

The development and evolution of crossveins in insect wings

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ABSTRACT

The formation of crossveins in *Drosophila* was an important early case study in understanding the role of the environment in the development and evolution of morphological structures. More recent work has shown that signalling processes play a crucial role in the formation of crossveins in *Drosophila* and that the interaction of a heat shock factor, Hsp90, with components of signal transduction pathways may account for the sensitivity of these structures to environmental perturbations. A new model for the development of crossveins is presented that divides the formation of crossveins into 3 separate stages. First, the number and placement of the crossveins is determined by signalling along the proximal-distal axis of the wing. This signal may involve the *cdc42* gene product and the Jun-N-terminal Kinase signal transduction pathway. Then, during the second stage, an inductive signal from the dorsal wing epithelium is sent to the ventral wing epithelium at locations specified by the first signal. The second signal appears to involve the BMP-like signalling pathway. Finally, in the third stage, a domain of vein competent cells is defined by the signalling from the EGF-receptor-Map Kinase signal transduction pathway, and the exact location of the veins is eventually determined within that domain by Notch-Delta signalling interactions. By altering components of these 3 stages, insects can independently regulate the presence or absence, the number and placement, and the thickness and flexibility of the crossveins. This capacity for the modulation of crossvein structure in many different ways may have contributed to the evolution of different modes of insect flight.

Key words: Crossveins; cell signalling; wing development; insect wing biomechanics; genetic assimilation.

INTRODUCTION

Insect wings are composed of only 2 cell layers, one each on the dorsal and ventral wing surfaces. Wing veins are formed within one or the other of these 2 cell layers, though they can pass through from one wing surface to the other (Fig. 1*a*, Comstock, 1918; Stark et al. 1999). In addition to their role as supporting structures of the wing, the wing veins contain tracheae, blood lacunae and nervous tissue. Wing veins can be divided into 2 basic types, longitudinal veins that extend from the wing hinge distally to the wing margin, and crossveins that extend between longitudinal veins. In the holometabolous insects, longitudinal veins form first, while the crossveins that connect the longitudinal veins develop somewhat later (Waddington, 1940; Sturtevant & Bier, 1995; Sturtevant et al. 1997).

When an insect is in flight, its wings must generate both thrust and lift during all phases of the wing beat

if the insect is to maintain a constant speed and altitude. In order to produce lift during all phases of the wing beat, the degree of camber (convex curvature along the anterior-posterior axis) of the wing must be carefully regulated (Vogel, 1981). Insect wings change curvature continuously in order to modulate air flow across the wing to create the appropriate wing camber at any given point in the wing beat (Ennos, 1989*b*; Brodsky, 1994; Grodnitsky, 1999). The rapidity of the wing beats in most insects requires that this regulation of wing camber be a material property (called flexural stiffness) of the wing itself because the flight musculature cannot contract fast enough to produce the necessary changes in wing curvature (Vogel, 1988). Of particular importance to determining the flexural stiffness of the wing are the crossveins (Ennos, 1989*a*). Insects of different sizes and insects with different modes of flight have different lift requirements (Ellington, 1995; Lehmann & Dickinson, 1998; Dudley, 2000) and an important mechanism of

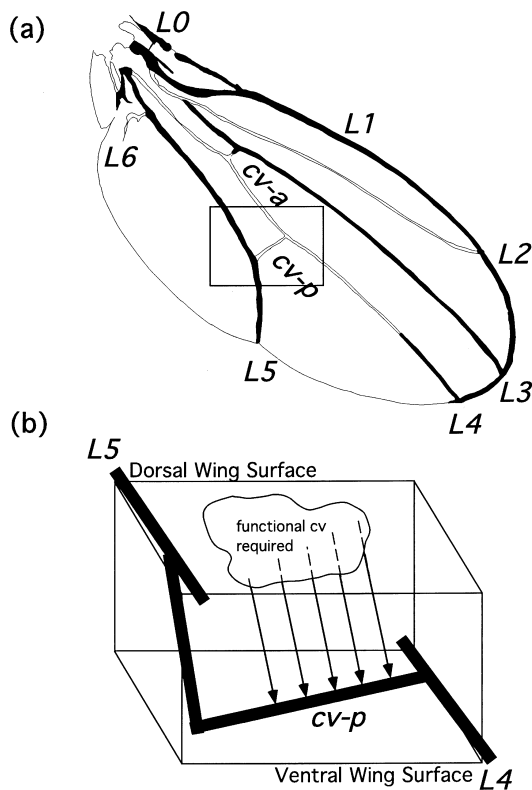


Fig. 1. (a) The wing vein morphology of a wild-type *Drosophila melanogaster* wing. The veins that are found on the dorsal surface of the wing are shown as filled lines, while those on the ventral surface are shown as unfilled lines. Longitudinal veins are numbered L0–L6, while the anterior and posterior crossveins are labeled as cv-a, and cv-p, respectively. The rectangle indicates the region enlarged in 3-dimensional perspective in (b), which shows the inductive signal from the dorsal wing epithelium which is required for the formation of the posterior crossvein in the ventral epithelium (Garcia-Bellido, 1977). This signal requires the presence of functional *crossveinless* gene product.

altering the amount of lift a wing can generate is to alter the number, placement, thickness, or flexibility of the crossveins (Ennos, 1989a).

In spite of the importance of crossveins for insect flight, we are only beginning to understand the development of crossveins in one insect, *Drosophila melanogaster*, and we know virtually nothing about crossvein development in other species (Stark et al. 1999). However, because there are clear homologies between each of the veins in *Drosophila* and the veins of other insects, what is learned about *Drosophila* wing vein development may be applicable to other insects (Comstock, 1918; Biehs et al. 1998). Here I review what is known about crossvein development in *D. melanogaster*. This species has 2 crossveins: the anterior crossvein (which corresponds to the radial–medial crossvein in other insects) that contains components from both the dorsal and ventral epithelia, and the posterior crossvein (which corresponds to the medial crossvein in other insects) and that is

formed entirely from the ventral wing epithelium (Diaz-Benjumea et al. 1989). I also present a model for crossvein formation in *Drosophila* that incorporates this developmental data and that suggests mechanisms by which selection on components of different cell signalling processes may have resulted in both changes in crossvein morphology and corresponding changes in the flexural stiffness of the insect wing.

THE DEVELOPMENTAL BIOLOGY OF CROSSVEINS

Environmental influences on crossvein development

Early attempts to understand crossvein development in *Drosophila* focused on the effects of environmental perturbations on the formation of crossveins (Waddington & Begg, 1952; Bateman, 1959; Milkman, 1960; Mohler & Swedberg, 1964; Mohler, 1965; Thompson, 1967). Typically, a group of genetically heterogeneous flies were collected in a given locality and taken into the laboratory. After some small number of generations, the descendants of these flies were exposed to high temperatures (as high as 40 °C) for several hours during a critical period of development (21–24 h after the onset of pupariation). When these heat-shocked flies emerged from the puparium, they were scored for the presence or absence of the posterior crossvein, and individuals with disrupted crossveins were mated to one another to establish the next generation. Over time, not only were there increases in frequency of crossveinless flies after heat shock, but crossveinless individuals began to appear in these lines even in the absence of heat shock. This phenomenon, called genetic assimilation, has Lamarckian overtones, and it was initially described as the tendency of selection to not only increase the frequency of a favoured character, but also to stabilise the development of that character (Waddington, 1953). The more conventional and now generally accepted explanation for the genetic assimilation phenomenon is that the laboratory manipulations not only resulted in the selection of alleles that favored the production of crossveinless phenotypes under heat shock but also coincidentally involved the selection of already existing alleles that were predisposed to produce these phenotypes without heat shock. Regardless of the explanation for the genetic assimilation phenomenon, these experiments showed the complex way that genotype and environment can interact to produce a phenotype, leading Waddington (1957) to produce one of the most enduring visual metaphors in biology, the epigenetic landscape, in

which environmental and developmental factors interact to determine a trajectory for the nascent soma.

Recently, a possible mechanism has been suggested by which these crossveinless phenocopies may have been produced (Rutherford & Lindquist, 1998). The mechanism involves Hsp90, a heat-shock factor that is responsible, under typical environmental conditions, for stabilising a variety of heat-sensitive components of both cell surface-receptor and nuclear receptor mediated signal transduction pathways (Picard et al. 1990; Xu & Lindquist, 1993; Holley & Yamamoto, 1995; Stepanova et al. 1996). Conditions such as heat shock that cause general protein damage can divert Hsp90 from its normal targets to other partially denatured proteins, thus linking environmental perturbations to developmentally important signalling systems (Rutherford & Lindquist, 1998). In certain genetic backgrounds, heterozygous mutations in the gene that encodes Hsp90 produce ectopic crossvein phenotypes similar to those observed in some of the earlier genetic assimilation experiments (Waddington, 1957; Bateman, 1959). These phenotypes were also shown to be heritable and Rutherford & Lindquist (1998) suggest that these phenotypes were due to cryptic genetic variation in components of signalling pathways that was expressed to a greater extent due to the presence of defective Hsp90 protein. This suggests that signalling processes may play an important role in the formation of crossveins, a hypothesis that is supported by the ectopic crossvein phenotypes exhibited by hypomorphic mutations (which reduce gene product activity) in *cdc42* (Genova et al. 2000), a gene that may play a role in the Jun-N-Terminal Kinase signal transduction pathway (Coso et al. 1995).

Signalling processes and crossvein development

Additional evidence suggesting the importance of signalling in crossvein formation comes from work by Garcia-Bellido (1977). He found, by means of genetic mosaic analysis, that clones of cells in the dorsal wing epithelium that lacked functional *crossveinless* (*cv*) gene product prevented the formation of the posterior crossvein even though this vein is formed entirely from the ventral epithelium of the developing wing. Similar clones of cells in the ventral wing epithelium that also lacked *cv* gene product had no effect on the formation of the crossvein. It can be inferred from these results that an inductive signal that originates in the dorsal epithelium and must be received by the ventral epithelium in order for the posterior crossvein to form (Fig. 1*b*). The *cv* gene product is necessary for

the wild-type transmission of this inductive process (Garcia-Bellido, 1977). This type of mosaic analysis has not yet been extended to include other loci with similar phenotypes (Diaz-Benjumea & Garcia-Bellido, 1990), but such an extension is likely to provide additional information about the different roles of these genes. A somewhat similar vein induction mechanism, which involves the *Vein off* gene product, has been suggested for the formation of longitudinal veins that form from the ventral wing epithelium (Sturtevant & Bier, 1995).

Another body of evidence that signalling plays an important role in the formation of crossveins comes from studies of BMP-like signalling in the *Drosophila* wing and has been reviewed by Conley et al. (2000). Manipulations of many different components of this signalling pathway result in the loss of crossveins. Homozygotes of hypomorphic alleles of the BMP-like ligand *Glass Bottom Boat* (*Gbb*) show a complete loss of both the anterior and posterior crossveins (Khalsa et al. 1998). Also, the presence of a single mutant copy of *Gbb* or of another BMP-like ligand *Decapentaplegic* (*Dpp*) in combination with 2 mutant copies of the *crossveinless-2* (*cv-2*) gene result in an enhancement of the loss of crossvein *cv-2* mutant phenotype (Conley et al. 2000). Similarly, overexpression of the *short gastrulation* gene product, that inhibits BMP-like signals by binding and sequestering BMP-like ligands and preventing them from activating their receptors, blocks the formation of the crossveins (Yu et al. 1996, 2000). Finally, mutants in the *tolkin* gene, whose product is normally involved in the cleavage and inactivation of *short gastrulation* protein, block the formation of crossveins (Nguyen et al. 1994; Finelli et al. 1995), as does overexpression of a dominant negative form of *Saxophone*, a receptor for BMP-like ligands in *Drosophila* (Haerry et al. 1998).

BMP-like signalling promotes vein-cell fates indirectly by activating the vein gene *rhomboid* (*rho*) that participates in yet another signalling system, the EGF-receptor-Map Kinase (Egfr-MapK) signal transduction pathway (Yu et al. 1996). The Egfr-MapK pathway in turn defines domains of the wing that are competent to produce vein tissues and also promotes the differentiation of vein tissues (Sturtevant et al. 1993). High levels of Egfr-MapK signalling appear to drive the expression of *Delta* along the incipient wing veins, and *Delta* induces *Notch* target gene expression and inhibits vein formation in neighbouring cells (Sturtevant & Bier, 1995; de Celis et al. 1997; Huppert et al. 1997). Mutations in components of these pathways have phenotypes that include the loss of vein material or the thickening of veins (Diaz-

Benjumea & Garcia-Bellido, 1990). In wild-type *Drosophila*, the Egfr-MapK and Notch signalling pathways together determine the thickness of the wing veins and control the transitions between the veins and the intervein regions of the wing.

MODEL FOR CROSSVEIN FORMATION

A model for crossvein development can be divided up into 3 stages. In the first stage, signalling along the proximal–distal axis of the wing might be used to determine the number and location of the incipient crossveins (Fig. 2*a*). This signal could involve *cdc42* and the Jun-N-Terminal Kinase signal transduction pathway (Agnès et al. 1999). The phenotypes of *cdc42* mutants suggest that crossvein number and crossvein placement are intimately related because ectopic crossveins appear at discrete intervals along the

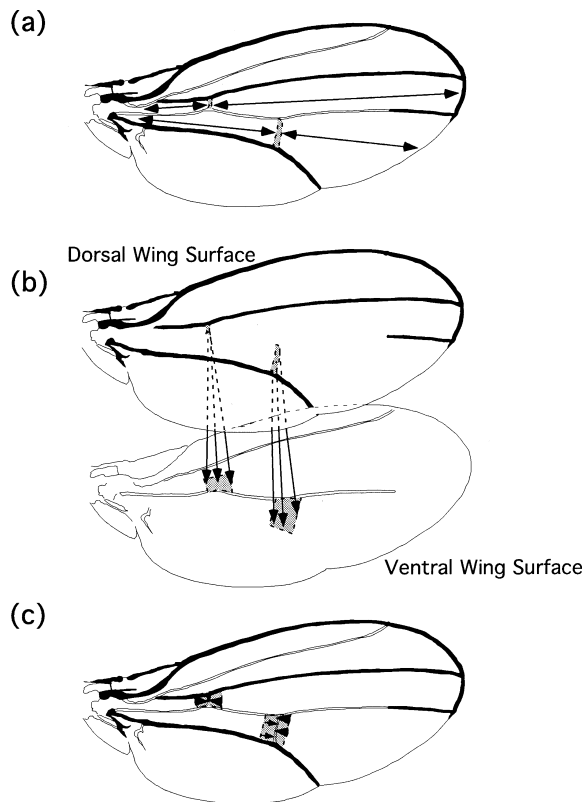


Fig. 2. Three-stage model for crossvein formation. In the first stage (*a*), the number and position of the crossveins along the proximal–distal axis is specified. Gene products that may play a role in this stage include *cdc42*, Hsp90, and components of the Jun-N-terminal Kinase signal transduction pathway. In the second stage (*b*), the presence or absence of crossveins is determined by a signal from the dorsal wing epithelium that is required for the formation of vein tissue in the ventral wing epithelium. This process involves *cv*, *cv-2*, *dpp*, *gbb*, and other components of the BMP-like signalling pathway. In the final stage (*c*), domains of vein competent cells is specified by the Egfr-MapK signal transduction pathway. Vein cell fates are then restricted to the incipient vein by Notch-Delta signalling. Together the Egfr-MapK and Notch-Delta signals regulate the thickness (and perhaps the flexibility) of the crossveins.

proximal–distal axis of the wing and the addition of individual ectopic crossveins results in shifts in the location of the naturally occurring anterior and posterior crossveins with respect to other landmarks on the wing (J. Marcus and C. Klingenberg, unpublished data). This stage of crossvein formation may be sensitive to environmental perturbations as suggested by the ectopic crossvein phenotypes that have been observed in association with heat shock (Waddington, 1957; Bateman, 1959) and in association with defective Hsp90 (Rutherford & Lindquist, 1998).

In the second stage, cells in the dorsal wing epithelium at positions along the proximal–distal axis specified by the signalling processes of the first stage induce cells in the ventral wing epithelium to take on vein cell fates (Fig. 2*b*). This stage corresponds to the inductive process first described by Garcia-Bellido (1977) and the signals involved may correspond to the BMP-like ligands mentioned by Conley et al. (2000). The placement of the BMP-like signalling system after the specification of the location of crossveins is supported by the fact that while manipulations of the *dpp* and *gbb* signalling pathways often result in the loss of crossveins (Conley et al. 2000), there are no reports of such manipulations resulting in the production of ectopic crossveins. Finally, there have been suggestions that *dpp* transcription is upregulated by *cdc42* activity and the Jun-N-Terminal Kinase signal transduction pathway during wing development (Martin-Blanco et al. 1998; Agnès et al. 1999). Since losses of crossvein phenocopies are common in populations of heat-shocked flies (Waddington & Begg, 1952; Bateman, 1959; Milkman, 1960; Mohler & Swedberg, 1964; Mohler, 1965; Thompson, 1967), this may also be a temperature-sensitive signalling process.

In the third stage of the model, the cells of the ventral wing epithelium upregulate *rho* and initiate signalling through the Egfr-MapK pathway to define a domain of vein competency (Yu et al. 1996). Notch signalling then restricts vein cell fates to those cells in which Egfr-MapK signalling is greatest (Sturtevant & Bier, 1995; de Celis et al. 1997; Huppert et al. 1997) and it is these cells that ultimately form the posterior crossvein and the ventral portion of the anterior crossvein (Fig 2*c*). Components of these signalling systems may also show temperature sensitivity because thick vein phenotypes have been observed in association with defective Hsp90 (Rutherford & Lindquist, 1998).

This model, while speculative in many details, provides a framework with which one can think about

the formation of crossveins. It can also be used to create experimentally testable predictions. For example, if this model is correct, then components of the BMP-like signalling pathway are downstream of *cdc42* activity. Double homozygous mutant flies for a hypomorphic *cdc42* mutation (which has an ectopic crossvein phenotype in single homozygotes) and for *cv-2* mutations (a component of the BMP-like signalling pathway, and which has a crossveinless phenotype in single homozygotes) show only the *cv-2* loss of crossvein phenotype (J. Marcus, unpublished data). This suggests that *cv-2* is downstream of (or in a parallel pathway to) *cdc42*, as the model predicts.

CONCLUSIONS

This model for crossvein development clearly shows that there are many parallels between longitudinal vein formation and crossvein formation, even though these structures form at different times during development (Waddington, 1940, Sturtevant & Bier, 1995; Sturtevant et al. 1997). For example, the ventral components of both longitudinal veins and crossveins appear to require an inductive signal from the dorsal wing epithelium (Garcia-Bellido, 1977; Sturtevant & Bier, 1995). Both types of vein also require BMP-like signalling in order to form normally (Conley et al. 2000). Finally, both types of vein utilise the Egr-MapK pathway to specify vein-competent domains of cells, and then restrict that domain using Notch-Delta signalling interactions (Sturtevant et al. 1993; Sturtevant & Bier, 1995; de Celis et al. 1997; Huppert et al. 1997). While there have been many studies which have emphasized that genes with important roles in developmental processes are reused to perform a different function later in development (e.g. Castelli-Gair & Akam, 1995; Abouheif et al. 1997), it is less commonly pointed out that many genes perform similar roles repeatedly during development. The signalling pathways that are involved in longitudinal vein and crossvein formation are good examples of sets of genes that are utilized in similar ways, but in spatially and temporally distinct developmental processes, to ultimately produce similar structures.

This 3-stage model for crossvein development also has some interesting implications for the evolution of insect wing function. Each stage includes at least one potentially independent signalling process and by modulating each of these signals insects might be able to independently alter the presence or absence of crossveins, the number and placement of the crossveins along the proximal–distal axis, and the thickness and flexibility of the crossveins. This multiplicity of

mechanisms provides a large number of combinations of crossvein properties that could be selected to alter the flexural stiffness of an insect wing (Ennos, 1989*a*). Allelic variation in the components of these signalling processes may have provided the necessary variation in flexural stiffness of the wing to allow for the evolution of different modes of flight in insects.

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