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Butterfly wings shaped by a molecular cookie cutter: evolutionary radiation of lepidopteran wing shapes associated with a derived Cut/*wingless* wing margin boundary system

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SUMMARY Butterflies and moths show a remarkable diversity of specialized wing shapes, yet little is known about the molecular basis of wing shape determination. To learn more about this process we examined the expression of dorsoventral (DV) boundary candidate genes in developing wings of several species of Lepidoptera. We found that the transcription factor Cut and mRNA for the signaling molecule *wingless* (*wg*) are strongly co-expressed in a discrete zone around the larval wing disc margin. Surprisingly, the expression boundary of Cut and *wg* clearly presages complex future adult wing shapes, including the hindwing tails of swallowtail butterflies, very early in final-instar wing disc development. During pupal wing development the cells in this zone undergo apoptosis, thereby defining the actual margin of the adult wing. Comparison with gene expression in beetle and fly wings suggests that this delineation of a topologically independent boundary running parallel to the DV boundary is a derived feature of Lepidoptera. We propose that the developmental decoupling of wing margin determination and DV boundary formation was a major developmental innovation that facilitated the radiation of specialized wing shapes in moths and butterflies.

INTRODUCTION

Moths and butterflies are remarkable for the array of highly derived wing shapes they display. One of the most obvious adaptive benefits of this wing shape variation is for aerodynamic specialization (e.g., hovering vs. gliding flight), but there are many other striking examples of wing shape adaptations that include false heads and antennae for predator deflection, serrated wing edges for enhancing bark and foliage crypsis, and false petioles for leaf mimicry. There are also other bizarre and beautiful wing shape characteristics of debated and/or unknown function, most famous of which are the hindwing tails found in many moth and butterfly groups, including many species of the swallowtail butterfly family Papilionidae. No other insect order shows a diversity of extreme wing shape innovations approaching that seen in Lepidoptera. This begs the question: what characteristics of lepidopteran wing development have permitted this unprecedented morphological radiation? To begin addressing this question at a molecular level, we are working to develop a better understanding the early pattern formation process underlying lepidopteran wing shape determination.

Butterfly wing imaginal discs develop as flat bilayered epithelial buds that resemble miniature adult wings (Fig. 1A). The wing discs remain relatively small until the fifth (and final) larval instar, at which time they start growing rapidly as tracheae ingress from the base of the discs, between the epithelial layers, to form the wing vein precursors (Süffert 1929; Nijhout 1991). This bud-like mode of wing development is probably ancestral in holometabolous insects, as it bears a general resemblance to development of larval wing anlagen in beetles and ants (Quennedey and Quennedey 1999; Abouheif and Wray 2002; Sameshima et al. 2004; Tomoyasu et al. 2005).

Although early butterfly wing development appears to be fairly typical for holometabolous insects, there is one interesting aspect of the process that is possibly unique to Lepidoptera—the differentiation and eventual degradation of the "peripheral tissue" (Fig. 1, A and B) around the developing wing margin (Süffert 1929; Dohrmann and Nijhout 1988; Kodama et al. 1995). In early fifth-instar wing discs, before tracheal ingression, a border lacuna forms roughly parallel to the disc margin (Fig. 1, A and B). Tracheae invade this border lacuna late in fifth-instar larval development (Miner et al. 2000; Reed et al. 2007), and within 2–3 days after pupation the peripheral tissue between the border lacuna and the edge of the pupal wing epithelium undergoes cell-death. Detailed observations of this process in the butterfly *Pieris rapae* suggest that cell death in the peripheral tissue is truly

programmed cell-death (apoptosis) and not necrosis (Kodama et al. 1995). Nijhout's analogy of the border lacuna acting as a wing-shape cookie-cutter is especially vivid in the wing discs of some swallowtail butterflies, where the lacuna forms a dramatically shaped boundary that presages hindwing tails (Nijhout 1991).

While histological studies have been performed on the differentiation and subsequent apoptosis of the peripheral tissue, little is known about the molecular basis of this process. In order to gain a better understanding of peripheral tissue specification we took a comparative gene expression approach using the detailed understanding of dorsoventral (DV) boundary formation in *Drosophila melanogaster* wing discs. *D. melanogaster* larval wings develop much differently than those of most other holometabolous insects, in that axis specification occurs in an epithelial monolayer (Fristrom and Fristrom 1993; Pastor-Pareja et al. 2004). During pupation, this monolayer everts, folding back on itself to form a bilayer that then represents the dorsal and ventral surfaces of the wing. Before eversion occurs, however, the DV boundary that will become the adult wing margin is determined through the activity of a complex cascade of transcription factors and signaling molecules. Briefly, Notch (N) signaling in early DV boundary formation is thought to induce initial *wg* expression in response to reciprocal signaling between presumptive dorsal cells expressing Serrate and Fringe and presumptive ventral cells expressing active Delta (Panin et al. 1997). Later, during a second phase of pattern formation, a well-defined DV boundary is established and maintained through the activity of the N and *wg* signaling pathways (Fig. 1C), where N is activated in response to feedback between the border cells expressing Cut and *wg* and the adjacent cells expressing higher levels of Delta and Serrate (deCelis and Bray 1997; Micchelli et al. 1997; Irvine and Rauskolb 2001).

This detailed knowledge of *D. melanogaster* wing margin development provides a roster of candidate genes to assess for potential roles in lepidopteran wing margin development. It has previously been shown that *wg* is transcribed along the edge of larval butterfly (Fig. 1D) and moth wing discs (Carroll et al. 1994; Sato et al. 2008), supporting the idea that some aspects of DV boundary formation are conserved between butterflies and flies. In this study, we further test this hypothesis by more carefully examining *wg* expression over time while also looking at the expression of N and Cut. We also explore the relationship between DV boundary gene expression and delineation of the peripheral tissue boundary. To gain a preliminary phylogenetic perspective on the evolution of wing margin determination in Holometabola, we examine the expression of Cut and N in the developing wings of the beetle *Zophobas morio*. Finally, we speculate on how innovations in the lepidopteran DV boundary formation process were likely to have facilitated the radiation of wing shapes in moths and butterflies.

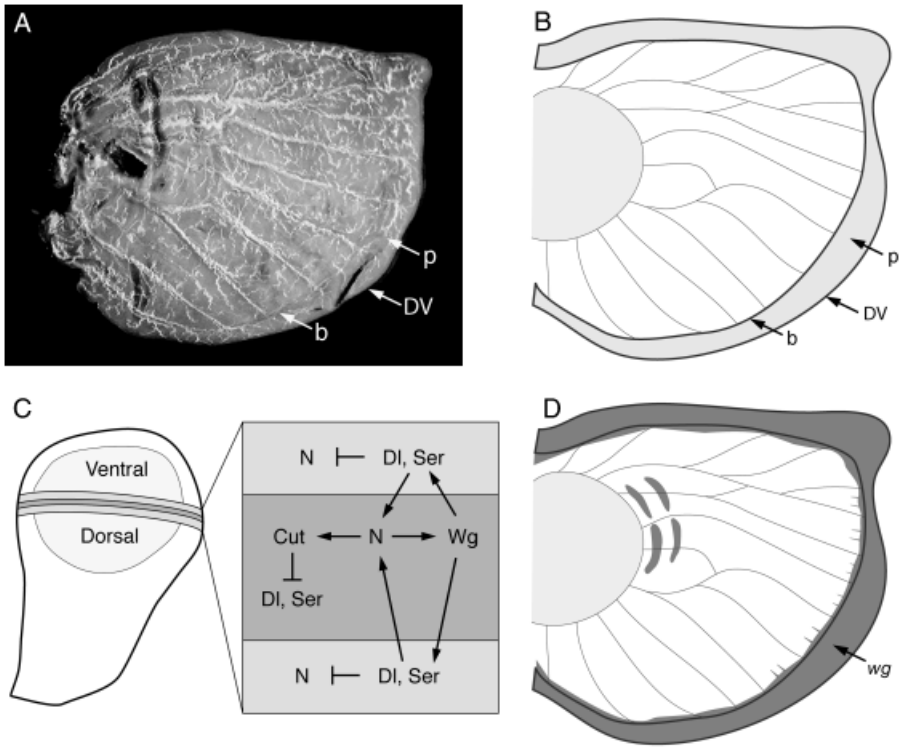

Fig. 1. Dorsoventral (DV) boundary formation and wing margin morphology in late-stage butterfly and fly wing discs. (A) A late fifth-instar butterfly wing disc. The border lacuna (b) defines the boundary of the future adult wing. The DV boundary (DV) and peripheral tissue (p) of the wing disc are marked. (B) A cartoon of a late fifth-instar wing disc showing the border lacuna (b), dorsoventral wing disc boundary (DV), and peripheral tissue (p). (C) A model of DV boundary maintenance in late third-instar *Drosophila melanogaster* wing discs. The expression of Notch (N), which activates Cut, is maintained through a feedback loop of wingless (*wg*), Delta (Dl), and Serrate (Ser) signaling. Model after Gonzalez et al. (2006). (D) An illustration of *wg* transcription in a late fifth-instar *Junonia coenia* wing disc as previously reported by Carroll et al. (1994).

MATERIALS AND METHODS

Immunohistochemsitry

Vanessa cardui (Nymphalidae), *Agraulis vanillae* (Nymphalidae), *Junonia coenia* (Nymphalidae), *Battus philenor* (Papilionidae), *Bombyx mori* (Saturniidae), *Manduca sexta* (Sphingidae), and *Z. morio* (Tenebrionidae) wings were dissected, fixed, and stained using a previously described protocol (Brunetti et al. 2001). The 2B10 monoclonal mouse anti-Cut (Developmental Studies Hybridoma Bank, Iowa City, IA, USA), C17.9C6 monoclonal mouse anti-Notch-intracellular domain (Developmental Studies Hybridoma Bank), and polyclonal rabbit anti-Distalless (Panganiban et al. 1997) were used for antibody stains. Primary antibodies were detected with Cy3 goat anti-mouse secondary antibodies (Jackson Immunoresearch, Inc., West Grove, PA, USA), and selected tissue samples were counterstained with the nuclear marker DAPI (Invitrogen Corp., Carlsbad, CA, USA). Negative controls using only the secondary antibody showed no signal. All tissue samples were visualized on a laser confocal microscope or a fluorescent light microscope using automated mosaic image stitching.

In situ hybridization

wg in situ hybridizations in *V. cardui* and *B. philenor* wing discs were performed using a previously published protocol (Reed and Nagy 2005), with the exception that riboprobes were not hydrolyzed. Riboprobes were generated using a partial cDNA clone of *J. coenia wg* generated using the WG1 and WG2 primers (Brower and DeSalle 1998). Sense probe negative controls showed no signal.

Apoptosis assay

Following Galant et al. (1998) we used acridine orange staining to assess patterns of apoptosis in pupal wing tissues. Insects were dissected in phosphate-buffered saline with 1% Triton (PBT) and 150 μg/ml acridine orange. Dissected wings were incubated in the PBT+acridine orange solution for 5 min, washed in PBT for 10 min, and immediately visualized and digitally photographed on a fluorescent light microscope. *V. cardui* pupal wings were stained and imaged while still attached to the pupal cuticle.

RESULTS

Cut, N, and *wg* expression along the imaginal disc margin

Strong Cut expression was consistently observed in the peripheral tissue of early (Fig. 2A) and late (Fig. 2, B, E, and I) fifth-instar butterfly wing discs. In early fifth-instar wing discs N was observed to be upregulated across the presumptive wing epithelium, with significantly lower expression levels in the peripheral tissue (Fig. 2C). Conversely, in late fifth-instar wing discs, N expression was generally higher around the edge of the discs (Fig. 2D), including the peripheral tissue, but showed a strong pattern of Cut-complementary upregulation along the peripheral tissue border (Fig. 2, E and F). The boundary between the opposing Cut and N expression patterns in late fifth-instar wing discs was along the interior edge of the border lacuna and its tracheae (Fig. 2F). We observed expression of Cut in presumptive peripheral tissue as early as the fourth instar (Fig. 2G). At this stage, however, N expression levels were fairly ubiquitous across the disc (Fig. 2H) and did not show an obvious spatial relationship with Cut expression. Cut expression was localized to cell nuclei, as expected for a transcription factor (Fig. 2I). The N antibody, which recognizes the intracellular domain of the protein, was found to be localized to the apical cell membranes and to intracellular bodies associated with nuclei (Fig. 2, J and K), consistent with its role in signal reception and transduction.

Transcription of *wg* in fourth-instar discs was primarily along the distal margin (Fig. 2L). In late fifth-instar discs expression was upregulated in the entire peripheral tissue (Fig. 2M) with particularly high expression in the border lacuna itself (Fig. 2N). This border lacuna *wg* transcription occurred in cells on both sides of the molecular N/Cut boundary (Fig. 2O), meaning that some cells in the presumptive adult wing epithelium—outside of the peripheral tissue—expressed *wg*. Another notable feature of *wg* expression was the discrete anterior–posterior boundary line in hindwing peripheral tissue (Fig. 2, N and O)—an expression pattern we have also noted in late fifth-instar *J. coenia* wing discs (not shown).

Cut expression associated with apoptosis in pupal wing peripheral tissue

Morphologically, *V. cardui*, undergoes differentiation of pupal peripheral tissue in a manner similar to that described in other butterflies (Fig. 3A). Acridine orange staining in 36 h pupal wings (Fig. 3B) verified that peripheral tissue cell-death occurs in a patchy pattern similar to what has been previously described in the butterfly *P. rapae* (Kodama et al. 1995). As in larval wing discs, Cut expression is strongly localized to the peripheral tissue in early pupal wings (Fig. 3, C and D). During the same time frame, N is expressed in cells proximal of the peripheral tissue boundary (Fig. 3E). We also observed strong relative upregulation of N in evenly spaced parallel rows of scale precursor cells (Galant et al. 1998; Reed 2004). DAPI stains reveal that presumptive adult wing epithelium is highly organized, with emergent parallel rows of enlarged nuclei associated with high N expression, while the peripheral tissue shows little analogous cellular organization (Fig. 3, D and E).

Cut and *wg* expression presage complex swallowtail butterfly wing shapes

To determine whether Cut expression in imaginal discs is associated with the development of complex wing shapes, we examined its expression in fifth-instar hindwing discs of the papilionid *B. philenor* (Fig. 4A). We chose this species because of its highly derived adult hindwing shape that includes a tail extension, an anal fold, and a distinctly undulating wing margin—all features commonly found throughout the

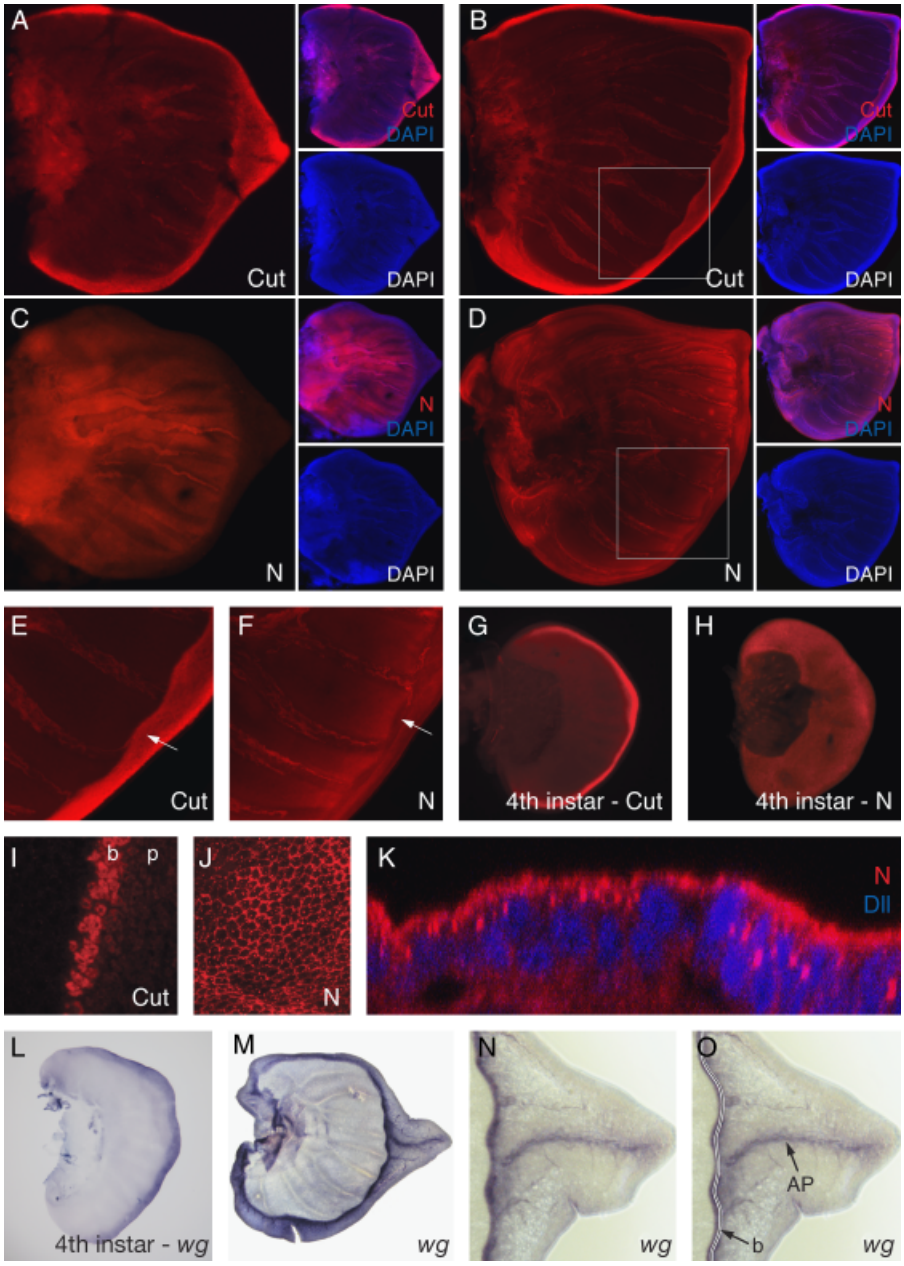

Fig. 2. Cut, N, and *wg* expression in larval wing discs of *Vanessa cardui* (unless otherwise noted). Note that signal at the base of the wing discs and in presumptive wing veins is due to tracheal autofluoresence. (A) Cut expression in a mid fifth-instar hindwing disc. (B) Cut expression in a late fifth-instar forewing disc. (C) N expression in a mid fifth-instar hindwing disc from the same individual providing the wing shown in (A). (D) N expression in a late fifth-instar forewing disc from the same individual providing the wing shown in (B). (E) Detail of Cut expression shown in (B). (F) Detail of N expression shown in (D), note the close opposing fit of the Cut expression pattern shown in (E). White arrows mark homologous wing positions between the left and right wings shown in (E) and (F). (G) Cut expression in fourth-instar wing discs shows discrete expression in the presumptive peripheral tissue. (H) N expression in the fourth-instar wing discs is fairly ubiquitous, showing only a slight upregulation along the presumptive peripheral tissue boundary. (I) Cut staining is localized in nuclei of border cells in late fifth-instar wing discs of *Agraulis vanillae*. (J) N staining is localized to cell membranes and in intracellular bodies in late fifth-instar wing disc epithelium of *Junonia coenia*. (K) A z-stack projection of a lateral epithelial section shows localization of N to apical cell membranes and to bodies located adjacent to or within nuclei of *J. coenia*. Nuclei here are marked by immunostaining of the transcription factor Distalless. (L) *wg* transcription in a fourth-instar hindwing occurs around the margin of the wing disc before formation of the border lacuna. (M) *wg* transcription in a mid fifth-instar hindwing occurs in the peripheral tissue. (N) *wg* expression is especially high along the border lacuna and also in an apparent anterior–posterior boundary in the peripheral tissue. (O) Border lacuna expression of *wg* occurs in cells flanking both sides of the peripheral tissue boundary. p, peripheral tissue; b, peripheral tissue boundary; AP, apparent anterior–posterior boundary.

swallowtail butterfly family Papilionidae. Indeed, we observed Cut (Fig. 4B) and *wg* (Fig. 4C) expression in the peripheral tissue of fifth-instar *B. philenor* hindwing discs that clearly predicts all three of these morphological features.

Cut expression marks peripheral tissue in moths

To assess how phylogenetically widespread the relationship between peripheral tissue specification and Cut expression is in Lepidoptera, we examined Cut expression in fifth-instar wings discs of the moths *B. mori* (Fig. 4D) and *M. sexta* (Fig. 4E). We found that expression was very similar to that observed in butterflies; Cut is highly expressed in the peripheral tissue, but does not extend proximally past the border lacuna.

Cut and N mark DV border cells in beetle wing anlagen

We examined expression of Cut and N in developing beetle wing anlagen (Fig. 4, F–J) in order to make preliminary

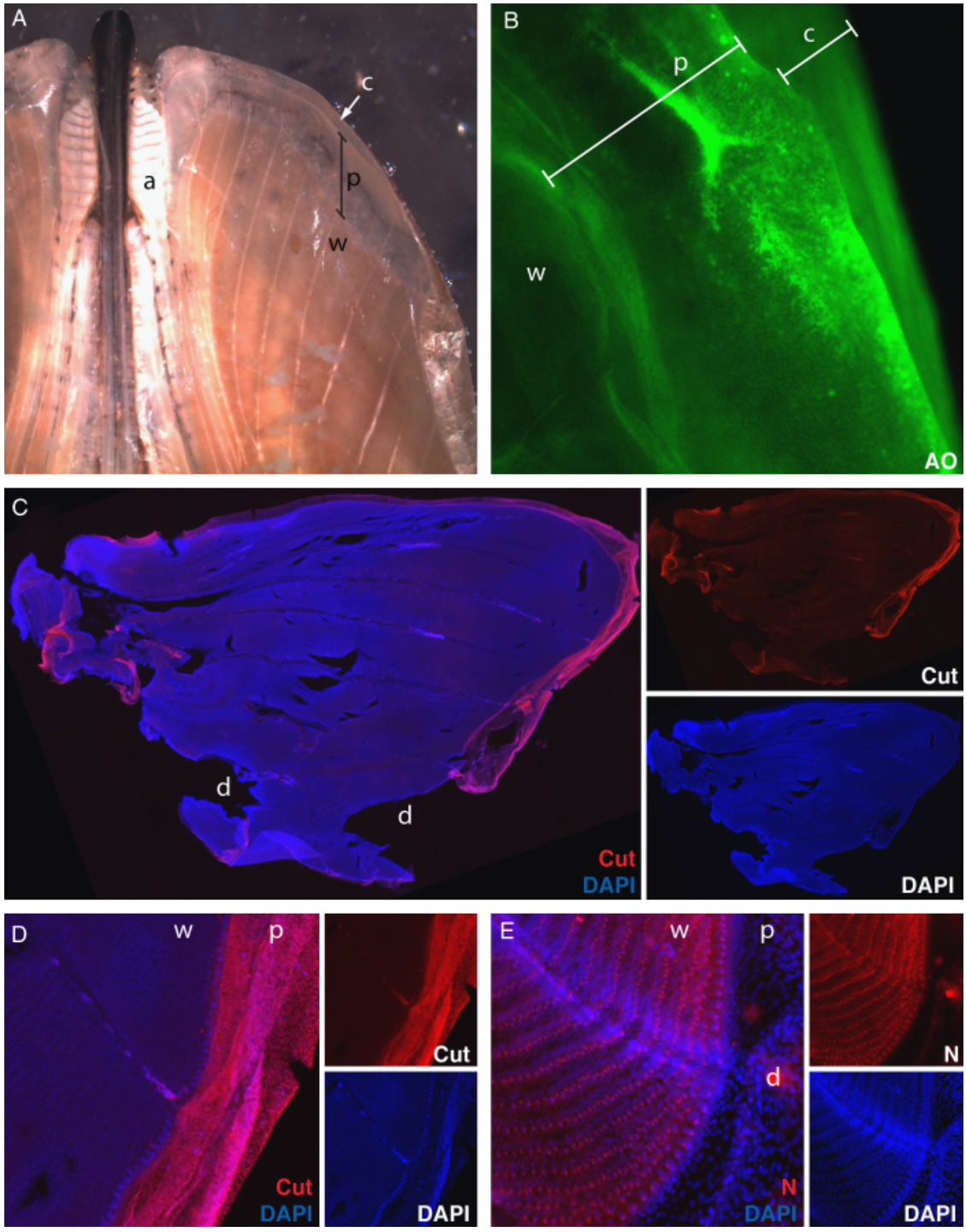

Fig. 3. Gene expression and cell death in the early pupal forewing margin of *Vanessa cardui*. (A) Ventral view of a developing forewing 36 h after pupation. The reflective sheen is the peripodial membrane. (B) A patchy pattern of cell death in the peripheral tissue is observed in this acridine orange stained wing margin 36 h after pupation. The wing tissue remains attached to the cuticle in this preparation, and the diffuse non-cellular signal is due to cuticular autofluorescence. (C) Cut expression is restricted to the peripheral tissue in this whole-mount of a 24 h pupal wing. Note that the posterior and proximal sections of the wing are slightly damaged (d). (D) Detail of Cut expression in the peripheral tissue of a forewing 24 h after pupation. (E) Detail of N expression restricted to epithelium proximal of the peripheral tissue boundary. c, cuticle; p, peripheral tissue; w, presumptive adult wing epithelium; a, antenna; d, damage or debris.

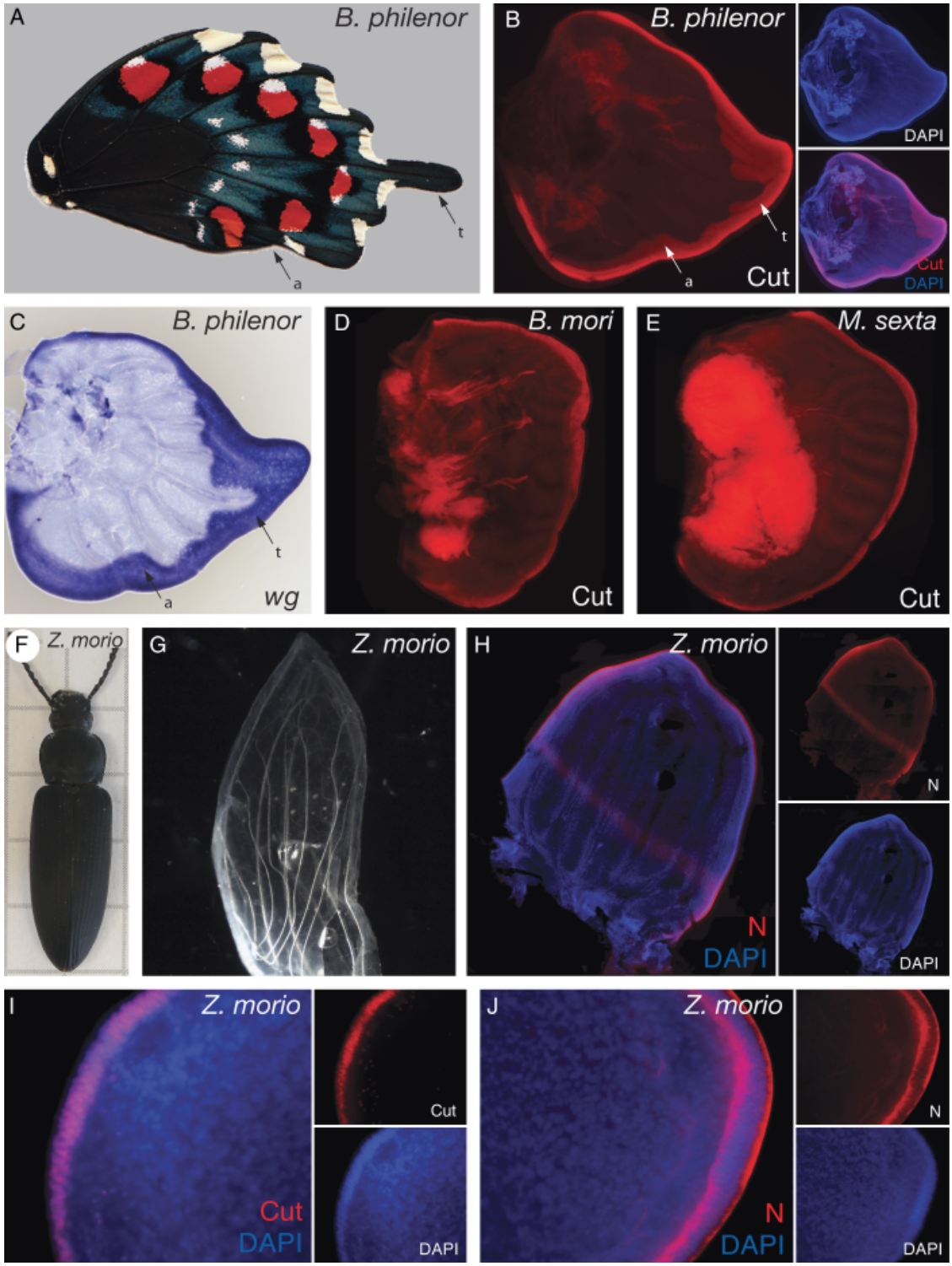

Fig. 4. In the (A) *Battus philenor* hindwing, (B) Cut expression, and (C) *wg* expression in fifth-instar wing discs presage formation of the wing tail (t) and the anal fold (a). Cut expression occurs in the peripheral tissue of fifth-instar wing discs of (D) the silkmoth *B. mori* and (E) the tobacco hornworm moth *Manduca sexta*. N and Cut expression in (F) *Z. morio* (G) elytral anlagen support a border cell mode of DV boundary formation in beetles. (H) In a day 4 *Z. morio* pupal elytron N expression occurs along the margin as well as in a conspicuous stripe of expression perpendicular to the proximodistal axis. (I) Cut expression in day 2 *Z. morio* pupal elytra occurs in a discrete line of border cells along the DV boundary. (J) N expression in day 2 *Zophobas morio* pupal elytra occurs in a border cells along the DV boundary, similar to Cut. Wings in (I) and (J) are right and left wings from the same individual.

inferences about ancestral expression patterns in holometabola. Unlike in Lepidoptera, we found that N was distinctly upregulated along the margin of the wing anlagen, as well as in a conspicuous, well-defined stripe, perpendicular to the proximodistal axis, crossing the entire wing (Fig. 4H). Closer observation of the pre-pupal wing margin showed N expression confined to a discrete strip of DV boundary border cells around the wing margin (Fig. 4J). We found that Cut was coexpressed with N in these DV boundary border cells (Fig. 4I).

DISCUSSION

Derived features of lepidopteran wing margin development

Broadly, we found little evidence that the *early* stages of DV boundary formation differ significantly between the handful of holometabolous insects considered in this study. We found that *wg* expression during early wing margin development is similar between Lepidoptera, Hymenoptera, and Diptera, with expression occurring along the DV boundary starting early in wing development. Also, we found that early expression of Cut along the DV boundary of developing wing discs appears to be generally conserved between Lepidoptera, Diptera, and Coleoptera. Lastly, early N expression levels are relatively ubiquitous across the early wing discs of both Lepidoptera and *D. melanogaster*, again implying evolutionary conservation.

Later in development, however, there are three aspects of wing margin determination that appear to differ between orders. The first of these differences relates to patterns of N expression. In *D. melanogaster*, N is expressed fairly ubiquitously during late wing disc development. Contrast this with the expression in the beetle *Z. morio* where N is expressed in a discrete strip of border cells around the margin of developing wings, along with Cut (Fig. 4, I and J). Contrast also with Lepidoptera, which shows a temporally varying trend throughout development to have higher expression of N in the presumptive wing epithelium (Fig. 2C) and along the interior boundary of the border lacuna (Fig. 2F). Unfortunately, the functional significance of these differences between orders is difficult to surmise because N expression levels are not necessarily correlated with N activity. As studies in *D. melanogaster* have shown, the strength of N signaling is more dependent on expression of its ligands Delta and Serrate and their modulator Fringe, than the expression levels of N itself (deCelis and Bray 1997; Micchelli et al. 1997; Panin et al. 1997). So, although intriguing, the possible evolutionary and functional ramifications of these differences in N expression remain ambiguous.

A second and more remarkable difference in wing margin development between Lepidoptera and other insects is the relative independence between the contour of the wing disc

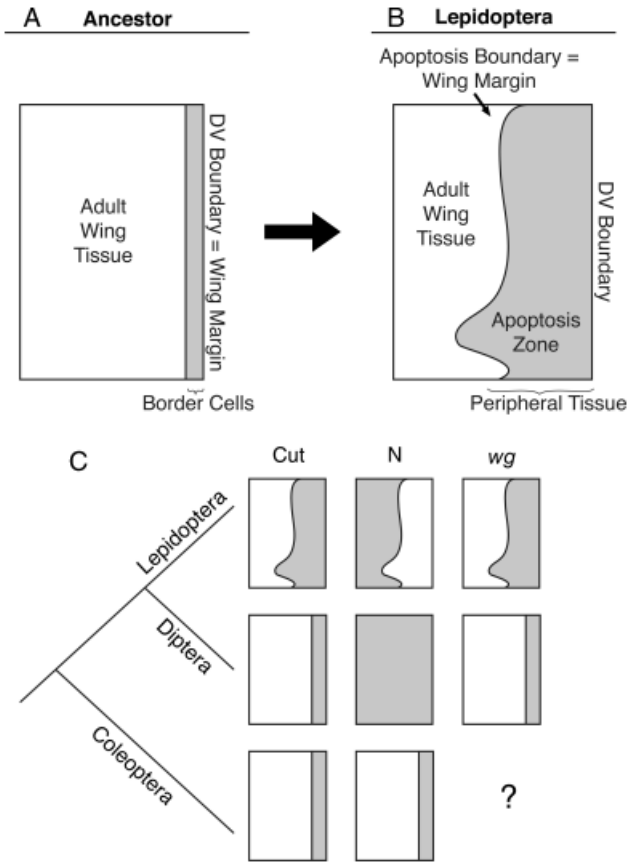

Fig. 5. A model of wing margin developmental evolution in holometabolous insects. (A) We speculate that the lepidopteran peripheral tissue was evolutionarily derived from an ancestral border cell field via several innovations, including a spatial expansion of the field, independence of the interior boundary contour, and apoptosis of all cells in the field to form the wing margin. (B) Cut, N, and *wg* wing margin expression in the context of Holometabola phylogeny. Note that the novel features in Lepidoptera include the expression of N interior to the peripheral tissue boundary and a complex contour of the peripheral tissue boundary unrelated to the contour of the DV boundary.

DV boundary and the contour of the inner boundary of Cut and *wg* expression (Fig. 5A). The narrow strip of cells representing the DV boundary in developing beetle and fly wings appears to be a typical border cell boundary—i.e., a specialized group of cells along a discrete developmental border (Irvine and Rauskolb 2001). In contrast, the field of Cut and *wg* expression in Lepidoptera is relatively wider than the border cell fields of Coleoptera and Diptera and can have a very complex inner contour, as is dramatically illustrated in the swallowtail butterfly (Fig. 4, B and C). Unlike a formal border cell field, then, the lepidopteran peripheral tissue appears to represent two topologically distinct boundaries instead of one—the wing disc margin (the DV boundary) and the adult wing margin.

A third major difference between Lepidoptera and the other orders appears to be the differentiation and subsequent apoptosis of the peripheral margin tissue. Based on the previously published studies we are aware of, this phenomenon seems to be unique to Lepidoptera. Published images of developing ant and beetle wings (Quennedey and Quennedey 1999; Abouheif and Wray 2002; Sameshima et al. 2004; Tomoyasu et al. 2005), as well as our own observations (Fig. 4G), do not reveal obvious morphological signs of anything analogous to the lepidopteran peripheral tissue in the other major orders of Holometabola. We reach this conclusion with the important caveat that the studies we cite do not focus on wing margin development per se, and represent a very limited sampling of Holometabola. Consequently, more thorough work on these and other taxa, including *Drosophila*, could very well reveal something that would contradict this conclusion. We further infer that the association between Cut expression and wing disc margin apoptosis is a novel feature of lepidopteran development. Cut is a transcription factor implicated in cell-type specification in numerous tissues (Nepveu 2001), however we are not aware of any previously described connection between Cut and apoptosis regulation. The remarkably strong correlation between Cut expression and extensive apoptosis in butterfly wing discs leads us to speculate that Cut may play some role in activating programmed cell death in this context.

A novel boundary decouples wing shape and DV boundary formation

In this study, we have shown that several aspects of lepidopteran DV boundary formation differ from what is seen *D. melanogaster* and other holometabolous insects (Fig. 5). While this in itself provides an interesting story of developmental divergence, we speculate that there may be a more significant evolutionary implication of these differences. Namely, that the novel features of lepidopteran wing margin development permitted a *decoupling* of wing margin and DV boundary determination. We further speculate that this decoupling freed lepidopteran wing shape determination from various constraints of larval and pupal wing development, allowing wing shape to undergo rapid and radical diversification in Lepidoptera. This potential example of a strip of border cells evolving into a developmental compartment flanked by two relatively independent boundaries could present a useful case study for how novel developmental boundaries originate and facilitate subsequent morphological diversification.

Hopefully further functional and comparative work will shed light on how the shape of the lepidopteran Cut/*wg* boundary is defined so we can better understand the evolution and development of wing shape itself. In terms of getting a handle on the functional basis of wing shape determination in Lepidoptera, it could be fruitful for investigators to take advantage of natural genetic polymorphism in wing tail phenotype (Clarke and Sheppard 1960a, b; Clarke et al. 1968) in order to genetically map and positionally clone the underlying genes—a strategy that has worked well for identifying color pattern polymorphism loci in *Heliconius* butterflies (Papa et al. 2008). Of comparative interest, apart from simply increasing taxonomic sampling to include additional insect orders, it would be informative to look at other insect lineages that have shown unusual patterns of wing shape diversification. For instance, developmental studies on groups like the thread wing antlions (Neuroptera: Nemopteridae) or *Mormolyce* fiddle beetles (Coleoptera: Carabidae) might reveal what others kinds of alterations to wing margin development might facilitate an evolutionary "escape" from the stereotyped insect wing.

Acknowledgments

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