution

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## Abstract

With the exception of a few moth and butterfly species, gene-editing tools in Lepidoptera have been lagging behind other well-studied insects. In order to elucidate gene function across the order, it is necessary to establish tools that enable such gene manipulation. CRISPR/Cas9 is a promising technique and here we review the recent progress made in implementing the technique in butterflies; from broad patterning of the wing, to the development of specific colours in particular wing sections, to eyespot formation. The often species-specific responses to the CRISPR/Cas9-induced mutations in candidate genes, underscore the significance of these genes in the wide evolutionary

diversification of butterfly wing patterns. We further discuss potential caveats in the interpretation of the resulting mutant phenotypes obtained through CRISPR/Cas9 gene editing. Finally, we discuss the possibilities CRISPR/Cas9 offers beyond mere knockout of candidate genes, including the potential for the generation of transgenics that will further elucidate the developmental genetic basis for wing pattern evolution.

## ABBREVIATIONS

<b>CDS</b>	coding DNA sequence
<b>CRISPR</b>	clustered regularly interspaced short palindromic repeats
<b>DSB</b>	double-stranded break
<b>Indels</b>	insertion/deletions
<b>KO(s)</b>	knockout(s)
<b>RNAi</b>	RNA interference
<b>sgRNA</b>	synthetic guide RNA
<b>TALENs</b>	transcription activator-like effector nucleases
<b>ZFNs</b>	zinc-finger nucleases



## 1. FUNCTIONAL GENOMICS IN LEPIDOPTERA: AN INTRODUCTION

The developmental evolution of butterfly wing patterns is a major model for the evolution of morphological novelties and the cooption of particular developmental genes therein (Brakefield et al., 1996; Deshmukh et al., 2017; Fujiwara and Nishikawa, 2016; Jiggins et al., 2017; Monteiro, 2015; Nijhout, 1991, 1996; Ohno and Otaki, 2015). Despite having identified many of the key genes involved over the years, and elucidated their expression patterns and allelic variability, we thus far lacked the tools to adequately study their function. Although some progress had been made in moths, in particular for the silk moth *Bombyx mori* (Kolliopoulou and Swevers, 2014), Lepidoptera as a whole are somewhat unique as a major model system in the field of evolutionary developmental biology to have been lacking such functional tools. Lepidopterans appear to have a very poor response to RNA interference (RNAi) (Kolliopoulou and Swevers, 2014; Terenius et al., 2011), which some have argued is a result of high levels of expression of dsRNases present endogenously within the order (Shukla et al., 2016), and because they display poor intracellular transport of the injected RNA (Joga et al., 2016). The development of RNAi and its application in moths have largely been driven by a desire to develop

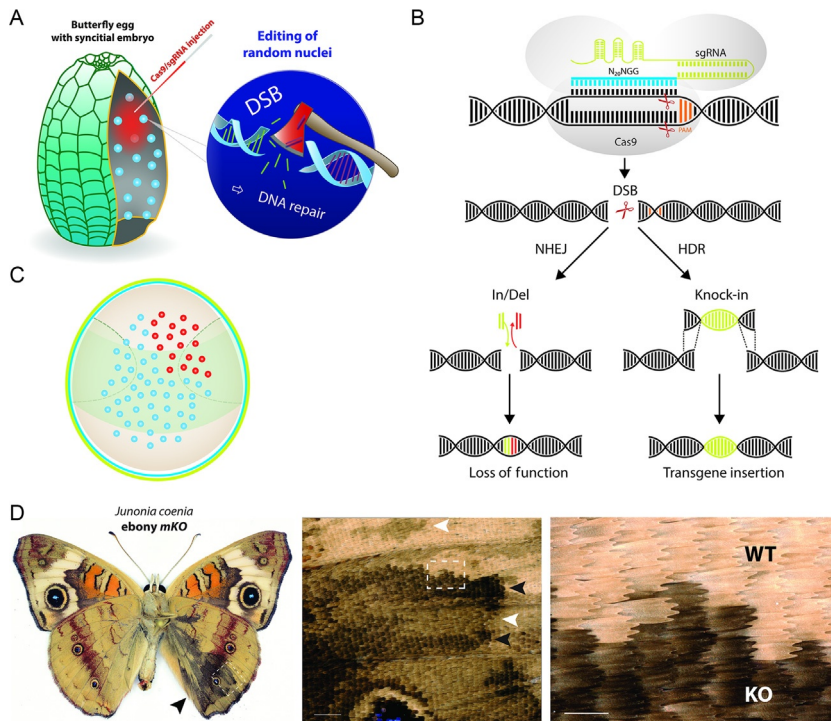
efficient pest control and safe guarding commercially interesting species such as *B. mori* (Kolliopoulou and Swevers, 2014). Very few moth studies have deployed RNAi in an evolutionary developmental biology context, and those that have done so use it in the context of early embryogenesis (Nakao, 2012, 2015). It is only very recently that two RNAi-based techniques showed promise for studying butterfly wing patterning and its evolution: (1) RNAi driven by an inducible heat-shock promoter in transgenic *Bicyclus anynana* butterflies (Monteiro et al., 2013; Ozsu et al., 2017) and (2) a novel RNAi technique (in combination with localised electroporation) in *Papilio* butterflies, which has been deployed in the study of wing patterning mimicry (Fujiwara and Nishikawa, 2016; Komata et al., 2016; Nishikawa et al., 2015).

Several other techniques, most notably zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs), are available that enable the study of gene function in insects through knockout (KO)/knockdown, and again these have recently been developed in moths largely with a view to pest control; for example, disruption mating behaviour in the moth *Ostrinia furnacalis* by targeting odorant receptor coreceptor (*Orco*) (Yang et al., 2016). TALEN-mediated genome editing has indeed been well established now for *B. mori* (Daimon et al., 2014; Nakade et al., 2014; Takasu et al., 2010, 2013, 2016). For butterflies, few examples exist for the use of ZFNs. They were successfully employed to study the significance of *cryptochrome 2* for migration in the Monarch butterfly *Danaus plexippus* (Merlin et al., 2013), but the technique has not been deployed for the study of wing patterning. Both ZFNs and TALENs require the engineering of site-specific nucleases, which is time consuming, costly, and technically challenging (Gaj et al., 2013). The use of transposon-based transgenics has been used successfully in *B. mori* (Tamura et al., 2000) and has been extended to transgenic lines of *B. anynana* for evo-devo research via the PiggyBac-mediated integration (Marcus et al., 2004; Monteiro et al., 2013; Ozsu et al., 2017; Ramos and Monteiro, 2007; Tong et al., 2014). This approach, however, requires aggressive injection, screening, and line maintenance efforts that prevent its routine use for testing gene function. Finally, virus-mediated transformation techniques have been used with some success for gene overexpression in butterfly wings (Beldade and Peralta, 2017; Dhungel et al., 2013, 2016; Lewis et al., 1999), but here again, the difficulty of the method in question has prevented its routine use in spite of having yielded the first successful butterfly wing genetic modification results almost 20 years ago (Lewis et al., 1999).

The clustered regularly interspaced short palindromic repeat/CRISPR-associated (CRISPR/Cas9) system has recently been developed to study gene function. CRISPR/Cas9 is an RNA-guided nuclease tool for the targeted introduction of double-stranded DNA cleavage (Harrison et al., 2014; Hsu et al., 2014; Jinek et al., 2014) (Fig. 1). To knockout expression of the target genes, a synthetic guide RNA (sgRNA) is introduced into the desired organism, in conjunction with Cas9 protein or plasmid/capped mRNA encoding the protein (for a practical guide for Lepidoptera, see Zhang and Reed, 2017). Cas9 protein delivery appears to be an order of magnitude more efficient than mRNA delivery (Kistler et al., 2015). A 17–20 nt region in the sgRNA complementary to a portion of the (coding DNA sequence) CDS of the target gene binds the DNA and directs the recruitment of Cas9, an endonuclease which induces double-stranded breaks (DSBs) at the desired site (Hsu et al., 2014). These DSBs result in insertion/deletions (indels) at the targeted loci as a result of ineffective repair by nonhomologous end joining. If a coding exon is targeted, most of the cleavage events yield a frameshift resulting in nonfunctional proteins in those cells carrying the mutations. The power of the CRISPR/Cas9 system as compared to other gene-editing methods lies in its programmability: a single sgRNA is sufficient to guide the binding of Cas9, and the N<sub>17–20</sub>NGG (PAM) motif required for this binding is readily available within most target sequences (Hsu et al., 2014). Furthermore, the availability of a rapidly increasing number of available lepidopteran genomes (Challis et al., 2016), CRISPR/Cas9 has the tremendous potential to blur the boundaries between model and nonmodel organisms when it comes to detailed studies of gene function and their evolution. Indeed, CRISPR/Cas9 has been shown to work across a very wide range of organisms and with a wide variety of applications in mind (Barrangou and Doudna, 2016; Hsu et al., 2014).

In Lepidoptera, soon after its inception CRISPR/Cas9 was developed for *B. mori* (Daimon et al., 2014; Liu et al., 2014; Ma et al., 2014; Nakade et al., 2014; Wei et al., 2014). Given the commercial importance of *B. mori*, a novel combination of techniques has recently been developed to target a baculovirus in this species. It uses the PiggyBac transposon to integrate a transgene containing the Cas9 protein under the control of a constitutive *ie1* promoter (in the baculovirus), while the gRNA expression cassettes are under the control of U6 promoters. This construct prevents the baculovirus from disrupting its host's development (Chen et al., 2017). In general, CRISPR/Cas9 in moths is again mostly used in a pest control context. Just like for *O. furnacalis* (Yang et al., 2016), the odorant receptor coreceptor (*Orco*) has also been targeted in *Spodoptera littoralis* to disrupt sex

pheromone and plant odour detection, in this case using CRISPR/Cas9 (Koutroumpa et al., 2016) rather than TALENs as was used in *O. furnacalis*. Disrupting pheromone communication as a means for pest control has been undertaken in *Helicoverpa armigera*, by targeting pheromone-binding protein



**Fig. 1** Targeted CRISPR/Cas9 mutagenesis for the generation of mosaic gene knockouts (mKO) in butterflies. Cas9/sgRNA protein duplexes are injected into early stage syncytial embryos (which is in the first few hours after egg laying for butterflies), presumably entering a fraction of the dividing nuclei (A). RNA-guided cleavage induces a double-stranded DNA break three nucleotides upstream of the PAM sequence (orange), leading to DNA-repair pathways (B). The egg of a butterfly is maternally prepatterned into a region that is fate mapped to become the extraembryonic tissue called the serosa, and a region in the shape of a band (here in faint green) where the germ band and thus the embryo will form (Carter et al., 2015; Ferguson et al., 2014). Upon cellularisation after the syncytial stage, those nuclei carrying a CRISPR-induced mutation (red; wild type in blue) in this band region will result in an observable mosaic mutant phenotype in the developing butterfly (e.g. in the wings as discussed in this chapter) (C). Generation of hypermelanic wing clones in an adult buckeye butterfly, 1 month after the syncytial delivery CRISPR targeting the eumelanin inhibitor gene *ebony* (*e*) (D). Arrowheads show mutant clones of various identity, possibly reflecting two allelic states (black:  $e^{-/-}$ ; white:  $e^{+/-}$ ). Scale bar: 100  $\mu$ m. Panel (D): From Zhang, L.L., Martin, A., Perry, M.W., van der Burg, K.R.L., Matsuoka, Y., Monteiro, A., Reed, R.D., 2017. Genetic basis of melanin pigmentation in butterfly wings. *Genetics* 205, 1537–1550.

(PBP1) (Ye et al., 2017) and the male pheromone antagonist Z11-16:OH (produced by females to avoid inopportune mating) (Chang et al., 2017) by means of CRISPR/Cas9. However, recently this species has been used to study pigmentation in Lepidoptera, by creating stable somatic and germline KO mutations of the ABC proteins, which transport pigment precursors from the cytoplasm (Khan et al., 2017). This study highlighted in particular the effects on eye pigmentation. Indeed, CRISPR/cas9 has also been used to investigate butterfly colour vision, by targeting rhodopsins in the Japanese yellow swallowtail (*Papilio xuthus*) and the painted lady (*Vanessa cardui*, Nymphalidae) (Perry et al., 2016). Given the fact that CRISPR/Cas9 is a relatively novel technique, the number of genes investigated in Lepidoptera is still small. In the context of embryonic and larval development the following genes have been targeted: the Hox genes *abdominal-A* (*abdA*) (*Plutella xylostella*, Huang et al., 2016; *Spodoptera litura*, Bi et al., 2016) and *Abdominal-B* (*AbdB*) (*P. xuthus* and *machaon*, Li et al., 2015, 2017) have been targeted, *wingless* (*B. mori*, Zhang et al., 2015), *frizzled* (*P. xuthus* and *machaon*, Li et al., 2015), *abnormal wing disc* and *fringe* (*B. mori*, Ling et al., 2015), and *FOXO* (*B. mori*, Zeng et al., 2017).

As can be expected for any technique that attempts to generate complete nulls of developmentally important genes (including ZFNs and TALENs), it is for the study of genes of importance in early embryogenesis, and larval growth, that this technique is not entirely without its limitations (at present). Introducing mutations early in development for key developmental genes is likely to be lethal to the animal before it reaches later developmental stages, as was shown, for example for *abd-A* and *Abd-B* (Bi et al., 2016; Huang et al., 2016; Li et al., 2015, 2017). Furthermore, interpreting mosaic phenotypes resulting from these injections is often challenging, as they result in partial phenotypes in the tissue of interest.

Although the technique of CRISPR/Cas9 may have some limitations and poses several technical challenges, it is currently the most promising technique available to facilitate the study of the functionality of candidate wing patterning genes in butterflies and relevant guidelines and protocols to that effect have been developed (Wang et al., 2015; Zhang and Reed, 2017). The developmental genes and their interactions underpinning wing patterning are at present still the most well-characterised aspect of development in butterflies (Deshmukh et al., 2017; Jiggins et al., 2017; Kunte et al., 2014; Monteiro, 2015; Monteiro et al., 2015; Nishikawa et al., 2015; Reed et al., 2011; Saenko et al., 2011; Wallbank et al., 2016). We will review here the insights we have gained, through CRISPR/Cas9, in the developmental

evolution of the following genes involved in butterfly wing patterning: (1) the major pre-patterning genes *WntA* (Mazo-Vargas et al., 2017) and *optix* (Zhang et al., 2017b), (2) pigmentation genes; *ebony* (Beldade and Peralta, 2017; Li et al., 2015), *yellow* (Perry et al., 2016; Zhang et al., 2017a) (as well as unpublished data on *Pararge aegeria*), *yellow-d*, *yellow-h2*, *yellow-h3*, *black*, *pale* (Zhang et al., 2017a), *Dopa decarboxylase Dcd* (Beldade and Peralta, 2017; Zhang et al., 2017a), and (3) genes involved in and affecting eyespot formation; *apterousA* (*apA*)/*apterousB* (*apB*) (Prakash and Monteiro, 2017), *spalt* (Zhang and Reed, 2016), and *Distal-less* (*Dll*) (Connahs et al., 2017; Zhang and Reed, 2016). We will then discuss where we should be aiming for in the field of the evolution and development of butterfly wing patterning. Ideally we should not only be aiming for knocking candidate genes out, but we should investigate specific polymorphisms of relevant genes in more detail, as well as the genes with which these genes interact and the control of their expression (e.g. through manipulation of regulatory regions). We will outline relevant approaches currently being developed.



## 2. INSIGHTS INTO BUTTERFLY WING PATTERNING AND EYESPOT DEVELOPMENT THROUGH CRISPR/CAS9

### 2.1 *WntA*: An Evolutionarily Malleable Genetic Pencil That Sketches Pattern Shapes

The gene *WntA* encodes a secreted ligand of the Wnt family, a molecular affiliation which makes it a good candidate for directing pattern formation processes. Indeed, its better-known relative *wingless* (*syn. Wnt1*) has been linked to pigment pattern formation in several insects (Koshikawa et al., 2015; Otsu et al., 2017; Werner et al., 2010; Yamaguchi et al., 2013) and is a hallmark of developmental signalling. Nevertheless, it is not by a candidate gene approach that *WntA* was identified as a wing patterning factor, but by forward genetics: more precisely, by the crossing of multiple morphs in three species of *Heliconius* butterflies, each differing by the shape of their colour patterns, followed by the Mendelian mapping of this variation at the genetic level by chromosome walking (Martin et al., 2012; Papa et al., 2013). Later, the identification of additional *WntA* alleles was extended to more *Heliconius* populations and species by a combination of linkage and association mapping techniques (Huber et al., 2015; Van Belleghem et al., 2017), and more surprisingly, to the 65 My distant *Limnitis arthemis*, which displayed adaptive variation at the *WntA* locus across the US Northeast (Gallant et al., 2014). In total, no less than 18 alleles have been mapped as



drivers of pattern shifts related to mimicry (Martin and Courtier-Orgogozo, 2017). Expression assays also suggested a key role for this gene in the induction of conserved pattern elements such as the Central Symmetry System, a stereotypical stripe that decorates the median region of many butterfly species (Martin et al., 2012; Mazo-Vargas et al., 2017). Nonetheless, it is only recently and with the use of CRISPR/Cas9 that this gene could be functionally validated. Mosaic KOs demonstrated an inductive role of *WntA* for the formation and positioning of many discrete pattern elements across seven species (Mazo-Vargas et al., 2017) (Table 1). We illustrate this principle here in the Gulf Fritillary butterfly, *Agraulis vanillae* (Fig. 2A). *WntA* shows an exquisitely complex expression pattern in last instar larval wing discs (Martin and Reed, 2014). In *A. vanillae*, it marks numerous spots corresponding to presumptive silver-spot patterns, each with an outline of black. Each *WntA*-positive silver pattern was lost or strongly reduced in the *WntA* KOs, demonstrating that *WntA* is necessary for the induction of these patterns (Mazo-Vargas et al., 2017). Seemingly *WntA*-negative silver-spots (outlined in yellow in Fig. 2A) expanded upon *WntA* KO, and conversely, contracted upon injection of heparin—a Wnt agonist (Martin and Reed, 2014; Mazo-Vargas et al., 2017). This suggests these upper hindwing silver spots are in fact inhibited by *WntA*, unlike the other silver spots of this butterfly. Thus depicting a complex picture of the *Agraulis* wing where identical patterns are under the antagonistic influence of the same pathway. While the mechanism behind this deserves further characterisation, it was observed that the *WntA*-negative patterns, in fact, are swept by a secondary wave of *WntA* expression in the late fifth instar stage, right before metamorphosis. From an evolutionary perspective, it is noteworthy that *Agraulis* patterns are thought to represent a highly derived, fragmented version of a more ancestral patterning system: the Nymphalid Ground Plan (NGP) (Nijhout and Wray, 1988). The combination of *WntA* expression and loss-of-function experiment validates this hypothesis. Indeed, in species representatives of the NGP, such as *Junonia coenia* (Fig. 2B), *V. cardui* (Fig. 2C), and *P. aegeria* (Fig. 2D), larval wing disc stripes of *WntA* expression delineates the position of signature components of the NGP such as the Central Symmetry System (in blue in Fig. 2B–D) and External Symmetry System (in green) (Table 1). Upon *WntA* KO, the Central Symmetry Systems were missing in the three species, and their External Symmetry Systems underwent a distal shift (Mazo-Vargas et al., 2017). Thus, *WntA* emerges as a key and conserved organising factor of the NGP, and underwent numerous changes in its expression to reshape the pattern boundaries and positions of more



**Table 1** Summary of CRISPR Experiments Targeting Wing Patterning Genes to Date

Gene Targeted	Expression in Wing Tissue	Species Studied and Observed Mutant Phenotype
<i>Dll</i> <sup>a,b</sup>	5th instar: Eyespot centre and first ring + border of wing	Species studied: <i>Bicyclus anynana</i> , <i>Vanessa cardui</i> , and <i>Junonia coenia</i> For <i>B. anynana</i> : Exon 3 (Homeobox containing exon)—Missing eyespots, missing scales, lighter wing colouration, bisected eyespots, missing border patterning. Exon 2 (including 5'UTR)—Exon skipping producing a N-truncated protein with an intact homeobox, resulting in ectopic eyespots. For <i>V. cardui</i> : Increase in eyespot size or number. Effects on multiple noneyespot traits: parafoveal and margin colour pattern elements, and pigmentation. Hyperpigmentation mutants and elongated eyespots observed in <i>J. coenia</i>
<i>WntA</i> <sup>c</sup>	5th instar: In Nymphalinae and Satyrinae: Basal, Central and Marginal symmetry systems. In Heliconiinae: Expression associated with black melanic regions in forewings and hindwings. In Danainae: Expression associated with wing vein margins	Species studied: <i>J. coenia</i> , <i>Pararge aegeria</i> , <i>V. cardui</i> , <i>Heliconius erato demophon</i> , <i>H. sara sara</i> , <i>Agraulis vanillae</i> , and <i>Danaus plexippus</i> In Nymphalinae and Satyrinae: Loss of patterning in regions associated with <i>WntA</i> expression (i.e. Basal, Central and Marginal symmetry systems) Distalisation of distal parafoveal elements. In <i>V. cardui</i> , loss of forewing eyespots In Heliconiinae: Distalisation of pigmented wing bands as a result of the loss of proximal pattern boundary in <i>WntA</i> -positive zone In the derived <i>A. vanillae</i> , both pattern inhibition and pattern expansion observed In Danainae: Expansion of white coloured scales in cells adjacent to wing veins
<i>optix</i> <sup>d</sup>	Early pupa	Species studied: <i>H. erato</i> , <i>A. vanillae</i> , <i>V. cardui</i> , and <i>J. coenia</i> Replacement of colour pigments (ommochromes) with melanin. Structural colouration defects, resulting in induction of blue iridescence in <i>J. coenia</i> . <i>Optix</i> appears to play a fundamental and deeply conserved role in regulating both pigmentary and structural coloration in butterfly wings
<i>apterous</i> ( <i>A</i> and <i>B</i> ) <sup>e</sup>	5th instar: Expressed uniformly in dorsal side of wing disc	Species studied: <i>B. anynana</i> . Severe mutants display complete lack of wings. Mosaic mutants display ventralisation of dorsal structures and patterns (note: Apterous A absent from future eyespot)

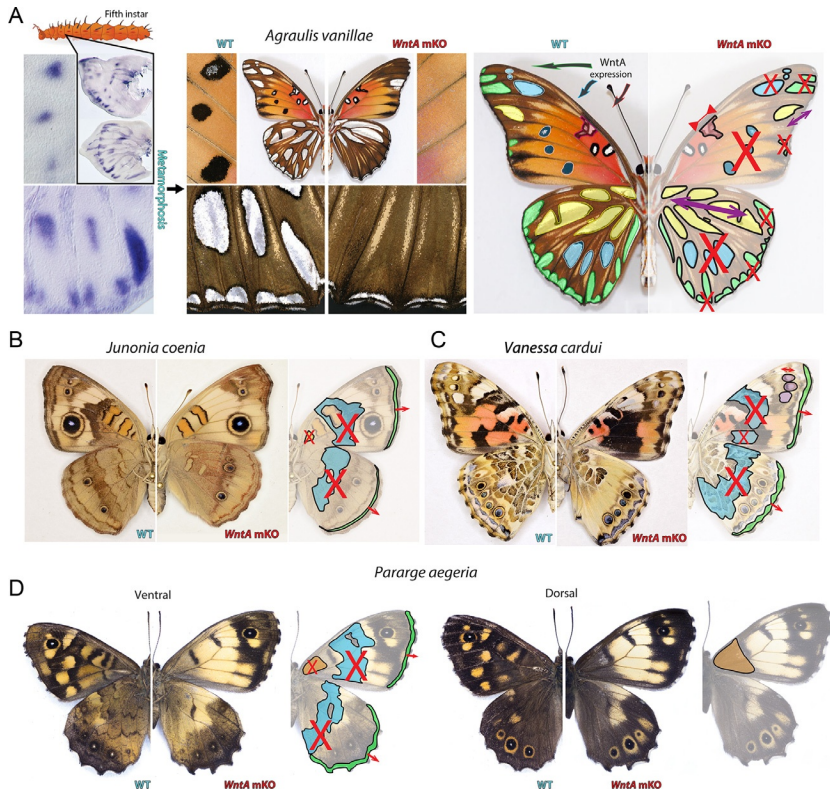
Continued

**Table 1** Summary of CRISPR Experiments Targeting Wing Patterning Genes to Date—cont'd

Gene Targeted	Expression in Wing Tissue	Species Studied and Observed Mutant Phenotype
<i>spalt</i> <sup>a</sup>	Pupa	Species studied: <i>J. coenia</i> and <i>V. cardui</i> . Missing eyespots in both forewings and hindwings
<i>ebony</i> <sup>f-h</sup>	Late pupa	Species studied: <i>Papilio xuthus</i> , <i>B. anynana</i> , and <i>J. coenia</i> . Darkening of wing scales (depending on background of scales)
<i>yellow</i> <sup>h,i</sup>	Late pupa	Species studied: <i>P. aegeria</i> , <i>B. anynana</i> , and <i>V. cardui</i> . Lack of black melanisation in wing scales, which may extent to eyespots ( <i>P. aegeria</i> )
<i>Yellow-d</i> <sup>h</sup>	Late pupa	Species studied: <i>V. cardui</i> . Promotion of orange–brown pigmentation in specific ventral forewing patterns
<i>Ddc</i> <sup>a,g,h</sup>	Late pupa	Species studied: <i>B. anynana</i> and <i>V. cardui</i> . Strong depigmentation of black scales
<i>pale</i> <sup>h</sup>	Late pupa	Species studied: <i>P. xuthus</i> and <i>J. coenia</i> . Cuticular defects, scale development defects, lack of melanisation in wing scales and thorax
<i>black</i> <sup>h</sup>	Late pupa	Species studied: <i>V. cardui</i> . Increased melanisation of wing scales and pupae

<sup>a</sup>Zhang and Reed (2016).<sup>b</sup>Connahs et al. (2017).<sup>c</sup>Mazo-Vargas et al. (2017).<sup>d</sup>Zhang et al. (2017b).<sup>e</sup>Prakash and Monteiro (2017).<sup>f</sup>Li et al. (2015).<sup>g</sup>Beldade and Peralta (2017).<sup>h</sup>Perry et al. (2016).<sup>i</sup>Zhang et al. (2017a).

Genes targeted along with respective expression in wing tissue and resulting phenotype are summarised.



**Fig. 2** Context dependent (species, wing region) inductive roles of *WntA* in nymphalid butterflies. Summary of *WntA* expression and KO effects on the ventral wing patterns of *Agraulis vanillae* (A), *Junonia coenia* (B), and *Vanessa cardui* (C). Panel D shows KO effects for both a ventral and dorsal of *P. aegeria*, highlighting the dorsal basal melanic patch on the right. See main text for details. Coloured layouts represent areas of larval *WntA* expression. Symbols show the effects of *WntA* loss-of-function (crosses: pattern removal; red arrows: contractions and shifts; purple arrow: expansion).

derived species such as *A. vanillae*, which underwent a drastic dislocation of its symmetry systems. This principle applies to even more derived nymphalid butterflies such as *Heliconius* and *Danaus* (Mazo-Vargas et al., 2017), which are not presented here for brevity.

## 2.2 CRISPR Mutants Highlight a Modular Organisation of Wing Patterns

A particularly salient outcome of the *WntA* loss-of-function phenotypes is the fact that they affect a fraction of discrete pattern elements. In other words, some pattern elements are *WntA* dependent, while other ones are

*WntA* independent, thus making apparent a highly modular architecture behind wing organisation. Allowing an uncoupling in the control of parts, modularity enables evolutionary flexibility, including plastic responses (Breuker et al., 2006; West-Eberhard, 2003). Here we illustrate this principle using serendipitous insights from the *WntA* mKO phenotypes of the Speckled Wood butterfly *P. aegeria*. Of interest here is a wing section which remains largely melanised while other areas become less melanised after KO; the dorsal basal forewing area (Fig. 2D). This in contrast to the corresponding ventral area. The dorso-basal patch thus appears *WntA* independent, an interesting finding from an evolutionary ecology perspective. Many butterflies, including *P. aegeria* (Berwaerts et al., 2001), rely on overall dorsal wing melanisation, but this basal part of the wing in particular, to heat up in order to become active (Wasserthal, 1975). *Pararge* wing pattern variation has been well documented (Schwanwitsch, 1935), and not only does there appear to be genetic variation between populations associated with melanisation (i.e. populations from Northern Europe being darker) (Vandewoestijne and Van Dyck, 2011), but there is also a large degree of developmental plasticity in melanisation in response to temperature and diet (Nylin et al., 1995), not least because the production of melanin is costly and subject to various resource trade-offs in this species (Talloen et al., 2004). One study concentrated on the size of the dorsal yellow areas in the border and central symmetry system, rather than the overall degree of melanisation. The size of the dorsal yellow areas has been associated with a degree of crypsis and thermoregulation, and associated differences in flight behaviours (Van Dyck and Matthysen, 1998). Males with less melanised wings and larger yellow patches spend significantly more time basking in sunlit patches, and darker males fly more frequently, suggesting an adaptive advantage to melanisation with respect to thermoregulation and patrolling behaviour (Van Dyck et al., 1997).

## 2.3 *optix*: A Master Regulator of Pigmentation and Iridescence

While *WntA* appears to instruct the formation of patterns associated with specific symmetry systems, another gene, *optix*, has also repeatedly been mapped in association with red and orange ommochrome colour patterns (Baxter et al., 2008; Huber et al., 2015; Reed et al., 2011; Van Belleghem et al., 2017). *WntA* encodes a signalling molecule, while *optix* encodes a homeobox transcription factor, which is expressed later in wing development, in a pattern that prefigures the red bands and stripes observed

in many *Heliconius* species (Martin et al., 2014; Reed et al., 2011). Transcriptomic profiling by RNAseq in *V. cardui* showed an upregulation of *optix* in orange sections of the wings at the onset of pigment synthesis (Zhang et al., 2017a). Furthermore, *optix* is also expressed in wing areas associated with nonpigmentation traits in several species, such as derived scales hypothesised to be involved in wing coupling or in the spreading of male pheromones (Martin et al., 2014). Similar to *WntA* however, no functional evidence was available to demonstrate its direct involvement in ommochrome pigmentation or other aspects of scale cell determination until now.

Recently, CRISPR/Cas9 was used to knock *optix* out in four different species of butterflies to gauge its role in the development of wing patterning and its role in the evolutionary diversification of wing patterns. The species tested were *Heliconius erato*, *A. vanillae*, *V. cardui*, and *J. coenia* (Zhang et al., 2017b) (Table 1). The results confirmed that *optix* plays a fundamental role in ommochrome pattern development, where mosaic mutants displayed a complete replacement of ommochromes with melanins, resulting in largely black and grey butterflies (Table 1). Surprisingly, *optix* KOs revealed this function is probably not only shared among the *Heliconius* butterflies, but also among the more distantly related *V. cardui* and *J. coenia*, where the same phenotype was observed despite a divergence of ~75–80 Mya, suggesting a deep evolutionary origin for this trait in nymphalid butterflies.

Furthermore, the *optix* CRISPR mosaics had several structural abnormalities associated with specific scale types; the so-called wing conjugation scales. It appeared that *optix* KOs resulted in a complete transformation of wing conjugation scales to normal scales in all four species, illustrating that *optix* not only functions as an ‘or’ switch for ommochrome pigmentation, but also has a conserved role in scale type determination. Furthermore, in *J. coenia*, *optix* KOs induced strong colour iridescence in wing scales which are occupied by ommochrome pigments in the wild type, indicating that *optix* functions as a repressor of structural iridescence in this species. In summary, *optix* KOs reveal a conserved role for the gene in aspects of scale morphology and both structural and ommochrome colouration, which appears to have been repurposed throughout evolution to contribute to the diversity of wing patterns observed in nymphalid butterflies (Table 1).

The gene *optix* is not alone in having a hugely significant effect on the overall levels of melanin in the butterfly wing. An example is provided by that of the iconic case of industrial melanism in the peppered moth *Biston betularia* (Brakefield, 1990; Cook, 2003; Majerus, 1998). The overall degree of wing melanisation appears to be the in large part due to the *carbonaria*

transposable element increasing the abundance of *cortex* transcript (van't Hof et al., 2016). This gene is a cell-cycle regulator during early wing disc development and which also has been implicated in the mimetic radiation in *Heliconius* butterflies (in particular via its effects on the yellow wing areas) (Nadeau et al., 2016). Presumably *cortex* plays a key role in the development of specific pigment containing scale types per se (Nadeau et al., 2016). The functionality of the gene *cortex* is thus still poorly understood and it is hoped that CRISPR/Cas9 can elucidate how a cell-cycle regulator in (early) wing development can have such a profound effect on wing patterning across both moths and butterflies.

## 2.4 Pigmentation Genes

The many cases of evolution of melanisation patterns per se, both at the micro- and macroevolutionary level, as well as developmental plasticity in melanin production (for example the aforementioned case of *P. aegeria*), have been textbook examples of evolution in action (Majerus, 1998). Although the key regulatory mechanisms may have been eluding us for a long time (van't Hof et al., 2011, 2016), the downstream genes involved in pigment production itself, in particular the melanin pathway, have become well characterised (True, 2003; Wright, 1987). To date, quite a few genes underlying the production of pigments in the wings, in particular in relation to melanisation, have been functionally tested using CRISPR/Cas9 in several butterfly species (Table 1): *J. coenia* (*pale* and *ebony*, Zhang et al., 2017a), *V. cardui* (*yellow*, *ebony*, *Ddc*, *black*, *yellow-d*, *yellow-h2*, and *yellow-h3*, Zhang et al., 2017a), *B. anynana* (*yellow*, *Ddc*, and *ebony*, Beldade and Peralta, 2017; Zhang et al., 2017a), *P. xuthus* (*pale* and *ebony*, Li et al., 2015; Zhang et al., 2017a), and we present here the results of *yellow* mutants in *P. aegeria*. *Pale*, *yellow*, and *Ddc* have been shown as melanin-promoting factors (Beldade and Peralta, 2017; Zhang et al., 2017a), *ebony*, *black*, and *yellow* affect the hue of colour patterns that do not appear to be typical black DOPA-derived eumelanins (Zhang et al., 2017a). Below, we will discuss the main findings for each of these genes in turn. A further two genes, *yellow-h2* and *yellow-h3* have also been functionally tested using CRISPR/Cas9, but mortality in the pupal stage prevented characterisation of adult pigment phenotypes (Zhang et al., 2017a).

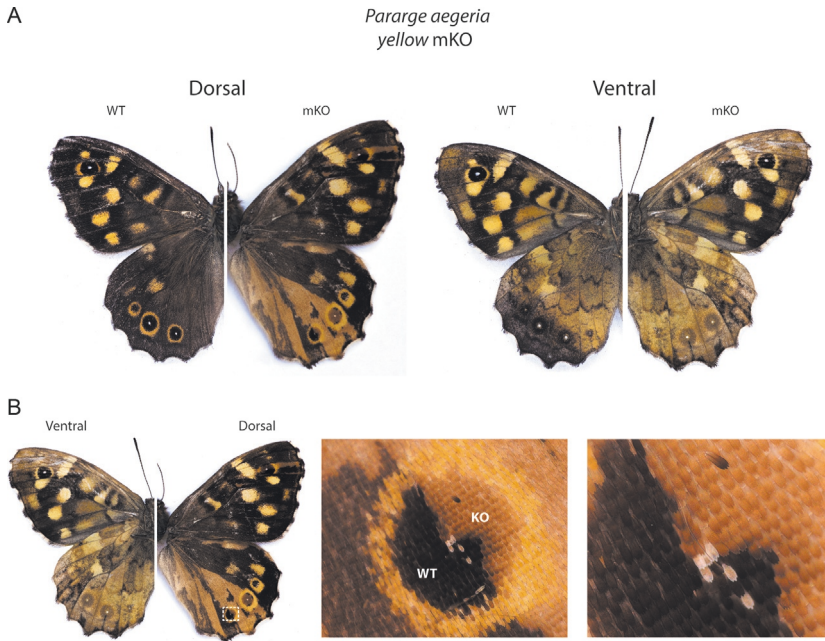
The gene *pale* encodes the tyrosine hydroxylase enzyme, which catalyses the formation of dihydroxyphenylalanine (DOPA) from tyrosine at an early step of the melanin synthesis cascade (Wright, 1987). In *J. coenia*, *pale*

mutants displayed severe cuticular defects including improperly expanded wings (Zhang et al., 2017a), presumably due to the role of DOPA in cuticle maturation and sclerotisation (Gorman and Arakane, 2010). Amelanism was also observed in *pale* mutants in thorax scales, wing scales, and in the inner ring scales of melanic eyespots, which became completely transparent (Zhang et al., 2017a) (Table 1).

The *yellow* gene family is large with a block of 3–5 genes highly conserved throughout insect diversification (Ferguson et al., 2011). Although the full function of many of the (pleiotropic) *yellow* genes is largely unknown (Ferguson et al., 2011), recent CRISPR/Cas9 functional analyses of *yellow*, *yellow-d*, *yellow-h2*, and *yellow-h3* have shown that these genes play a role in melanic pigmentation of butterfly wings (Perry et al., 2016; Zhang et al., 2017a). The gene *yellow* encodes a secreted extracellular protein required for production of black melanin pigments in *Drosophila* (Biessmann, 1985). KO of *yellow* in several species has been shown to result in loss of cuticular pigmentation (Wittkopp and Beldade, 2009). It has been shown in the butterfly *P. xuthus* (Futahashi and Fujiwara, 2007), for example, that 20-hydroxyecdysone (20E) hormone titres in the final larval instar regulate a number of (cuticular) pigmentation genes. A high titre of 20E leads to upregulation of *yellow*, whereas a low titre leads to downregulation of *ebony* and *Ddc*. Thus, cuticular pigmentation is significantly controlled by the exposure to 20E and its removal. The deployment of 20E in this context may very well be a mechanism underpinning the observed developmental plasticity in pigment production in many butterfly species as in-depth RNA-seq studies have indicated (Connahs et al., 2016; Daniels et al., 2014).

For *P. aegeria* mutations in *yellow* were produced by coinjecting two sgRNAs flanking the conserved major royal jelly protein (MRJP) motif. Examination of injected individuals revealed ectopic loss of black pigmentation across both forewings and hindwings (Fig. 3), in accordance with previously reported results in other butterfly species (Perry et al., 2016; Zhang et al., 2017a) (Table 1). The affected cells in the dorsal and ventral part of the wings were often asymmetrical, suggesting independent migration of affected cells during wing development. In the affected cells, areas of previous dark brown pigmentation became pale yellow, while black pigmented scales in the eyespots turned brown. Regions of yellow around the eyespots and in the forewings remained unaffected suggesting a crucial role for this gene in the synthesis of black melanin, while having no effect on lighter pigments of *P. aegeria*. Interestingly, darker areas on the ventral side of the forewings seemed to be differently affected in terms of colouration as compared





**Fig. 3** Knockout of the *yellow* locus in *Pararge aegeria*. Wild-type *P. aegeria* (left) shown alongside a representative yellow KO individual (right) for both dorsal and ventral wing surfaces (A). The effects of the *yellow* KO were also observed in one of the hindwing eyespots (i.e. HW-ocellus 2, indicated by a *dashed box*) (B).

to the dorsal side, where the yellow around the hindwing eyespots is affected, while seeing no obvious effects on the yellow around the dorsal forewing eyespot. Loss of pigmentation in the abdomen and thorax of some individuals was also observed.

Through the microinjection of sgRNA complementary to the *yellow* CDS, mutations were introduced that resulted in cells carrying mKO phenotypes for the gene. In accordance with recently published results in other butterflies (Perry et al., 2016, Zhang et al., 2017a), *yellow* mKOs results in a loss of melanisation on the wings of *P. aegeria*. These results are also in accordance with expectations from phenotypes observed in *Drosophila* (Wittkopp et al., 2002), as well as other insects including *Tribolium castaneum* (Arakane et al., 2010), *Oncopeltus fasciatus* (Liu et al., 2016), and *B. mori* (Futahashi et al., 2008). The *yellow* KO results in *P. aegeria* thus support a conserved role for this gene being involved in melanin pigmentation. It is interesting to note that the mKO phenotypes were observed in both hindwings and forewings, as well as in the dorsal and ventral sides of the wings (Fig. 3). This

is similar to the findings reported in *Drosophila*, but different to the RNAi phenotypes observed in *Tribolium* and *Oncopeltus*, where only hindwing defects were noted (Arakane et al., 2010; Liu et al., 2016). This discrepancy is also in accordance with a recently published study, where several species of butterflies with *yellow* mutants also contained both forewing and hindwing defects (Zhang et al., 2017a). These differences suggest that deployment of *yellow* has diverged throughout insect evolution. Furthermore, the effects of the *yellow* mKO did extend to the eyespots as well. Half of one eyespot on the hindwing (ocellus 2 in *P. aegeria*) lost its melanic scales in the area bordered by the unaffected yellow ring.

Zhang et al. (2017a) used CRISPR/Cas9 to functionally test the role of *yellow-d* in *V. cardui* wing scale pigmentation. Like *yellow*, *yellow-d* affected melanin patterns (Table 1). But unlike *yellow*, *yellow-d* also showed red-specific expression resulting in buff coloured pattern elements becoming a more chocolate-ochre hue. *Ddc* encodes the melanin synthesis enzyme DOPA decarboxylase (Wright, 1987). In *B. anynana*, CRISPR/Cas9-mediated *Ddc* KO in early embryos resulted in mosaic adults with pigment removed from the eyespot black disc (Beldade and Peralta, 2017) (Table 1). In *V. cardui*, *Ddc* mutants displayed pigment phenotypes similar to those observed for *yellow* (Zhang et al., 2017a). Unlike *yellow* however, *Ddc* KO in *V. cardui* also changed brown and tan pigments on the ventral wing surface (Zhang et al., 2017a). *V. cardui* larvae furthermore showed reduced melanin pigmentation, but *Ddc* KO typically resulted in high mortality, with many larvae failing to hatch from their eggs (Zhang et al., 2017a) (Table 1).

The gene *ebony* encodes the N-beta-alanyldopamine (NBAD) synthase enzyme, an important component of melanin biosynthesis (Wright, 1987), which is expressed in the larval, pupal, and adult stages of butterflies (Beldade and Peralta, 2017; Li et al., 2015; Zhang et al., 2017a). Mutant *ebony* *P. xuthus* larvae obtained with CRISPR/Cas9 showed enhanced melanic pigmentation (Li et al., 2015). In *V. cardui*, late-stage pupal wing phenotypes were observed whose red, white, and brown colour patterns produced an almost inverse colouration of the wild-type wing, but this phenotype could not be recovered in the adult stage due to high pupal mortality (Zhang et al., 2017a). KOs of *ebony* in *P. xuthus*, *B. anynana*, *J. coenia*, and *V. cardui* results in darkening of adult wing patches, evident across a range of colour pattern types including buffs, tans, and light and dark browns (Beldade and Peralta, 2017; Li et al., 2015; Zhang et al., 2017a) (Table 1).

Disruption of *black* leads to beta-alanine deficiencies, which limits NBAD scelerotin production resulting in an excess of dopamine, and causes

cuticular melanisation (Wright, 1987). In *V. cardui* phenotypes with enhanced melanisation in pupal cuticles and wing scales was observed (Zhang et al., 2017a). In wing scales, *black* KO appeared to affect the same colour pattern elements as *yellow-d*, but without the marked yellow overtones (Zhang et al., 2017a) (Table 1 and Fig. 1D).

## 2.5 Genes Involved in Eyespot Development

Arguably, butterfly eyespots are one of the most well-studied wing pattern elements, with a large literature exploring their ecology, evolution, and development (Monteiro, 2015; Nijhout, 1991). To date, CRISPR/Cas9 has been used to study the function of three major transcription factor genes expressed in association with developing eyespots; *dll* (Connahs et al., 2017; Zhang and Reed, 2016), *spalt* (Zhang and Reed, 2016), and *apA/B* (Prakash and Monteiro, 2017) (Table 1). Previous functional analyses of the appendage-development gene *Dll* using germline transformation experiments in *B. anynana*, and baculovirus-mediated gene transfer experiments in *J. orithya* (Dhungel et al., 2016) demonstrated an eyespot-promoting role for this gene, a role which had been inferred before (Beldade et al., 2002; Carroll et al., 1994). The first use of CRISPR/Cas9 in an eyespot gene instead found an eyespot-repressing role for *Dll* (Zhang and Reed, 2016). Connahs et al. (2017) later solved that discrepancy by using CRISPR/Cas9 to target two different *Dll* exons. Disrupting the homeodomain in exon 3 produced missing eyespots, demonstrating its role as a positive eyespot regulator. In contrast, they found that targeting the exon 2 as what had been done in *V. cardui* produced a N-truncated Dll proteins with an intact homeodomain, which resulted in *B. anynana* butterflies with ectopic eyespots. This result is consistent with the previous study but changes its interpretation: the proper loss-of-function experiment is done by targeting exon 3 and reaffirms an eyespot activator role for *Dll*. It would appear that Zhang and Reed (2016) had actually generated a gain-of-function effect perhaps by truncating *Dll* from a repressor domain. Thus, these two studies offer an elegant case study showing that CRISPR somatic mutations can generate allelic heterogeneity depending on the target sites, a property that should induce caution, and also creativity among butterfly genome editors.

The transcription factor, *spalt* is one of the earliest factors expressed in eyespot foci (Brunetti et al., 2001). Zhang and Reed (2016) used CRISPR/Cas9 to test the hypothesis that *spalt* is a regulator of eyespot colour patterns in *J. coenia* and *V. cardui* (Table 1). Deletion of *spalt* resulted in

the loss and/or reduction of eyespots demonstrating that this gene plays a role in promoting eyespots (Zhang and Reed, 2016). Interestingly, a loss of an eyespot on one wing surface could occur without affecting an eyespot on the opposing surface, demonstrating that eyespot determination is dorsoventrally decoupled to a large degree (Zhang and Reed, 2016, and see *yellow* and *WntA* results for *P. aegeria* presented in Figs 2D and 3). The gene *apA*, the homologue of the dorso-ventral wing selector gene *ap* in *Drosophila*, is expressed on the dorsal surface of larval wings in *J. coenia* (Carroll et al., 1994). Expression of the paralogue *apB*, and the role of both homologues in wing development and patterning was unknown. Using CRISPR/Cas9, Prakash and Monteiro (2017) functionally tested the role of *ap* by disrupting the homeodomain and LIM domain of the homologue *apA*, and the LIM domain of *apB*. This study demonstrated that *apA* functions as both a repressor and modifier of ventral wing colour patterns, as well as a promoter of dorsal sexual ornaments in males (Table 1). In contrast however, no striking transformations of dorsal to ventral identity were observed in *apB* mutants, suggesting that only the *apA* homologue functions as a dorsal surface selector in *B. anynana*. Furthermore, *apA* has been implicated as a repressor of eyespot development in *B. anynana*; *apA* KO mutants had additional eyespots on the dorsal surface of hindwings (Prakash and Monteiro, 2017).

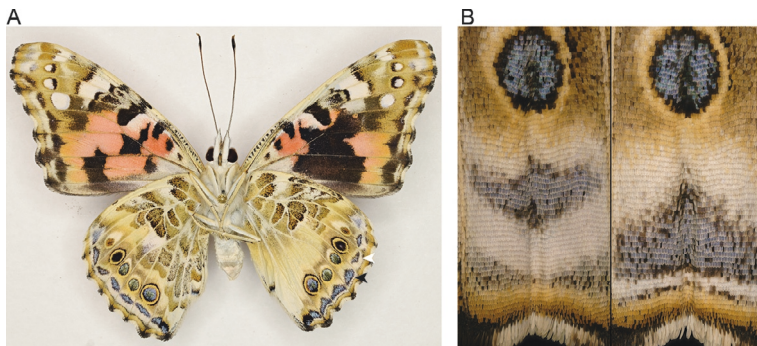


### 3. BENEFITS AND CAVEATS OF MOSAICISM

A demonstrably high biallelic KO rate makes it potentially feasible to characterise mutant phenotypes in injected individuals. Mosaicism is an essential property of the technique (Fig. 1C), as it allows some ‘escaper’ individuals to survive from the potentially deleterious effects of a given KO during embryogenesis and larval life, and yield healthy adults with a mutant (wing) phenotype. This inevitably comes with caveats: in short mosaicism prevents the *rigorous* (1) spatial delineation of mutant clones and (2) determination of their allelic states (and thus whether an allele is dominant or recessive). A pigment synthesis gene is expected to show *cell autonomous* effects, and in the case of *ebony* in *J. coenia* (Fig. 1D), it is even possible to see two degrees of hypermelanism that likely reflect a mosaic of heterozygous and homozygous mutant states. But making such assumptions is more problematic for developmental regulatory genes, which could show (1) *noncell autonomous* effects (propagation of phenotypic effects in genotypically wild-type cells), and idiosyncratic dominant, recessive, or other allele-dependent effects. Based on the rugged and elongated shape of iridescent *optix* mutant

clones in *J. coenia* (comparable to the shape of *ebony* mutants) (Zhang et al., 2017b), we can reasonably assume this gene is *cell autonomous*. In contrast, *WntA* mutants show more rounded boundaries, or can affect patterns at a distance. For instance, parafoveal elements were pushed towards the wing margin in *WntA* mutants while they do not show *WntA* expression (Fig. 4). These outcomes suggest a cell autonomous effect, as could be expected from a signalling molecule.

Connahs et al. (2017) took advantage of the visible cell autonomy of *Dll* KOs on scale pigmentation to map the spatial boundaries of the mutant clones on the wing, and implement the inferred initial conditions to test their mathematical model of eyespot pattern formation. Here again, the mosaicism was taken as an advantage. A particularly salient result that deserves attention is that when they attempted to genotype individual mutant and wing clones from a same surface by deep sequencing (MiSeq), they could not recover well-separated alleles. Based on this preliminary result and on our own experience, we now believe the direct genotyping of wing tissues cannot be used to map spatial clone boundaries or determine allelic state. There are several possible explanations for this. First, genotyping requires the amplification of DNA, which could capture heterogeneous cell populations irrelevant to the differentiation of wing scales, such as interstitial epithelial cells, neurons, or even circulating cells such as haemocytes. Second, it is possible that the



**Fig. 4** Region-specific and distal effects of *WntA* mKO. Example of a *V. cardui* *WntA* mosaic mutant shown on its ventral side. The right half of this individual (left side of the picture) is wild type; its left forewing and hindwings are wild type anteriorly to M2, and mutant posteriorly to M2 (A). Arrowheads denote WT and mutant parafoveal elements above (white) and below (black) the M2 vein. Displacement effect of *WntA* mKO on the grey-blue parafoveal elements of *V. cardui* (right) compared to wild type (left), here in ventral hindwings (B). *WntA* is expressed at the margin in last instar larval wing discs and likely organises distal patterns via morphogenetic signalling activities.

epithelial cells of butterfly wings rapidly delaminate before being absorbed by the insect body, as described in *Drosophila* (Togel et al., 2008). Given these concerns, and at the same time the remarkable heuristic power of targeted mutagenesis, we advise both caution and creativity in the interpretation of phenotypic and genotypic data derived from mosaic mutants.



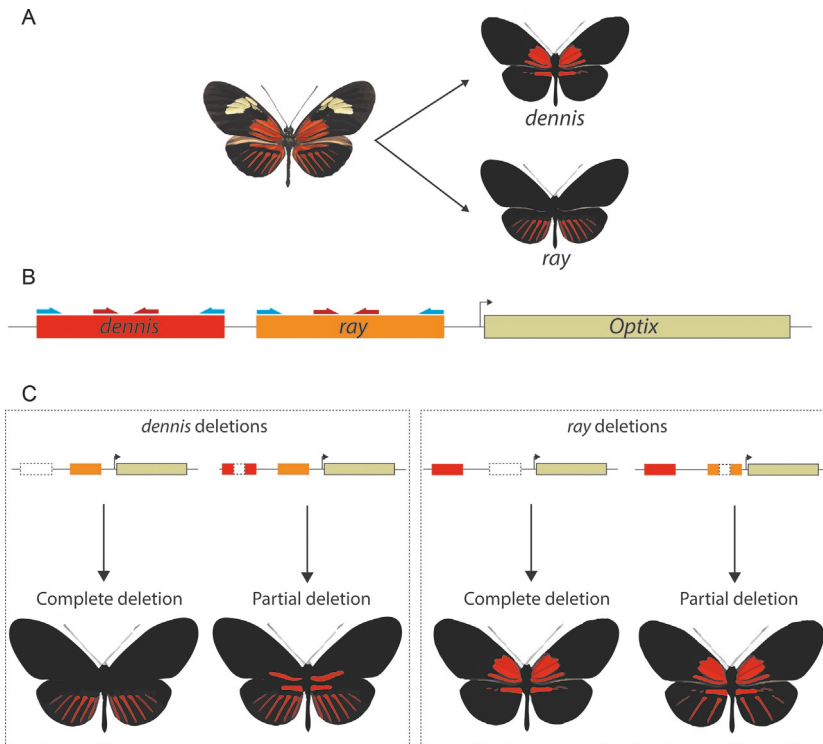
## 4. BEYOND KNOCKOUTS OF CANDIDATE GENES

CRISPR/Cas9-mediated KOs have been a major step forward for those wishing to elucidate the functional role of a number of candidate genes. However, KOs are only the beginning, and a number of research groups are developing CRISPR/Cas9-based techniques to go beyond KOs. For example, as discussed elsewhere in this chapter, mosaic phenotypes may not always be easily interpretable and/or informative of function and in such cases it is desirable to generate stable transgenic lines. A potential positive side effect of generating stable transgenic lines from CRISPR/Cas9-induced mosaic founders is the ability to recover an allelic series of the targeted locus. Mosaicism in germline of the injected founders can lead to transmission of an array of mutant alleles in the next generation (Wei et al., 2014; Yen et al., 2014). In butterflies, the successful transmission of an allelic series of mutants was first demonstrated in *D. plexippus* using ZFNs (Merlin et al., 2013). Germline transformation in general has also been achieved with *Pieris rapae* (Stoehr et al., 2015) and *B. anynana*, using a PiggyBac construct (Chen et al., 2011). The complexity of such germline mutations could be further increased by coinjecting a cocktail of several different sgRNAs targeting the same locus. The utility of an allelic series of protein-coding genes may not be intuitive; however, it could provide a crude but efficient tool to probe the function of noncoding regulatory loci. This approach has been used to functionally validate upstream regulatory elements of the *tyrosinase* gene in mice (Seruggia et al., 2015).

Such an approach might be feasible to begin dissecting the *cis*-regulatory elements associated with the reported red pigment patterns in *Heliconius* species (Nadeau et al., 2012, 2013; Reed et al., 2011; Wallbank et al., 2016). Genomic studies in *H. melpomene* and its relatives have further narrowed the location of these noncoding regulatory elements down to a few kilobases (Wallbank et al., 2016), and chromatin accessibility profiles and methods are now available in *Heliconius* to narrow down sites that are differentially active between tissues and stages (Lewis et al., 2016). Targeting these regions simultaneously with several sgRNAs, spaced out across the length of the



locus, could potentially generate butterflies carrying regulatory alleles of various lengths (including complete deletions) (Fig. 5). Phenotypic analysis of this panel of alleles in heterozygous ( $F_1$  generation) and/or in homozygous ( $F_2$  generation) state could aid in identifying the functional elements of these regulatory regions and further refining the location of the causative regions underlying differences in red patterns. Such an approach could also be applied to putative regulatory regions of *WntA* (Martin et al., 2012) (Fig. 5). Moreover, targeting regulatory regions of the genes, instead of



**Fig. 5** The *optix* regulatory elements experiment. The red bands in many *Heliconius* species are decoupled into two developmental modules known as the *dennis* and *ray* phenotypes (A). These are controlled by two separate *cis*-regulatory regions located upstream of the *optix* gene (B). By inducing different deletions using combinations of sgRNAs (red and blue arrows in B), the contribution of each enhancer to colour pattern formation could in theory be established (C). Complete enhancer deletions should result the abolishment of each respective *dennis* or *ray* phenotype, while partial deletions would allow further dissection of the *cis*-regulatory logic controlling red pattern formation. Such a phenotype analysis would be effective if same/similar alleles could be brought together in homozygous state in an  $F_2$  generation.



the protein-coding regions, potentially alleviates issues of lethality associated with complete functional KO of pleiotropic and early embryonic genes (Huang et al., 2016). However, it should be borne in mind that genetic variation and enhancer dominance may limit to an extent obtaining quick and easy-to-interpret results.

More precise manipulations such as integrating inducible expression cassettes; for conditional expression (Chen et al., 2011), insertion of small protein tags (e.g. V5 epitope), or larger fluorescent proteins for expression assays, and direct allelic replacement (to identify causative nucleotide(s)) would also benefit from the generation of stable transgenic lines. Such applications rely on using the CRISPR/CAS9 system alongside the cells' innate HDR machinery which has shown to be conducive for successfully generating knock-ins in butterflies (Zhang and Reed, 2017). These initial efficiencies of HDR-mediated knock-ins appear to be low but could potentially be increased by in vivo linearisation of donor DNA (Irion et al., 2014), the use of single-stranded oligodeoxynucleotides (ssODNs are particularly useful for introducing small changes (Ran et al., 2013), including the Easi-CRISPR technique (Quadros et al., 2017)), suppression of the innate NHEJ machinery (NHEJ and HDR machinery compete to repair DSBs; Singh et al., 2015) and perhaps even avoiding the HDR machinery altogether (Auer et al., 2014). It is likely that finetuning of these and other parameters are likely to lead to a further increase in knock-in efficiency in butterflies. In the future, the application of such experimental strategies may largely be limited by generation times, mating strategies, maintenance of inbred lines, and the ability to rear large numbers in the lab—factors that will vary by species.

The technique of CRISPR/Cas9 has proved a breakthrough for the study of butterfly wing pattern and eyespot development, and their evolution. Exciting new results are being produced at an accelerated rate and further advances of the technique, coupled with other techniques such as transcriptomics to identify novel relevant genes, will rapidly provide us with detailed insights into how the wide variety of beautiful butterfly wings develop and evolve.

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## FURTHER READING

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