

Parallels in leg and wing proximal-distal patterning in Holometabola

Running title: PD Patterning in Holometabola

Jeriel C. H. Lee¹ (jeriel_lee@u.nus.edu)

Tirtha Das Banerjee¹ (tirtha_banerjee@u.nus.edu)

Antónia Monteiro¹ (antonia.monteiro@nus.edu.sg)

¹Department of Biological Sciences, National University of Singapore

Key words: Proximal-distal patterning, *Bicyclus anynana*, *Drosophila melanogaster*, leg, wing, leg gap genes

ABSTRACT

The wings and legs of insects are both appendages that develop along a proximal-distal (PD) axis and likely share many underlying patterning mechanisms. Comparisons between the two appendages have been detailed in *Drosophila melanogaster*, a derived insect where both traits develop initially as imaginal discs. Here, we visualise and compare the expression of prominent PD developmental genes in the embryonic legs and larval wings of a lepidopteran species, *Bicyclus anynana*. We examine the domains of twelve leg gap genes that subdivide legs into distal, medial, or proximal domains, three morphogens, and two genes that refine the PD axis after initial specification. Our results reveal high spatial congruence in the order of PD gene expression between the two appendages. Notably, we observe a distinct loss of the medial domain in the *Bicyclus* wing compared to the leg, providing evidence for the evolutionary re-patterning of these structures. Comparisons with *Drosophila* further highlight conserved versus lineage-specific regulatory architectures. These findings suggest a deeply conserved PD patterning logic across Holometabola, while pointing to divergent mechanisms that likely facilitated the morphological innovation of butterfly wings.

INTRODUCTION

Novel complex traits often use core patterning mechanisms, or gene-regulatory networks (GRNs), that are also used to pattern other (more primitive) traits in the same body. For example, petals in flowers use a modified leaf genetic program for their development (Sablowski, 2015), the novel turtle carapace uses a fused and restructured rib cage (Hirasawa *et al.*, 2013), and new lobes in the male genitalia of *Drosophila* emerge from the same genetic program that produces breathing spiracles in larvae (Glassford *et al.*, 2015). For insect wings, however, a trait with remarkable patterning complexity that evolved ~400 million years ago, it remains unclear which ancestral GRN was reused to produce them. While wings are positioned dorsally in the body, they likely share patterning mechanisms with the more primitive ventral legs, as both appendages grow along a Proximal-Distal (PD) axis. Key modifications to transform a tube-like leg into a sheet-like wing have also been proposed in *Drosophila*, which mostly involve wings acquiring a well-defined dorsal-ventral axis via the recruitment of *apterous* (*ap*), a distal leg PD patterning gene, into a novel dorsal compartment (Williams *et al.*, 1994; Williams & Carroll, 1993).

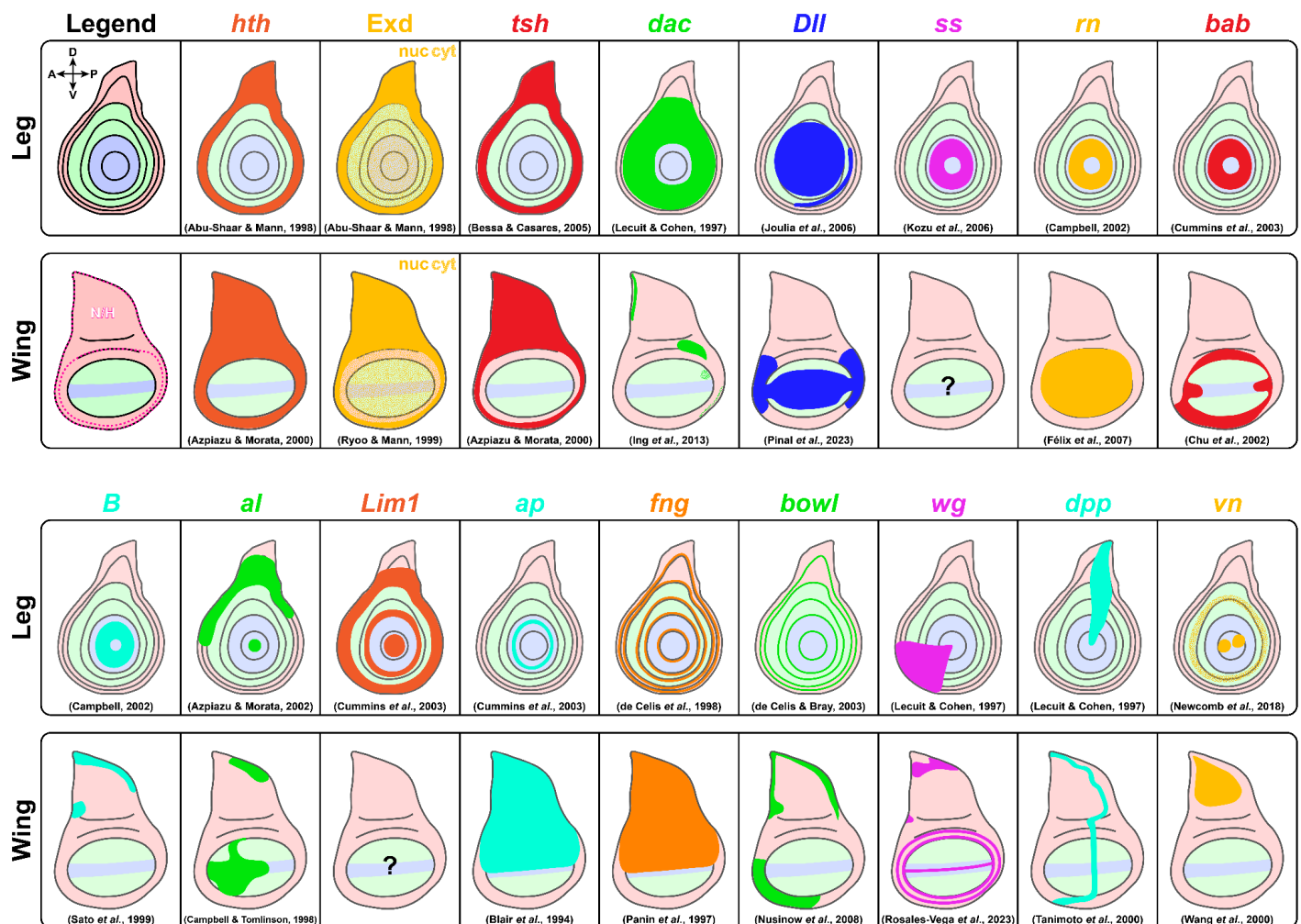


Figure 1: Review of LGG and LGG-related gene expression domains in both *Drosophila* third-instar leg and wing imaginal disc. The PD domains in the leg and wing disc and the notum-hinge region (pink dashed outline; N/H) in the wing have been outlined in the first box (Red = Proximal; Green = Intermediate; Blue = Distal). The other axes of both appendages are specified at the top left of the leg legend (D = Dorsal; V = Ventral; A = Anterior; P = Posterior). Relative areas of expression are highlighted for each gene, with references below each illustration.

The PD patterning of thoracic legs has been extensively studied in *Drosophila* among other invertebrates (Fig. 1). In flies, leg PD patterning starts with the juxtaposed expression of two ligands, Wnt family ligand Wingless (Wg/Wnt1) and TGF- β family ligand Decapentaplegic (Dpp), in ventral and dorsal domains of the imaginal disc, respectively (Lecuit & Cohen, 1997). These long-range morphogens induce the expression of two leg gap genes (LGGs), *Distal-less* (*Dll*), at the centre of the disc (distal end), and *dachshund* (*dac*), in a more medial

domain, and repress the LGG *homothorax* (*hth*) more peripherally in the disc (proximally along the PD axis). In turn, these broadly expressed genes, when disrupted, lead to a corresponding regional deletion or “gap” in the adult leg (Rauskolb, 2001). Outside *Drosophila*, Wg and Dpp gradients also establish the PD axis in *Glomeris marginata* (Prpic, 2004), *Trioblium castaneum* (Grossmann *et al.*, 2009), *Triops longicaudatus* (Nulsen & Nagy, 1999), and *Gryllus bimaculatus* (Niwa *et al.*, 2000), and the three LGGs, *hth*, *dac*, and *Dll*, have an equally conserved role in patterning the proximal, intermediate, and distal regions of the leg, respectively, in many arthropod and crustacean species (references within Ober & Jockusch, 2006).

In contrast to legs, where PD patterning has been extensively characterised in many insects, wing patterning studies lack comparable depth and diversity. In flies, the activities of Wg and Dpp are still central to initiating and organising the distal development and growth of wings (Bosch *et al.*, 2017; Swarup & Verheyen, 2012). Subsequently, several LGGs are expressed in wings in similar PD domains as in legs, such as *hth* (Azpiazu & Morata, 2000, 2002), *teashirt* (*tsh*) — another proximal LGG (Erkner *et al.*, 1999; Wu & Cohen, 2002), and two distal gap genes, *rotund* (*rn*) (St Pierre *et al.*, 2002), and *Dll* (Gorfinkiel *et al.*, 1997), supporting the hypothesis that most animal appendages use a common shared PD patterning network (Panganiban *et al.*, 1997). Despite these parallels, important differences between leg and wing patterning programs have also been documented (Fig. 1, Tripathi & Irvine, 2022; Williams *et al.*, 1994), albeit never directly compared outside of flies. Furthermore, comparative work on proximal-distal axis specification in insect appendages outside of *Drosophila* remains scarce.

Evidence that wings and legs share a fundamental appendage patterning program is supported by the ectopic expression of selector genes, which induce a change in appendage fate in *Drosophila*. *Dll* overexpression in the larval wing imaginal disc is sufficient to transform wings and halteres into leg-like appendages (Gorfinkiel *et al.*, 1997), while ectopic *vestigial* (*vg*) expression in leg imaginal discs is sufficient to induce these to grow into flat, wing-like outgrowths (Kim *et al.*, 1996). In addition, these ectopic outgrowths displayed proper PD axis specification with fate-altered wings showing a femur-like proximal, tibia-like medial, and tarsi-like distal segments (Gorfinkiel *et al.*, 1997), and fate-altered legs having a “wing” margin align with the distal tip of the modified leg (Kim *et al.*, 1996). Hence, a common PD axis patterning program is likely underscoring these similarities.

Here, we test the hypothesis of a common PD leg-wing patterning program in insects by characterising the spatial-temporal expression of twelve prominent LGGs in both embryonic thoracic legs and larval wings of the model butterfly *Bicyclus anynana* (*Bicyclus*). These LGGs are further categorised as broad-domain LGGs involved in proximal (*hth*, *extradenticle* (*exd*), and *tsh*), intermediate (*dac*), and distal patterning (*Dll*); and narrow-domain LGGs involved mainly in the distal subdivision of the tarsus and pretarsus: *spineless* (*ss*), *rn*, *bric-à-brac* (*bab*), *Bar* (*B*), *ap*, *aristaless* (*al*), and *Lim1*. We also explored the expression domains of five LGG-related genes, which include two genes involved in PD boundary refinement after initial PD axis specification by the broad-domain LGGs, *fringe* (*fng*) (Rauskolb, 2001) and *bowel* (*bowl*) (de Celis Ibeas & Bray, 2003), as well as three crucial morphogens involved in establishing PD domain boundaries, *wg*, *dpp*, and *vein* (*vn*) (Estella *et al.*, 2012).

RESULTS

Expression of LGGs in embryonic legs

We first examined the expression of five broad-domain LGGs: *hth*, *exd*, *tsh*, *dac*, and *Dll*, in 48 h embryonic thoracic legs (TLs).

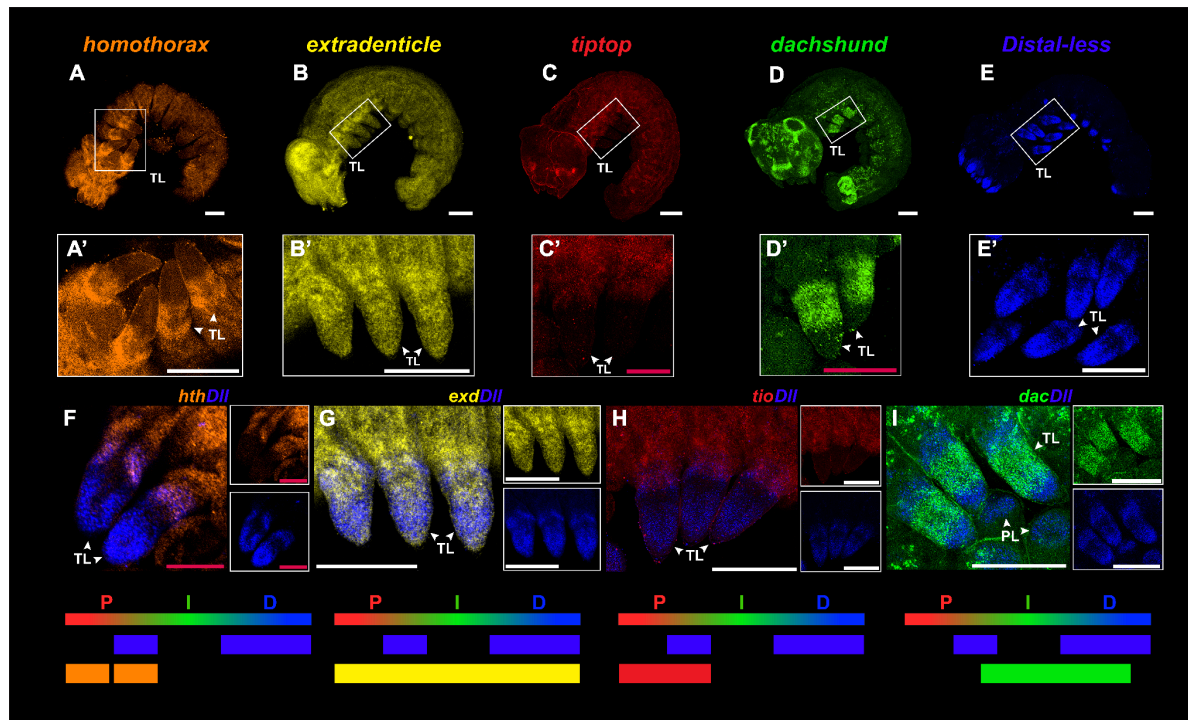


Figure 2: Expression of broad-domain LGGs in thoracic legs (TLs) of *Bicyclus* 48 h embryos. (A', B', C', D', E') are leg close-ups of (A, B, C, D, E), respectively (PL = prolegs). For each merged figure, a summary illustration is included at the bottom with an overview of the respective leg expression areas along the PD axis (Red domain = proximal, P; Green domain = intermediate, I; Blue domain = distal, D). (A, A') *hth* is expressed as two bands in the leg proximal domain. (B, B') *exd* stainings show ubiquitous expression throughout the leg. (C, C') *tiptop* (*tio*) is the single *tio/tsh* ortholog in lepidopterans (Zhang *et al.*, 2020) and is expressed in the entire proximal region of the leg. (D, D') *dac* expression is a single broad band in the intermediate domain of the leg. (E, E') *Dll* is expressed in two bands at the proximal-intermediate region and in the distal domain of legs. (A'-E') corresponding magnified boxed regions. *Dll* is also expressed in the prolegs. (F) Single and merged channels of *hth* and *Dll* show overlap between the more distal band of *hth* and the proximal band of *Dll*. (G) Single and merged channels of *exd* and *Dll*. (H) Single and merged channels of *tio* and *Dll* showing overlap only at the proximal *Dll* band. (I) Single and merged channels of *dac* and *Dll* show a small overlap between both ends of the *dac* band and the two bands of *Dll*. In this embryo, *Dll* expression can also be seen

strongly in the prolegs. All embryos are oriented from anterior to posterior (left to right). White scale bars: 100 μ m; red scale bars: 50 μ m.

These genes were expressed in slightly overlapping domains along the PD axis. For *hth*, we observed two distinct bands restricted to the proximal domain (Fig. 2A, A'). For *exd*, there was pan-tissue expression throughout the embryo (Fig. 2B, B', G). *tio* was broadly expressed in the entire proximal domain of the leg (Fig. 2C, C', H). *dac* had a single, broad band in the intermediate domain (Fig. 2D, D', I). *Dll* had two separate bands: a broader one at the tip (distal domain) and a narrower one in the proximal/intermediate domain (Fig. 2E – I). The more distal band of *hth* expression was co-expressed with the more proximal band of *Dll* (Fig. 2F), which was also co-expressed with *tio* (Fig. 2H). *dac* was expressed as a broad band within both *Dll* bands, with a small region of co-expression on both sides (Fig. 2I).

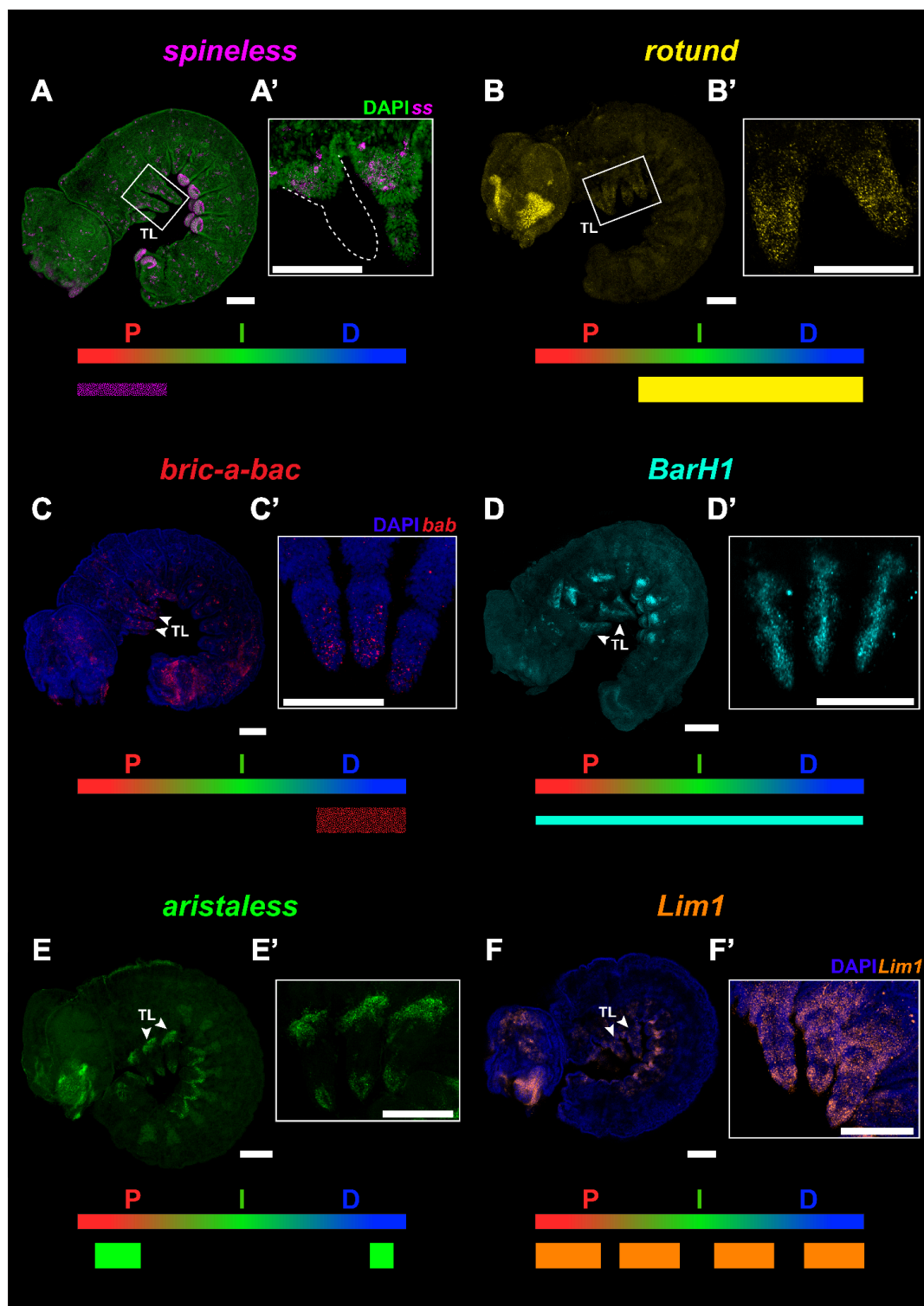


Figure 3: Expression of narrow-domain LGGs in the thoracic legs (TLs) of *Bicyclus* embryos. (A', B', C', D', E', F') are leg close-ups of (A, B, C, D, E, F), respectively, with a summarised illustration for each gene. Gene expressions that are not uniform bands around the leg epidermis are denoted by a thin bar, while sparse expression is represented by a speckled bar. Close-ups (A', C') are restricted to the leg epithelium layer. (A, A') *ss* is expressed at low levels in the anterior-proximal region of the leg epidermis. (B, B') *rn* is expressed uniformly throughout the intermediate and distal regions of the leg. (C, C') *bab*

expression is sparse and only at the distal region of the leg epidermis. **(D, D')** *BarH1* is expressed along the AP axis of the leg ventrally. **(E, E')** *al* is expressed in two bands at the proximal and distal regions. **(F, F')** *Lim1* is expressed in four bands of similar thickness throughout the leg. All embryos are oriented from anterior to posterior (left to right). Scale bars: 100 μ m.

Expression of narrow-domain LGGs in embryonic legs

Next, we explored the expression of narrow-domain LGGs, *ss*, *rn*, *bab*, *ap*, *Bar*, *al*, *Lim1*, in embryonic legs. *apterous-A* (*apA*) was used to represent *ap*, which is the only gene copy in *Drosophila* (Prakash & Monteiro, 2018), and *BarH1* was used to represent *Bar* as both paralogs play similar roles in distal fly leg patterning (Kojima *et al.*, 2000). *ss* was expressed in a few cells at the anterior-proximal region on the leg epithelium (Fig. 3A, A'). *rn* had a stronger expression compared to *ss*, along a broad domain that spanned the intermediate and distal regions of the leg (Fig. 3B, B'). *bab* was expressed sparsely in the distal domain of the leg (Fig. 3C, C'). *BarH1* was expressed strongly and ventrally along the AP axis, spanning all PD domains in the leg (Fig. 3D, D'). *al* was expressed in a strong proximal band, as well as in a much thinner band just before the tip of the leg (Fig. 3E, E'). *Lim1* was expressed along four strong, thick bands throughout the PD domains (Fig. 3F, F'). Lastly, *apA* was not expressed in the leg epidermis (Fig. S1A – A'').

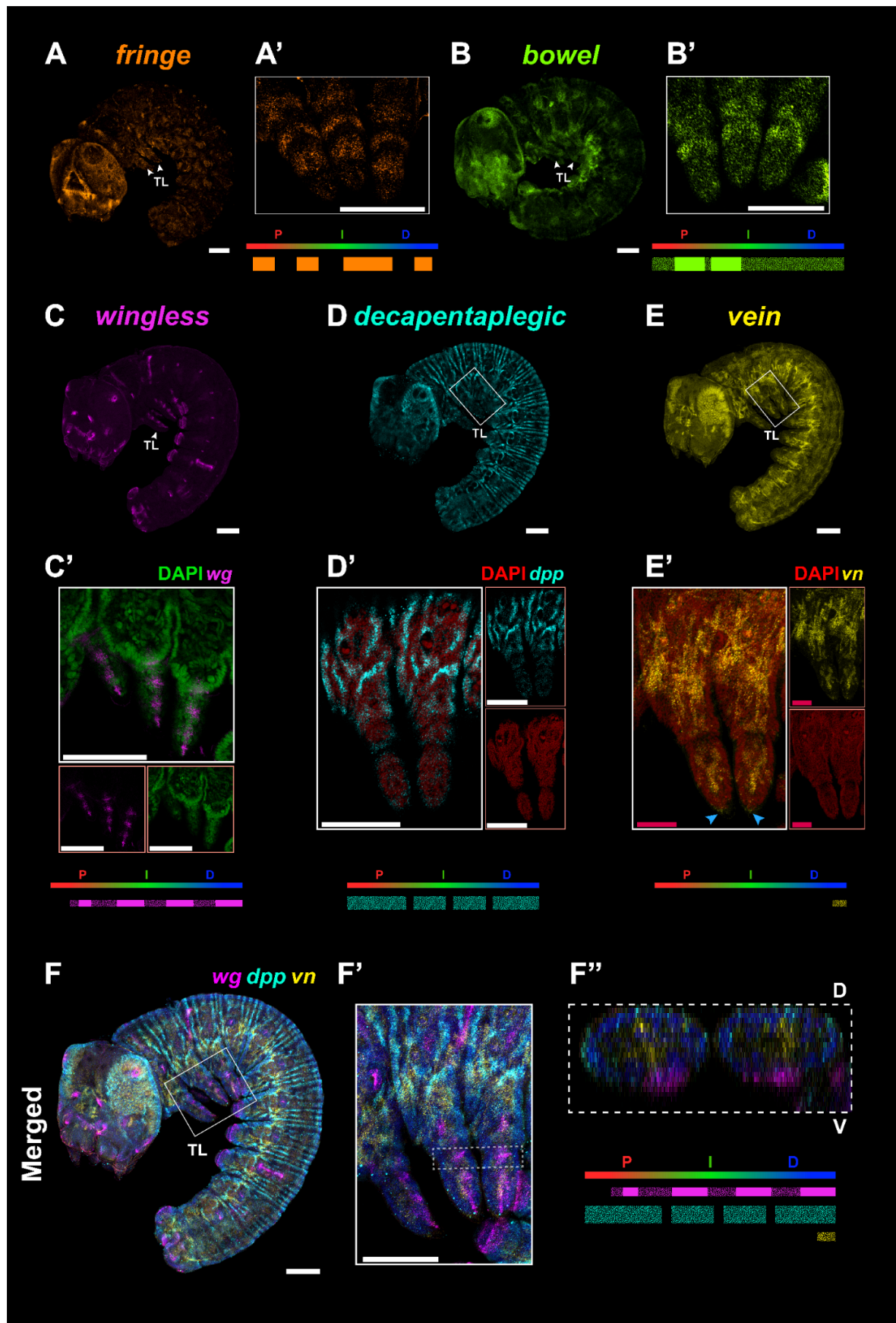


Figure 4: **Expression of LGG-related genes in the thoracic legs (TLs) of *Bicyclus* embryos.** For each panel, a summarised illustration is included at the bottom. All embryo orientations, annotations, and summary illustration interpretations follow that of Fig. 2. (A', B', C', D', E') are leg closeups of (A, B, C, D, E), respectively, and closeups (C', D') are restricted to the epithelium layer. (A, A') *fng* is expressed in four bands of varying thickness across the three domains in the leg. (B, B') *bowl* is expressed at a low level throughout the whole leg, with two stronger bands of expression in the proximal and intermediate regions of the leg. (C, C') *wg* is expressed along the leg AP axis ventrally, with varying degrees of expression. (D, D') *dpp* is expressed in a unique striped pattern sparsely in the leg. (E, E') *vn* is expressed strongly in the mesodermal layer, and is only present weakly in the epidermis at the very tip of the leg (blue arrowheads). (F, F') Merged image of *wg*, *dpp*, and *vn* morphogens. (F'') Cross-section of merged leg outlined by a dashed box in (F') showing *wg* present ventrally, *dpp* present sparsely throughout the leg epidermis, and *vn* only present in the middle mesodermal layer of the leg. All embryos are oriented from anterior to posterior (left to right). White scale bars: 100 μ m; red scale bars: 50 μ m.

Expression of LGG-related genes and *wg*, *dpp* and *vn* morphogens in embryonic legs

In addition to characterising the expression of important PD patterning genes, we also explored *fng* and *bowl*, which refine the PD axis after broad-domain specification, and the morphogens *wg*, *dpp*, and *vn*, responsible for establishing the PD axis of the leg, respectively. Four bands of *fng* expression could be seen along all PD domains with different thicknesses (Fig. 4A, A'). *bowl* had a constitutive, low-level expression throughout the entire leg, except for two strong bands at the proximal-intermediate region (Fig. 4B, B'). *wg* had minimally four bands of strong expression throughout the ventral PD axis, interspersed by sparser expression (Fig. 4C, C'). A unique striped expression pattern of *dpp* was observed along the entire embryonic epidermis, including weak expression in the leg (Fig. 4D, D'). *vn* was sparsely present in the leg epidermal layer at the very tip (Fig. 4E – E', F''). These three morphogens only overlapped in epidermal expression at the very tip of the leg (Fig. 4F, F').

Expression of LGGs in larval wings

We next investigated the expression of the LGGs in fifth instar larval wings at three different developmental time points. To accurately track wing developmental stage, we used venation and tracheal growth markers, translated to a numerical system: (0.00 – 0.75, early larvae, EL), mid (1.00 – 1.75, ML), and late (2.00 – 2.75, LL) larval wing stages for temporal gene expression data (Banerjee & Monteiro, 2020b; Reed et al., 2007). All larval wings were imaged during the fifth instar.

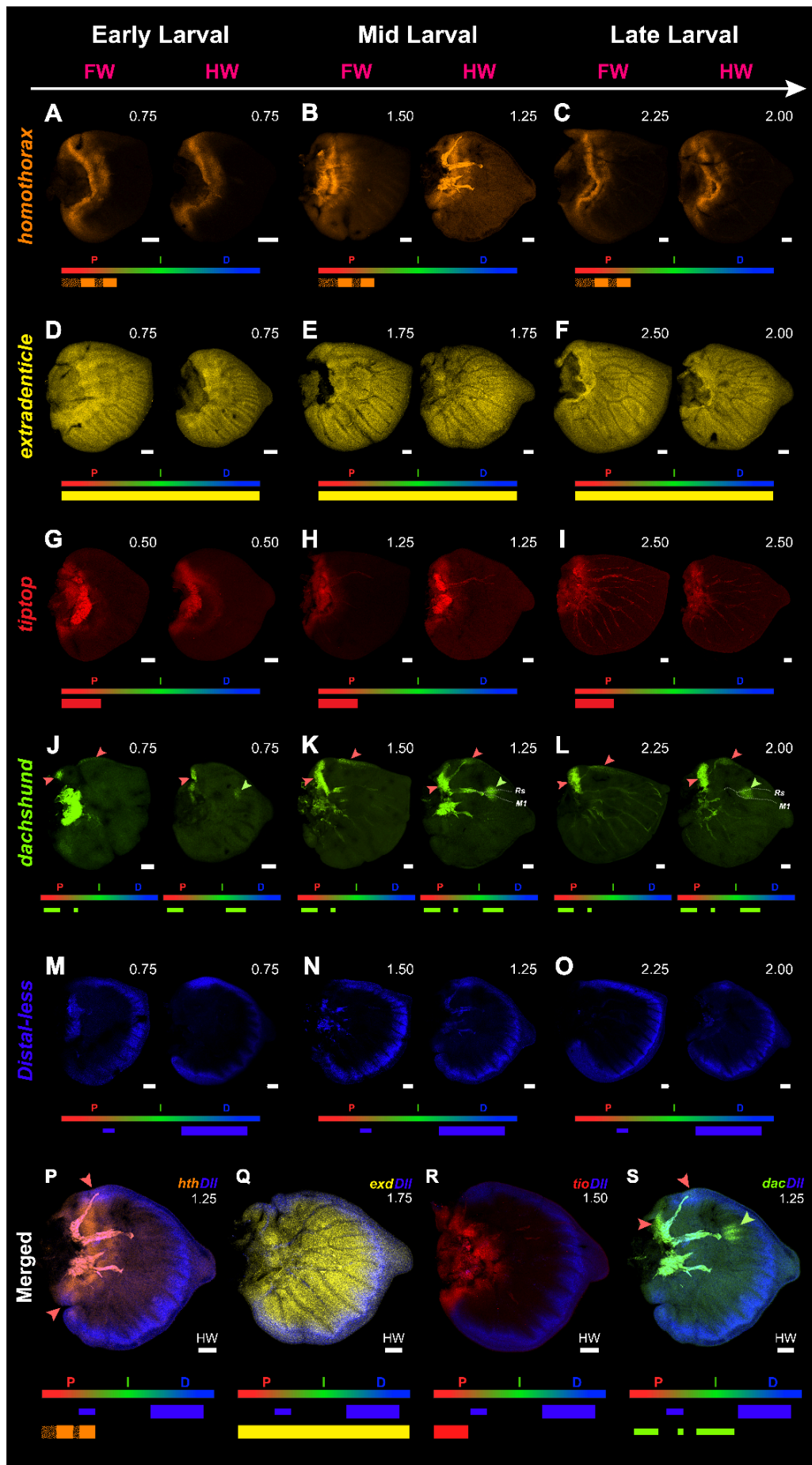


Figure 5: Spatial-temporal expression of broad-domain LGGs in *Bicyclus* larval wings. Each panel consists of a forewing (FW; left) and a hindwing (HW; right). Wings are oriented along proximal-distal (left to right) and anterior-posterior (top to bottom) axes, and staged (top right). For each panel, a summarised illustration is included at the bottom. Gene expressions that are not along a continuous AP band are denoted by a thin bar, while lower levels of expression are represented with a speckled bar. **(A – C)** *hth* is expressed at the proximal domain for all wing stages. **(D – F)** *exd* expression is ubiquitous during wing development. **(G – I)** *tio* is consistently expressed in the proximal region only. **(J – L)** *dac* varies across stages and wings. Red and green arrowheads represent expression in the proximal and intermediate domains, respectively. **(M – O)** *Dll* is consistently expressed along the wing margin at the distal region, and at both anterior and posterior ends of the proximal region throughout wing development. **(P – S)** Merged images of each gene with *Dll* in mid-larval hindwings. **(P)** *hth* and *Dll* colocalise at the anterior-most and posterior-most proximal region (red arrowheads). **(Q)** *exd* and *Dll* colocalise at the wing margin. **(R)** *Dll* expression stops just before *tio* expression in the proximal region. **(S)** Colocalization of *dac* and *Dll* in the anterior-proximal region (red arrowheads). Scale bar: 100 μ m.

Similar to the expression analysis in legs, we first explored the expression of broad-domain LGGs in larval wings. *hth* was expressed in two strong bands in the proximal region, with a lower level of expression throughout the entire proximal domain, at all developmental stages (Fig. 5A – C). *exd* expression was expressed throughout the wing at all stages (Fig. 5D – F, Q). *tio* was expressed in a band that occupied most of the proximal domain at all stages (Fig. 5G – I). *dac* was expressed in two regions in the forewing and three in the hindwing (Fig. 5J – L). Both fore- and hindwings expressed *dac* at the proximal-most and anterior-proximal region. At the same time, *dac* was expressed in a cluster of intermediate cells at the intersection of the *Rs* and *MI* veins in the middle of hindwings alone (Fig. 5J – L, right wings). Expression of *dac* in all domains became strongest by mid-larval wing development. *Dll* was expressed in a band in the distal domain, along the wing margin but excluding the peripheral tissue beyond the border lacuna, which will undergo apoptosis (Macdonald *et al.*, 2010), and extending into the proximal domain at anterior and posterior regions, at all wing stages (Fig. 5M – O). *Dll* expression extended inward during LL (Fig. 5O) to form the centres

of the future eyespots (Monteiro *et al.*, 2013). There was overlap between the proximal expression of *Dll* and the more distal *hth* band (Fig. 5P, arrowheads), the expression domains of *tio* and *Dll* abutted each other (Fig. 5R), and *dac* expression overlapped with *Dll* at the anterior-proximal region (Fig. 5S, red arrowheads).

Expression of narrow-domain LGGs in larval wings

Figure 6: Spatial-temporal expression of narrow-domain LGGs in *Bicyclus* larval wings. All wing orientations, annotations, and summary illustration interpretations follow that of Fig. 5. **(A – C)** *ss* is expressed in two relatively small cell clusters located at the proximal-most region (red arrowheads). **(D – F)** *rn* is expressed broadly in the entire intermediate and distal region except for the peripheral tissue **(G – I)** *bab* is expressed in one cell cluster in the proximal region, up to three clusters in the intermediate region as the wing reaches mid-larval development, and at the distal tip. **(J – L)** *apA* is expressed only in the dorsal cells of the wing, as shown in the DV cross-section below each wing. In forewings, strong *ap* expression is also seen in the anterior-dorsal region (blue arrowheads). **(M – O)** *al* is expressed consistently throughout larval wing development as a short band at the intermediate region and at the anterior-distal region. Scale bar: 100 μ m.

Next, we characterised the expression of the narrow-domain LGGs in fifth instar larval wings. Two small, distinct clusters of *ss* were observed at the proximal-most region across all larval wing stages (Fig. 6A – C). *rn* was consistently expressed in a broad domain spanning the intermediate and distal regions of the wing at all stages, with expression in the intermediate region being strongest (Fig. 6D – F). Multiple clusters of *bab* were seen throughout the three PD domains, with its onset in the early larval stage marked by strong localisation in a central cluster and at the wing tip. Peak *bab* expression was observed by the mid-larval stage, characterised by a central-proximal region and a triad of clusters in the intermediate domain. Comparison between the wing pairs revealed that the anterior-intermediate cluster was significantly more prominent in the forewing than in the hindwing (Fig. 6G – I). *apA* was expressed at a consistent baseline level throughout the dorsal side of the wing at all stages, as well as in a strong proximal band in all wings and at the anterior-distal region of all forewings (Fig. 6J – L). Two short, anterior bands of *al* were seen in the intermediate and distal domains of the wing at all stages. The distal band in forewings crossed the AP boundary, while the same band in hindwings did not. The intermediate band in hindwings also seemed slightly shorter than that of the forewing at each developmental stage (Fig. 6M – O). *BarH1* and *Lim1* were not expressed in the wing throughout its development (Fig. S1B, C).

Expression of LGG-related genes and *wg*, *dpp* and *vn* morphogens in larval wings

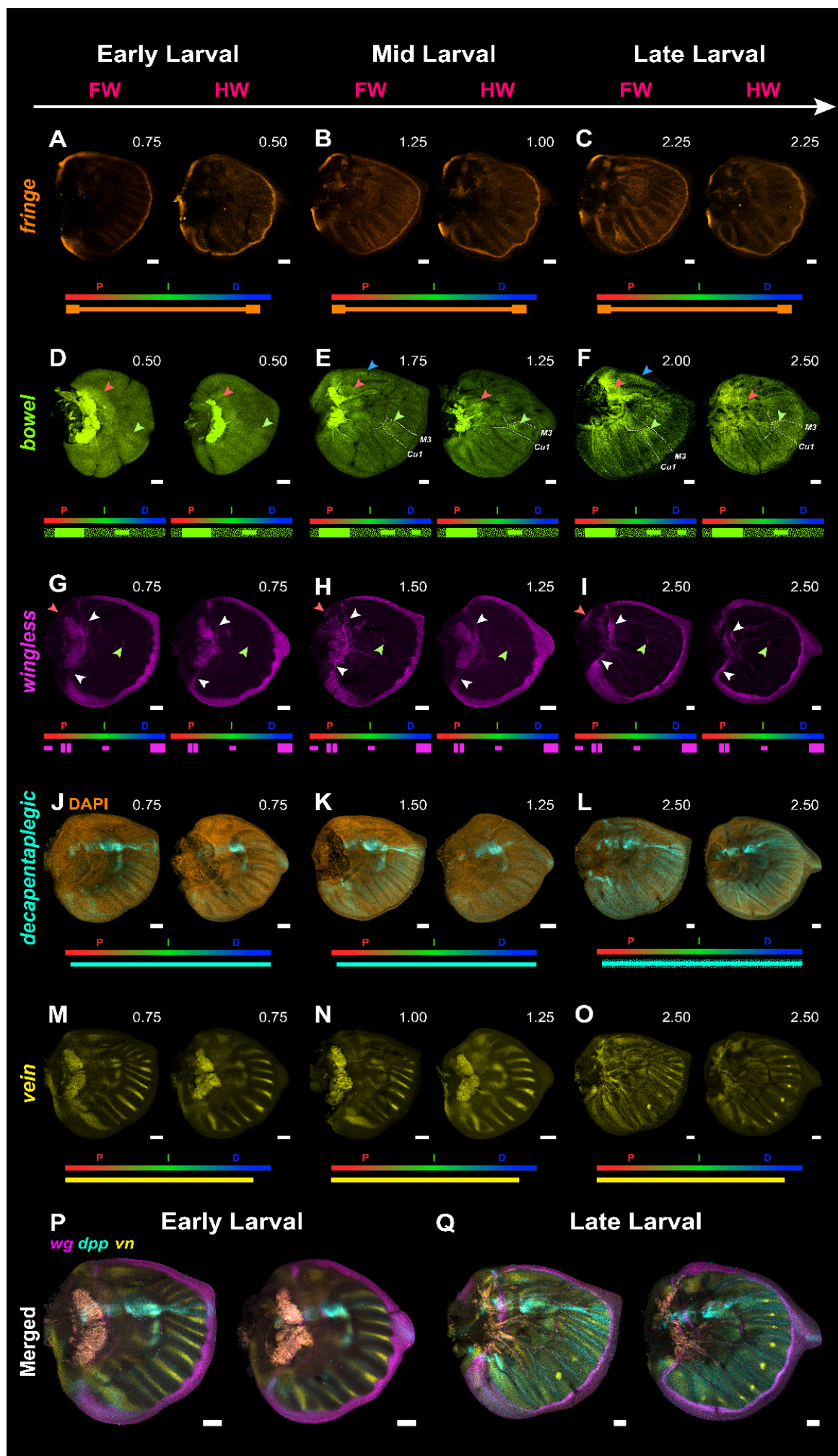


Figure 7: Spatial-temporal expression of LGG-related genes in *B. anynana* larval wings. All wing orientations, annotations, and summary illustration interpretations follow that of Fig. 5. Red, green, and blue arrowheads indicate expression in the proximal, intermediate, and distal domains, respectively. **(A – C)** *fng* is expressed at the proximal-most region and along the wing margin, and in the veins. **(D – F)** *bowl* is sparsely expressed throughout the wing, with strong expression areas at all three domains of the larval wing. **(G – I)** *wg* expression is present at all three domains throughout larval wing development: in the most peripheral tissue along the margin of the disc, in a thin proximal band that is present in both the dorsal and ventral layers (white arrowheads), and on top of a future cross-vein (green arrowhead). **(J – L)** *dpp* is expressed strongly along the AP boundary from proximal to distal throughout all larval stages, along a more posterior domain, and in an orthogonal short stripe in the center of the wing at earlier stages. *dpp* flanks the veins at later stages. **(M, N, O)** *vn* is expressed in the central intervein regions throughout the entire wing and also at the proximal-most region during all larval stages. Distal expression becomes restricted to the eyespots during late wing development. **(P, Q)** Merged images of *wg*, *dpp* and *vn* in early (G, J, M) and late (I, L, O) wings. Scale bars: 100 μ m.

We then looked at the expression of the two LGG-related genes and the three morphogens in larval wings. *fng* was expressed in a single, thin band along the distal region separating the wing margin from the peripheral tissue, in the wing hinge, and along the veins across all stages. *fng* expression along the veins also became stronger in older wings (Fig. 7A – C). Constitutive *bowl* expression was observed at low levels throughout the entire wing at all stages, with a stronger proximal band (Fig. 7D – F, red arrowheads) as well as at the intersection between the *M3* and *Cu1* vein. In addition, forewings alone had an anterior-distal region of strong expression by mid larval development (Fig. 7D – F, blue arrowheads). Consistent and strong *wg* expression was present throughout the peripheral tissue, in two thin proximal bands (one in the dorsal, the other in the ventral surface), in a cross-vein in the intermediate region of both fore- and hindwings, and in the anterior-proximal region of forewings (Fig. 7G – I). *dpp* was expressed in a strong band along the AP boundary, which splits into two at the distal region of forewings, in a more posterior band at earlier stages, in a short transversal band in the central-intermediate region of both wings, and flanking the

posterior veins during the early larval stage before extending to the anterior veins during the late larval stage (Fig. 7J – L; Banerjee & Monteiro, 2020). Lastly, *vn* was strongly expressed in all intervein regions throughout all PD domains (Fig. 7M – O) and was anti-colocalised with *dpp* throughout wing development (Fig. 7P, Q). In older wings, *vn* was refined to the eyespot centres.

DISCUSSION

In this study, we investigated the potential conservation in the expression of genes known to pattern the PD axis of the ventral appendage, in patterning the same axis of the dorsal appendage, the wing. We characterised the spatial expression of twelve LGGs and five LGG-related genes in embryonic thoracic legs, as well as across three developmental stages of fifth instar larval wings. The summary illustration below compares the relative areas of expression of all the genes in legs and wings, and contrasts them with previous expression data from *Drosophila* (Fig. 8).

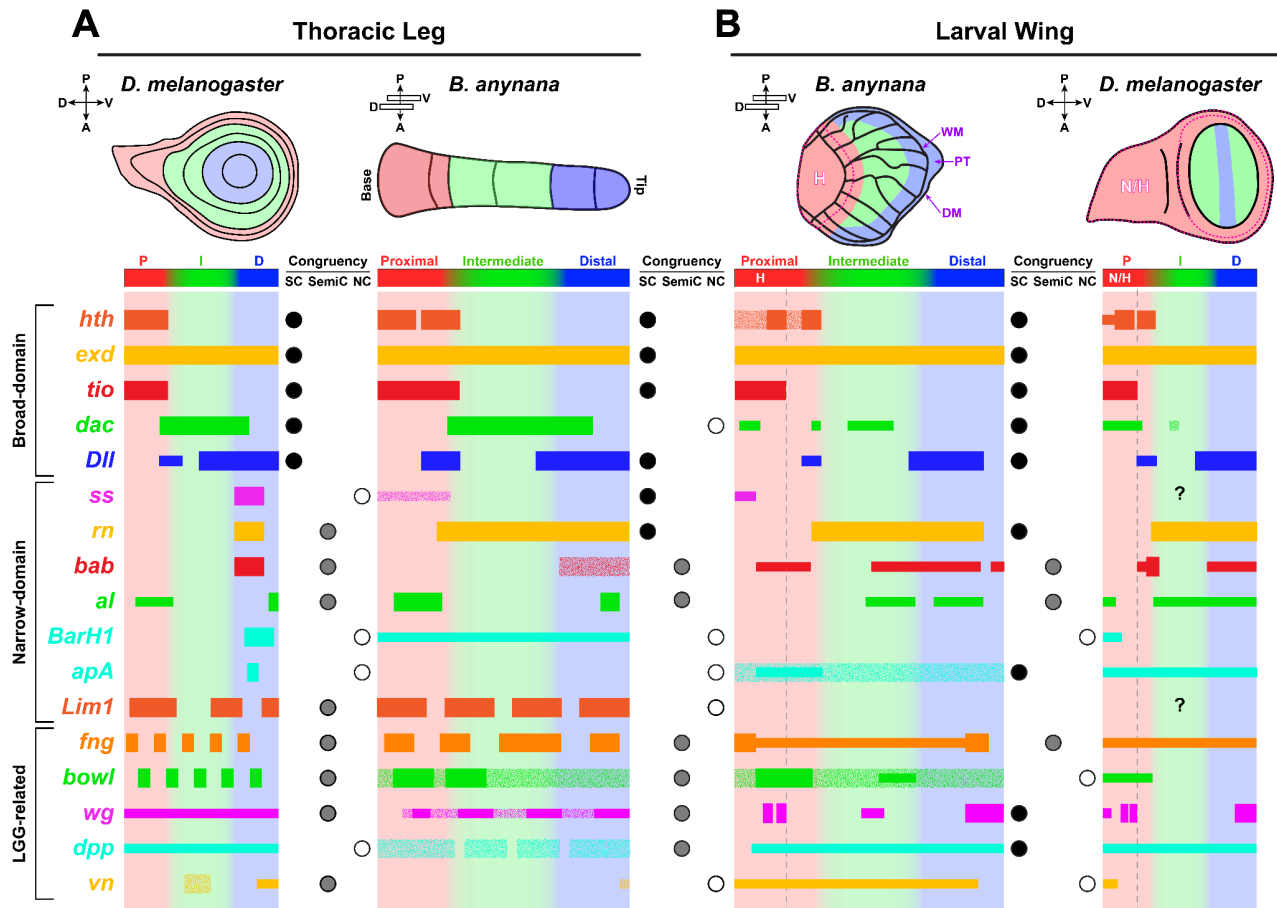


Figure 8: **Overall summary of LGG expressions in the 48hr embryonic thoracic leg and larval wing of *Bicyclus* and 3rd instar larval imaginal discs of *Drosophila*.** For all appendage illustrations and summary bars, red, green, and blue regions correspond to the proximal, intermediate, and distal domains, respectively. The other axes of each appendage are specified at the top left (D = Dorsal; V = Ventral; A = Anterior; P = Posterior). Genes that are not expressed as a band around the leg epidermis or along the AP axis in larval wings are denoted with a thin bar, while lower expression levels in both appendages are represented by a speckled bar. For *Drosophila* comparisons, the presence of gene expression in the proximal (red), intermediate (green), and distal (blue) domains is denoted with P, I, and D, respectively. The concentric ring-format gene expression data of Figure 1 have been mapped onto a linear axis: distal domains represent the central domains, proximal domains represent the peripheral domains. **(A)** Relative areas of gene expression in the *Bicyclus* 48h leg (right) and late third instar *Drosophila* leg imaginal disc (left). **(B)** Relative areas of gene expression in the *Bicyclus* mid-larval hindwing (left) and the *Drosophila* late third instar wing imaginal disc (right). The presumptive *Bicyclus* hinge region (H) and *Drosophila* notum/hinge (N/H) are outlined with pink dashed lines, while elements of the peripheral region were annotated with purple arrows (WM = Wing margin; PT = Peripheral tissue; DM = Disc margin). The spatial congruency of all the genes between *Drosophila* and *Bicyclus* legs and larval wings is shown in the colored circles between the summaries (black: Strongly congruent, SC; grey: Semi-congruent, SemiC; white: Non-congruent, NC).

Defining the PD domains in *Bicyclus* embryonic thoracic legs and larval wings

We used specific conserved marker genes from *Drosophila* appendages to define the proximal and distal domains of *Bicyclus* appendages, where the proximal domain includes the hinge region (Fig. 8B). We used *hth* as a marker of the proximal domain (Coulcher *et al.*, 2015; Dong *et al.*, 2001; Ruiz-Losada *et al.*, 2018; Schaeper *et al.*, 2013; Singh *et al.*, 2019), *Dll* as a marker of the distal domain (Jockusch *et al.*, 2004; Tripathi & Irvine, 2022), and *wg*, *hth*, and *tio* as markers for the hinge region (Bessa *et al.*, 2009; Rosales-Vega *et al.*, 2023). Specifically, the two proximal bands of *Bicyclus* *wg* seem to correspond to the inner and outer rings of *wg* expression that define the *Drosophila* hinge region (Fig. 7G – I; Rosales-Vega *et al.*, 2023). Based on these conserved markers, we defined the hinge, proximal, and distal domains, while the remaining region constitutes the intermediate domain of the appendage (Fig. 8B). This resulted in the proximal and distal domains lying adjacent to each other at the anterior and posterior margins (Fig. 8B), whereas the intermediate domain occupies a more central area that does not extend to the wing margins.

The majority of the LGGs are expressed in similar domains across the PD axis in legs and larval wings of *Bicyclus*

We categorised the extent of spatial congruency of the LGG expression patterns in both legs and larval wings into three main groups: strongly congruent, semi-congruent, and non-congruent LGGs (Fig. 8, central two columns). Our congruency comparison addresses whether the expression domains of these genes in both legs and wings are similar in relative position and intensity along the respective PD axes.

Six of the twelve LGGs, *hth*, *exd*, *tio*, *Dll*, *ss*, and *rn*, show similar expression patterns in both the embryonic leg and larval wing, and are hence classified as strongly congruent. Within this group, four out of five broad-domain LGGs are present.

Two additional LGGs, *bab* and *al*, have overlapping expression domains between the legs and wings while exhibiting differences. *bab* is expressed in the distal region of both legs and wings, but there are additional proximal and intermediate domains of expression in the wing. *al* is expressed in the distal tip in both legs and wings, but it is also expressed proximally in the leg and in an intermediate domain in the wing. Hence, since some elements of LGG expression are conserved, the expression of these genes is considered semi-congruent.

The last group of LGGs, *dac*, *apA*, *Lim1*, and *BarH1*, exhibits non-congruent expression, highlighting lineage-specific regulatory divergence. *dac* is strongly expressed in the intermediate region in legs, but it is not expressed in forewings or is reduced to a small cluster of cells in the intermediate wing domain in hindwings. *apA* was not expressed in the leg epithelium, while *Lim1* and *BarH1* were not expressed in the wing (Fig. S1B, C).

In addition to patterning the PD axis, certain genes were also involved in patterning specific cell types in both legs and wings. For example, *ss* and *bab* were expressed in sensory precursor cells present in the medial region of the legs and along the wing veins, while *ss* also has a sensory cluster at the proximal region, possibly where the future hair pencils develop.

Only proximal and distal PD patterning genes in the embryonic legs are likely reused to pattern the larval wing

From the set of strongly congruent genes between legs and wings, most are responsible for patterning the proximal and distal domains of the *Drosophila* appendages, with two genes, *exd* and *rn*, being exceptions. These two genes are also present in the intermediate domain of legs and wings, but their function is restricted to the proximal region for *exd* (Rauskolb *et al.*, 1995) and the distal region for *rn* (St Pierre *et al.*, 2002). No such conservation is seen for the remaining intermediate domain genes.

Divergence between legs and wings, in both *Drosophila* and *Bicyclus*, is mostly visible in the exclusion of *wg* from the dorsal leg domain, the exclusion of *dpp* from the ventral leg domain (and interrupted expression of *dpp* in *Bicyclus* legs), and the exclusion of *dac* from the majority of the intermediate region in wings. *dac* is a broad, strongly expressed intermediate domain marker in the legs, and is the only broad-domain LGG that is not expressed in a comparable larval wing band. In the *Drosophila* wing disc, *dac* is expressed in the body wall or notum, and at the proximal-intermediate domain of the leg proper in a smaller cluster of cells. Interestingly, *dac* repression contributes to leg-to-wing transdetermination in *Drosophila* and to the activation of wing-specific selectors (Ing *et al.*, 2013). In these studies, ectopic *wg* expression in the dorsal leg pouch, as observed in wings, enabled broad and robust *dpp* expression in that region (Theisen *et al.*, 2007). As a result, *dac* expression was repressed, and *vg*, a wing selector gene, became expressed within its vicinity. It is possible, thus, that the lack of a concentric ring of *dac* in *Drosophila*, or a continuous AP band in *Bicyclus*, is a prerequisite for wing appendage specification.

While the expression of most strongly congruent genes is conserved between *Drosophila* and *Bicyclus*, both *ss* and *rn* have diverged in their expression across the two species. Expression of *ss* in *Bicyclus* wings and legs approximates *ss* expression in *Drosophila* antennae, but not legs (Setton *et al.*, 2017). *ss* is expressed in a proximal domain in *Bicyclus* wings and legs and in a distal domain in the *Drosophila* leg. In *Drosophila*, *ss* is activated by Dll (D. M. Duncan *et al.*, 1998) while being proximally repressed by *Sex combs reduced* in the T1 segment (Tsubota *et al.*, 2008), and Antennapedia in the T2 and T3 segments (D. Duncan *et al.*, 2010). Such proximal repression might not take place in *Bicyclus*, as sparse *ss* expression overlaps *hth* expression. In *Drosophila* antennae, *hth* activates *ss* in this proximal region, and this regulatory interaction might be conserved in *Bicyclus* legs and wings. In addition, *rn* expression in *Bicyclus* legs and wings displays higher congruency compared to the legs and wings of *Drosophila*. Both *Drosophila* and *Bicyclus* express *rn* in both intermediate and distal domains in wings, whereas this gene is restricted to the distal domain in *Drosophila* legs. Regardless of expression differences between the two organisms, these PD patterning genes display highly similar expression patterns along the PD axis of their respective organisms.

Conservation and novelty of PD patterning in legs and wing via changes in the deployment of *wg*, *dpp*, and *vn* morphogens

In the *Drosophila* leg and wing imaginal discs, several morphogenetic gradients are involved in establishing the PD axis, the most prominent being Wg and Dpp. In the leg, these two long-range morphogens are expressed in stripes on opposite dorsal and ventral domains of the disc (Fig. 1) and are jointly responsible for activating *Dll* in the centre of the disc, where concentrations are highest, while repressing *dac* and *hth* (Lecuit & Cohen, 1997). In the intermediate domain, Wg and Dpp concentrations decrease below a threshold that allows *dac*

expression, and *Dll* also directly promotes *dac* expression in a part of its distal domain (Estella *et al.*, 2012; Giorgianni & Mann, 2011). *hth*, becomes restricted to the proximal part of the disc where it is activated by high proximal levels of Tio (Wu & Cohen, 1999). In *Drosophila* wings, *wg* is highly expressed along the DV margin and peripheral wing disc domain, and *dpp* is strongly expressed at the centre of the wing pouch, along the anterior-posterior axis. Through combinatorial control, they also promote distal markers and repress proximal markers similar to the leg, though wing-specific PD patterning genes such as *vg* and *scalloped* take a more prominent role in distal domain specification (Tripathi & Irvine, 2022).

The order of the proximal and distal LGGs in legs remains the same in *Drosophila* and *Bicyclus*, despite major differences in the expression of PD patterning morphogens, such as *dpp* (Fig. 8). In *Bicyclus*, the interrupted stripe of *dpp* expression seems to more closely resemble the *dpp* expression of other holometabolous and hemimetabolous insects, such as *Tribolium* and *Gryllus*, respectively (Niwa *et al.*, 2000; Pechmann & Prpic, 2022). This could be because *dpp* plays a less active role in axis formation relative to *wg* in all these insects, as corroborated by previous studies (Jockusch *et al.*, 2000; Niwa *et al.*, 2000).

Vn is another early patterning short-range morphogen that is crucial for PD patterning in *Drosophila* legs but has a different role in wings. *vn* is expressed at the distal centre of the leg imaginal disc of *Drosophila*, activated by Wg and Dpp signals (Galindo *et al.*, 2002), and is responsible for tarsal differentiation (Newcomb *et al.*, 2018). High Vn concentrations promote the expression of LGGs like *al* and *Lim1* (Campbell, 2005), which together with broad-domain LGGs like *Dll* promote *rn* through *dac* (St Pierre *et al.*, 2002), and refine *BarH1* (Kojima *et al.*, 2000) and *ap* expression domains (Pueyo *et al.*, 2000). *vn* also plays an

additional role in regulating the medial fate in *Drosophila* leg discs (Newcomb *et al.*, 2018). In *Drosophila* wing discs, however, Vn is expressed dorsally and is responsible for notum and dorsal compartment fate (Wang *et al.*, 2000). Hence, the morphogenetic functions of Vn differ between the *Drosophila* leg and wing imaginal discs.

In *Bicyclus* appendages, *vn* is expressed in completely different regions along the PD axis as compared to *Drosophila*. *vn* is expressed at the distal tip of the legs but has a complex expression in the wing: across all interveins and in an anti-colocalised pattern with *dpp*, suggesting a role in vein development and later in eyespot formation. *vn* is additionally expressed in a proximal domain, in the hinge region, similar to *Drosophila* wings.

The differences in *vn* expression, and likely function, between *Bicyclus* appendages could explain the non-congruence of some LGGs between legs and wings. The non-congruent group of genes mostly consist of narrow-domain LGGs, which respond to upstream EGFR signalling ligands, such as Vn, above some concentration threshold. It is possible that genes such as *BarH1*, which are expressed at intermediate Vn concentrations in the *Drosophila* leg (Campbell, 2005), were not activated in the wing due to the strong, broad expression of *vn*.

Evidence for a conserved Holometabolan appendage-specific PD patterning network

A comparative analysis of LGG expression in *Drosophila* and *Bicyclus* appendages revealed that the majority of LGGs are present in an appendage-specific order. In the legs of both species, most LGGs display some degree of spatial congruence, with the exception of the narrow-domain LGGs *ss*, *apA*, and *BarH1*. In the wing, *BarH1* is, so far, the only incongruent gene across species, as there is currently no available leg and wing data for *Drosophila* for *ss*

and *Lim1*. Interestingly, while *al* is mostly conserved in the wings of both organisms, there is an additional notum/hinge expression region in *Drosophila* that is not well studied (Campbell & Tomlinson, 1998). Hence, overall, these data suggest an evolutionarily conserved Holometabolous PD patterning network.

Previous research in hemimetabolous insects and crustaceans also supports the presence of a partially conserved appendage-specific PD patterning network for ventral appendages. For instance, similarities in broad-domain LGG (*hth*, *exd*, *dac*, *Dll*) expression can be seen in the legs of hemimetabolous insect orders, such as Orthoptera and Hemiptera (Angelini & Kaufman, 2005), and also in the gnathopods (grasping appendages) of crustaceans (Bruce & Patel, 2020). Future work that supplements this dataset with the same narrow-domain LGGs tested here would provide deeper insight into the extent of conservation of the PD patterning network for ventral and also for dorsal appendages.

The striking spatial congruency of LGG expression across legs and wings suggests that the proximal-distal patterning scaffold is a deeply conserved regulatory module shared between ventral and dorsal appendage development. While this ancestral scaffold provides the foundational positional coordinates, the recruitment of wing-specific selectors, including *vg*, *scalloped*, *nubbin*, and *Zn finger homeodomain 2*, was likely instrumental in the evolutionary transformation of a leg-like program into a wing program (Tripathi & Irvine, 2022). Moving forward, functional studies will be essential to determine if these spatial parallels are governed by identical upstream morphogenetic inputs or represent a case of regulatory convergence.

Conclusion

Our characterisation of twelve prominent LGGs, two LGG-related genes, and three morphogens, across the embryonic legs and larval wings of *Bicyclus* demonstrates that the PD axis is built upon a deeply conserved regulatory scaffold. The high spatial congruence of broad-domain LGGs suggests that these ancient positional coordinates are largely reused to organise dorsal appendages, especially in the proximal and distal domain. However, the observed divergence in intermediate domains, and in narrow-domain LGGs and the increased complexity of morphogen landscapes (*dpp* and *vn*) reveal regulatory changes that may be required to generate lineage-specific innovations, such as the intricate patterns of veins unique to insect wings. While this work establishes a comprehensive overview of PD patterning in butterfly appendages, it also highlights major differences from the current Holometabolan model, *Drosophila*. Ultimately, our findings require additional comparative and functional studies for a complete understanding of appendage PD patterning across the Holometabola.

MATERIALS AND METHODS

Rearing *Bicyclus anynana* and embryo collection

B. anynana butterflies were reared in the lab at 27°C, 60% humidity and a 12-12 h day-night cycle. Larvae and adults were fed corn leaves and mashed bananas, respectively. Corn leaves were placed inside adult *Bicyclus* cages when they were most active to promote oviposition. All embryos were incubated at 28°C for 48 h prior to dissection.

Hybridisation chain reaction (HCR) v3.0

Ultra-thin glass needles were first prepared via a needle puller (Narishige, PC-10). The 48 h embryos' outer membrane was then punctured with the needle before being fixed in 1×phosphate-buffered saline (PBS) with 10% Tween® 20 (PBST) supplemented with 4% formaldehyde for 30 minutes. Following fixation, the outer membrane was removed using a Dumont #5 fine forceps (Dumont, 11254-20), and embryos were transferred to 1×PBST. Dissection of 5th instar larval wings followed a previously described protocol (Banerjee & Monteiro, 2020a) in 1×PBS under a Zeiss Stemi 305 microscope (ZEISS) before being transferred into 1×PBST with 4% formaldehyde for 30 minutes. After washing with 1×PBST, the HCR procedure for embryos and larval wings followed an adapted, formerly detailed protocol (Bruce *et al.*, 2021; Choi *et al.*, 2018), following all buffer recipes from the original protocol. After incubation with the detergent solution at 37°C for 30 mins, three 3-minute 1×PBST washes were performed before another two 3-minute washes with 5×saline-sodium citrate with 10% Tween® 20 (SSCT), all at room temperature. The preparation of 1 mL probe hybridisation buffer (PHB) was optimally supplemented with 0.02 µM of DNA stock solution for each target gene. The gene sequences are available in Appendix A. From the PHB incubation step onwards until mounting, the sample remains at 4°C. After overnight incubation with PHB, samples were washed five times with probe wash buffer, followed by twice with 5×SSCT before a second incubation with amplification buffer supplemented with 6 nM of each fluorescent hairpin probe (Molecular Instruments Inc.) for 4 h. After four 20-minute 5×SSCT washes were performed, the samples were mounted in 60% glycerol (in 1×PBS). For embryos, an additional two glass slides were used as a spacer to flank the samples, preserving their three-dimensional integrity. Embryo and larval wings were imaged using the FLUOVIEW FV3000 Confocal Microscope (Olympus Corporation).

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ACKNOWLEDGEMENTS

We would like to thank Tan Lu Wee for help with the lab management, and Tong Yan from the DBS-CBIS confocal facility for access and training for the Olympus FV3000 confocal microscope.