# Correlations between spatiotemporal changes in gene expression and apoptosis underlie wing polyphenism in the ant *Pheidole morrisi*

#### Seba Jamal Shbailat, Abderrahman Khila, and Ehab Abouheif\*

Department of Biology, McGill University, Avenue Docteur Penfield, Montreal, QC, Canada H3A 1B1 \*Author for correspondence (email: ehab.abouheif@mcgill.ca)

SUMMARY Wing polyphenism, which is the ability of a single genome to produce winged and wingless castes in a colony in response to environmental cues, evolved just once and is a universal feature of ants. The gene network underlying wing polyphenism, however, is conserved in the winged castes of different ant species, but is interrupted at different points in the network in the wingless castes of these species. We previously constructed a mathematical model, which predicts that a key gene brinker (brk) mediates the development and evolution of these different "interruption points" in wingless castes of different ant species. According to this model, brk is upregulated throughout the vestigial wing discs of wingless ant castes to reduce growth and induce apoptosis. Here, we tested these predictions by examining the expression of brk, as well as three other genes up- and downstream of *brk*—*decapentaplegic* (*dpp*), *spalt* (*sal*), and engrailed (en)-in the winged reproductive and wingless

#### INTRODUCTION

Genes interact in complex ways with their environment during development (Gilbert and Epel 2009). Polyphenism, which is the ability of a single genome to produce alternative phenotypes in response to environmental cues, is a good model for studying how these complex interactions can change during development and evolution (Nijhout 1994; Brakefield et al. 1996; Evans and Wheeler 2001; Abouheif and Wray 2002; West-Eberhard 2003; Smith et al. 2008; Moczek and Rose 2009). Wing polyphenism in ants is a dramatic case of polyphenism-depending on environmental cues, such as temperature and nutrition, the gene network that regulates wing development either produces wings in the queen or male castes or is "interrupted" at specific points to produce the wingless soldier and worker castes (Abouheif and Wray 2002). A gene in the network is "interrupted" in wingless castes when we observe the following changes relative to the winged castes: (1) changes in the spatial pattern of its expression (Abouheif and Wray 2002); and/or (2) changes in the level (downregulation, upregulation, or even complete absence;

soldier castes in the ant Pheidole morrisi. We show that expression of these genes is conserved in the wing disc of winged castes. Surprisingly, however, we found that brk expression is absent throughout development of the vestigial soldier forewing disc. This absence is correlated with abnormal growth of the soldier forewing disc as revealed by En expression and morphometric analyses. We also discovered that dpp and sal expression change dynamically during the transition from larval-to-prepupal development, and is spatiotemporally correlated with the induction of apoptosis in soldier forewing disc. Our results suggest that, contrary to our predictions, brk may not be a key gene in the network for suppressing wings in soldiers, and its absence may function to disrupt the normal growth of the soldier forewing disc. Furthermore, the dynamic changes in network interruptions we discovered may be important for the induction of apoptosis, and may be a general feature of gene networks that underlie polyphenism.

Nahmad et al. 2008); and/or (3) changes in the timing of expression.

Wing polyphenism evolved just once and is a universal feature of all ant species (Hölldobler and Wilson 1990). Because wing polyphenism is the same across all ant species, we previously predicted that the mechanism through which wings are suppressed in the wingless castes of all ant species would also be the same (Abouheif and Wray 2002). However, we discovered that although the network regulating wing development in Drosophila is conserved in the winged castes of different ant species, the same network is interrupted at different points in the wingless castes in these species (Abouheif and Wray 2002). Here, we focus on the ant Pheidole morrisi because its interruption points are not only different from those of other species, but are also strikingly different both between and within its wingless castes. P. morrisi is composed of a winged male caste and three different female castes: winged queens (Fig. 1A), wingless workers (Fig. 1B), and wingless soldiers (Fig. 1C). The winged male caste is determined genetically, whereas the three female castes are determined environmentally (i.e., by temperature, photoperiod,

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Fig. 1. Growth and patterning of wing discs in winged and wingless castes of Pheidole morrisi. (A) Winged adult queen, (B) wingless adult worker, and (C) wingless adult soldier. Scale bar is 1000 µm. (D) Presence of functional wing discs during the last larval instar of winged queens and males (arrowheads). (E) Absence of imaginal wing disc development in workers (asterisks). (F) Presence of vestigial forewing disc (arrowhead), and absence of hindwing disc development (asterisk) in soldiers. (G) Schematic diagram of Drosophila and Pheidole wing discs. White marks the pouch, whereas yellow marks the hinge, pleura, and notum in Drosophila and hinge region in Pheidole. A-P boundary is shown in green and dorsal-ventral (D-V) boundary in red. (H-J) A representation of the wing patterning network in Drosophila, and its expression in winged and wingless castes of P. morrisi (Cohen 1993; Abouheif and Wray 2002; Carroll et al. 2005). Green boxes indicate conservation of gene expression, whereas red boxes indicate interruption of gene expression in the wingless caste. (K) The Dpp-signaling pathway is initiated by binding of Dpp ligand to Punt-Thickveins (Tkv) receptor complex, which leads to the phosphorylation of the R-Smad, Mothers against Dpp (Mad) (Raftery and Sutherland 1999; Held 2002; Affolter and Basler 2007). Phosphorylated Mad (pMad) binds to the co-Smad Medea and then pMad/Medea complex enters to the nucleus (Raftery and Sutherland 1999; Held 2002; Affolter and Basler 2007). pMad/Medea complex with the cofactor Schnurri (Shn) binds to the silencer region of brk to repress its expression (Muller et al. 2003). This generates a gradient of Brk which is complementary to Dpp gradient (Campbell and Tomlinson 1999; Jazwinska et al. 1999; Minami et al. 1999). The repression of brk is the primary mechanism by which the Dpp-signaling pathway activates the downstream target genes that pattern detailed structures along the A-P axis of the wing, such as sal, optomotor-blind (omb), vestigial quadrant ( $vg^Q$ ), and daughters against dpp (dad) (Campbell and Tomlinson 1999; Jazwinska et al. 1999; Minami et al. 1999; Affolter and Basler 2007). Arrows represent activation and T-shaped lines represent repression.

and nutritional cues) through the action of Juvenile hormone (JH; Passera and Suzzoni 1979; Wheeler and Nijhout 1983; Abouheif and Wray 2002). As a consequence of this caste determination, winged and wingless castes show dramatic differences in wing development. Queen and male larvae possess two pairs of large fore- and hindwing discs (Fig. 1D) that will develop into two pairs of functional wings. The wing disc of winged castes differs from that of *Drosophila* because it is already folded along its dorsal/ventral margin (Fig. 1G). In wingless castes, however, minor worker larvae do not develop any visible vestiges of these fore- or hindwing discs (Fig. 1E),

whereas soldier larvae possess one pair of relatively large vestigial forewing discs, but no visible hindwing discs (Fig. 1F). We have shown previously (Abouheif and Wray 2002) that the expression of the network in *P. morrisi* is conserved in the wing disc of winged castes—expression of *Ultrabithorax* (*Ubx*), *extradenticle* (*exd*), *engrailed* (*en*), *wingless* (*wg*), *scalloped* (*sd*), and *spalt* (*sal*) is conserved relative to their expression in *Drosophila* during the last larval instar (Fig. 1H). In the worker caste, none of these genes is expressed (Fig. 1I) in the expected positions of the vestigial fore- and hindwing discs (Fig. 1E), as well as in the expected position of the vestigial

hindwing disc in soldiers (Fig. 1F). This indicates that in these vestigial primordia the network is interrupted at a much earlier stage in development. In the vestigial soldier forewing disc, however, even though the expression of *Ubx*, *exd*, *en*, *wg*, and *sd* is conserved relative to the expression of these genes in winged castes (Fig. 1J), the expression of *sal* is interrupted (Fig. 1J). *sal* is expressed normally in the region of the disc corresponding to the future wing hinge, but is absent in that of the wing pouch.

We predicted previously that the evolution of interruption points in the wingless castes of P. morrisi and other ant species may be mediated through a key node in the network (Nahmad et al. 2008). By "key node," we mean that the suppression of wing development in wingless castes is mainly achieved through a key gene in the network, which functions to reduce growth and induce apoptosis. The full or partial development of wings on individuals in the soldier or worker castes is likely to be detrimental to the colony as a whole, because any development of wings would interfere with their ability to perform tasks in the colony. The negative consequences of wing development in soldier and worker castes is supported by the complete absence of wing development in any wingless castes in the  $\sim$  14,000 extant species of ants. Expression of the key node should therefore be under strong stabilizing selection, and should not change in the wingless castes of ants, whereas genes upstream or downstream of the key node may evolve neutrally or through selection as long as they do not perturb the ability of the key node to suppress wings in the soldier or worker castes.

There are several lines of evidence based on comparative or developmental genetic data in ants and Drosophila that support the gene brk as a key regulatory node in the network (Nahmad et al. 2008). brk is directly upstream of sal (the only interruption point known in the *P. morrisi* soldier forewing disc; Abouheif and Wray 2002). Furthermore, brk is part of the Dpp-signaling pathway (Campbell and Tomlinson 1999; Jazwinska et al. 1999; Minami et al. 1999; Raftery and Sutherland 1999; Held 2002; Martin et al. 2004; Carroll et al. 2005; Affolter and Basler 2007; Schwank et al. 2008; Fig. 1K), which in Drosophila plays an important role in regulating growth, patterning, and apoptosis. Dpp and Brk form inverse gradients that modulate cell proliferation and growth of the wing disc (Schwank et al. 2008). These opposing effects on growth by the Dpp/Brk inverse gradients convert an inherently low cell proliferation rate in the medial region and an inherently high proliferation rate in the lateral regions into an even proliferation rate across the entire wing disc (Schwank et al. 2008). Changes in the activity of Dpp/Brk inverse gradients can affect the overall size of the wing disc and adult wing (Campbell and Tomlinson 1999; Jazwinska et al. 1999; Minami et al. 1999; Martin et al. 2004; Schwank et al. 2008), and can also induce apoptosis (Moreno et al. 2002). Finally, brk and not *dpp* is predicted to be the key node because when

both *brk* and *dpp* signaling are ubiquitously expressed at the same time, wing discs and adult wings become significantly reduced in size (Martin et al. 2004; Schwank et al. 2008). This indicates that *brk* has a dominant and negative effect on growth and that the Dpp-signaling pathway indirectly regulates growth of wing discs via the repression of *brk* (Martin et al. 2004; Affolter and Basler 2007; Schwank et al. 2008).

We constructed previously a mathematical model to predict how brk may affect target gene expression and growth of the soldier forewing disc (Nahmad et al. 2008). Simulations of the model show that a direct 5- or 10-fold upregulation of brk by an unknown activator relative to that in the disc of winged castes can have a significant effect on reducing the expression of the target gene sal, as well as reducing the overall size of vestigial discs. Simulations also show that indirect upregulation of brk through a 5- or 10-fold downregulation of dpp can also significantly reduce sal expression and overall disc size. Therefore, we predicted that the expression of *brk* would be upregulated throughout the vestigial soldier forewing disc. This can occur either directly through an unknown activator or indirectly through downregulation of *dpp* (or interruption of other genes in the network). Here, we tested whether or not brk is a key node in the network that when upregulated reduces growth and induces apoptosis by determining the expression of four genes as well as programmed cell death in soldier forewing disc in P. morrisi.

#### MATERIALS AND METHODS

#### Ant collection

Colonies of *P. morrisi* were collected in Long Island, NY, USA. Colonies were kept in plastic boxes with glass test tubes filled with water constrained by cotton, and were fed a combination of mealworms, crickets, and Bhatkar–Whitcomb diet (Bhatkar and Whitcomb 1970). The colonies were maintained at 27°C, 70% humidity, and 12 h day:night cycle.

#### Staging, measurement, and statistical analysis

The criteria in Wheeler and Nijhout (1981) were used to identify the last larval and prepupal stages for both winged castes and wingless soldiers. Zeiss Microscope and Axiovision software (Carl Zeiss Canada Ltd., Toronto, Ontario, Canada) were used to measure the size (surface area in mm<sup>2</sup>) of wing discs, compartments, and expression domains of brk, as well, as the size (body length in mm) of both larvae and prepupae of winged castes and soldiers. To compare the relative sizes of brk expression domains in the anterior and posterior, we used an index called the "brk AP ratio," which is the size of the posterior domain divided by the size of the anterior domain of brk expression for each disc we measured. We then calculated the mean ratio of all discs that we measured and called this ratio the "mean brk AP ratio." Similarly, to compare the relative sizes of the anterior and posterior compartments as marked by En expression, we used an index called the "compartment AP ratio," which is the size of the posterior compartment divided by

the size of the anterior compartment for each disc we measured. We then calculated the mean ratio of all discs that we measured and called this ratio the "mean compartment AP ratio." The correlation coefficient (r) was calculated using SPSS software.

#### Gene cloning and sequence analysis

A fragment of P. morrisi dpp was amplified using RT-PCR, and 5' and 3' regions were then cloned using RACE (Invitrogen Canada Inc., Burlington, Ontario, Canada). A fragment of P. morrisi brk was amplified from genomic DNA. This gene has no introns as confirmed through PCR using cDNA. All degenerate and specific primers and PCR conditions for both genes are listed in Tables S1 and S2. GenBank accession numbers for P. morrisi dpp is HM045782 and P. morrisi brk is HM045781. P. morrisi Dpp and Brk were aligned using ClustalW to their respective orthologs in other animals.

#### Gene expression analysis

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In situ hybridization for dpp, brk, and sal was performed using Digoxigenin labeled anti-sense probes based on the protocol in Tautz and Pfeifle (1989). Double in situ for dpp and brk was performed using dpp Digoxigenin-labeled and brk-Fluorescein labeled probes according to the protocol in Hauptmann (2001). Antibody staining for Engrailed protein was performed using the anti-Engrailed antibody (Patel et al. 1989) following the procedure in Patel (1994). To confirm that the absence of *dpp* and *brk* expression is real, and not an artifact, larvae of winged castes were stained within the same tube as soldier larvae as a control for the expression pattern in the wing disc. Also, the staining reaction was overdeveloped for larvae of both winged castes and soldiers to ensure that there is no weak signal present in the vestigial discs. The same controls were used for the expression of all genes/proteins examined in the vestigial wing discs of soldiers during larval and prepupal development.

#### **TUNEL** assay

TUNEL assay was conducted according to the protocol in the kit (In situ Cell Death Detection Kit, AP, Roche Diagnostics Laval, Quebec, Canada) with the following modifications: dissected larvae and prepupae were treated with proteinase K (50 mg/ml) for 3 min. Larvae and prepupae were then post-fixed in 4% formaldehyde for 20 min.

#### RESULTS

#### Expression of *dpp* and *brk* is conserved between winged castes of P. morrisi and Drosophila

We first determined whether or not the sequence and expression of *dpp* and *brk* are conserved in the wing disc of winged castes. We cloned a 1271-bp fragment of dpp and a 2245-bp fragment of brk from P. morrisi. Amino acid sequences alignments unambiguously identified these two fragments as P. morrisi orthologs of Drosophila dpp and brk (Fig. 2). We then used these fragments to analyze the expression patterns of *dpp* and *brk* mRNA in the wing disc of winged castes. The expression of *dpp* during late larval and prepupal development (Fig. 3, D-F) is similar to its expression in the wing disc of Drosophila (Fig. 3A). Early dpp expression starts as a circle in the middle of the disc (Fig. 3B). Throughout larval development, the circle-like pattern starts to elongate, forming a stripe along the anterior-posterior (A-P) boundary (Fig. 3, C-E). This stripe of dpp expression persists during the prepupal development, and a circle domain of expression develops above it (Fig. 3F). brk expression in winged castes (Fig. 3, J–L) is also similar to its expression in the wing disc of Drosophila (Fig. 3G). brk expression is restricted to the lateral region of the anterior and posterior of the wing hinge (Fig. 3H, arrowheads), as well as the upper peripheral region

A	o o o	Fig
X1_BMP_2	CRRHPLYVDFSDVGWNDWIVAPPGYHAFYCHGECPFPLADHLNSTNHAIVQTLVNSVN-T	of
Hs_BMP_2	CKRHPLYVDFSDVGWNDWIVAPPGYHAFYCHGECPFPLADHLNSTNHAIVQTLVNSVN-S	the
Pm_Dpp	CRRHPLYVDFADVGWNDWIVAPPGYDAFYCHGDCPFPLADHLNSTNHAIVQTLVYSTKPS	
Bm_Dpp	CQRRPLFVDFAEVGWSDWIVAPPGYEAYFCQGDCPFPLADHLNGTNHAIVQTLVNSVDPA	cie
Dm_Dpp	CRRHSLYVDFSDVGWDDWIVAPLGYDAYYCHGKCPFPLADHFNSTNHAVVQTLVNNMNPG	of
Tc_Dpp	CRRRQMYVDFGSVGWNDWIVAPLGYDAYYCGGECEYPIPDHMNTTNHAIVQSLVNSMKPK	D
	*:*: ::******.***** **.*::* *.* :*:.**:* ***:**	mi
	oo o o	op
X1_BMP_2	NIPKACCVPTELSAISMLYLDENEKVVLKNYQDMVVEGCGCR	-
Hs_BMP_2	KIPKACCVPTELSAISMLYLDENEKVVLKNYQDMVVEGCGCR	saj
Pm_Dpp	MVPKACCVPTALSSISMLYLDEGNRVVLKNYQDMAVLGCGCR	(B
Bm_Dpp	LVPKACCIPTQLSPISMLYMDEHNQVALKNYQDMMVMGCGCR	ter
Dm_Dpp	KVPKACCVPTQLDSVAMLYLNDQSTVVLKNYQEMTVVGCGCR	an
Tc_Dpp	EVPGPCCVPTQLGQMSMLYLGSDGSVILKNYKEMVVVGCGCR	ac
	:* .**:** *. ::***: * ****::* * *****	bi
-		
В		(H
Dm_Brk	KMGSRRIFTPHFKLQVLESYRNDNDCKGNQRATARKYNIHRRQIQKWLQCESNLRSSVAN	or
Tc_Brk	GIGSRRIFAPHFKLQVLDSYRNDADCKGNQRATARKYGIHRRQIQKWLQVEGNLRSSVGK	(T
Pm_Brk	HDKDCRQNQRATARKYGIHRRQIQKWLQCEEQLRNSVEN	Ĝ
Bm_Brk	RAGSRRIFPPQFKLQVLEAYRRDSQCRGNQRATARKFGIHRRQIQKWLQAEPALRAALLR	tifi
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Fig. 2. Comparative sequence analysis of Pheidole morrisi Dpp and Brk and heir respective orthologs in other speies. (A) Amino acid sequence alignment of the C-terminal region of P. morrisi Opp (Pm; HM045782) with the C-terninal region of its orthologs from Xenpus laevis (Xl; BC100164), Homo apiens (Hs; AF040249), Bombyx mori Bm; FJ572058), Drosophila melanogaser (Dm; M30116), and Tribolium castneum (Tc; NM\_001039451). (B) Amino cid sequence alignment of the DNA binding domain (DBD) of Pm Brk HM045781) with the DBD of its orthologs from Dm (NM\_078514), Tc TC000748), and Bm (BGIBM-GA007697). All the sequences were idenified by blast searches in NCBI, with the exception of Brk sequences from Tc and

Bm, which were identified by blast searches in BeetleBase and SilkDB, respectively. Open circles represent the position of the seven conserved cysteine residues in Dpp C-terminal region, and asterisks mark identical residues between species in Dpp and Brk alignments. of the wing pouch during larval (Fig. 3, H–K) and prepupal (Fig. 3L) development. In addition, the domain of *brk* expression is larger in the posterior relative to the anterior (Fig. 3, H–L). Interestingly, during earlier stages, the posterior domain of *brk* expression is much larger than that of the anterior (Fig. 3H). Therefore, the expression of both *dpp* and *brk* is dynamic and is conserved relative to that in the wing disc of *Drosophila*. Furthermore, double in situ hybridization shows that the



expression domains of *dpp* and *brk* do not overlap (Fig. 3O) suggesting that Dpp and Brk proteins may form inverse gradients (Fig. 3N) similar to those in *Drosophila* (Fig. 3M).

#### In winged castes, *brk* expression is correlated to the relative sizes of the anterior and posterior compartments

We showed previously that in the last instar wing disc the expression of En, which marks the posterior compartment, is conserved in winged castes relative to Drosophila (Abouheif and Wray 2002). Interestingly, we found that during early developmental stages, the larger posterior domain of brk expression (Figs. 3H and 4) is correlated to the smaller size of the posterior compartment as shown by En expression (Figs. 3Q and 4). Later on in development, the size difference between the anterior and posterior domains of brk expression is dramatically reduced during development and become more comparable in size (Fig 4). Furthermore, the size difference between the anterior and posterior compartments is also reduced during development and the compartments become comparable in size (Figs. 3, R-U and 4). This raises the possibility that in P. morrisi brk may play a conserved role in repressing growth in which the reduction or expansion of brk expression domains in the anterior and posterior compartments may lead to size differences between the two compartments.

### In the vestigial soldier forewing disc, *dpp* and *brk* expression are absent in the last larval instar

The key node model for the evolution of interruption points in wingless castes predicts that relative to winged castes, the

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Fig. 3. Expression patterns of dpp, brk, and En in the wing discs of
Drosophila and winged castes of Pheidole morrisi during larval and
prepupal development. (A) Schematic diagram depicting dpp
expression in the middle of the Drosophila wing disc. (B-F) dy-
namics of dpp mRNA expression in the wing disc of winged castes
during early larval (B, n = 13 wing discs analyzed), mid-to-late
larval (C–E, n = 12 wing discs), and prepupal (F, n = 5 wing discs)
development. (G) A schematic diagram depicting brk expression in
the lateral regions of Drosophila wing disc. (H-L) Dynamics of brk
mRNA expression in the wing disc of winged castes during early
larval (H, n = 16 wing discs), mid-to-late larval (I–K, n = 30 wing
discs) and prepupal (L, n = 6 wing discs) development. Arrowheads
represent brk expression in the lateral regions of the disc. (M-N)
Schematic diagrams depicting the inverse gradients of Dpp (pur-
ple)/Brk (red) expression in the wing disc of Drosophila (M) and
winged castes of Pheidole (N). (O) Double in situ hybridization of
dpp (purple) and brk (red) in the wing disc of winged castes (n = 6)
wing discs). (P) Schematic diagram depicting En expression in the
posterior compartment of Drosophila wing disc. (Q-U) Patterns of
En expression in the wing disc of winged castes during early larval
(Q, n = 3 wing discs), mid-to-late larval (R-T, n = 8 wing discs),
and prepupal (U, n = 2 wing discs) development. All discs were
taken at the same magnification (20 \times ) and are oriented so that
hinge is to the top and posterior is to the right.
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Fig. 4. Size differences between anterior and posterior brk expression domains are correlated to size differences between anterior and posterior compartments. The Y-axis indicates the mean (black bars) and standard deviation (gray line) of the brk A-P ratio, as well as the mean (gray bars) and standard deviation (gray line) of the compartment AP ratio. The definitions of these ratios are described in "Materials and Methods." X-axis indicates wing discs of winged castes that were sampled during three time points in development: early wing disc development (surface area of discs sampled during this time point was up to  $0.025 \text{ mm}^2$ ); mid-to-late larval development (0.050-0.075 mm<sup>2</sup>); and prepupal development (0.100 mm<sup>2</sup> and over); as well as soldier forewing discs sampled during late last larval (up to 0.020 mm<sup>2</sup>) and beginningto-mid prepupal (0.021 mm<sup>2</sup> and over) development. The number of wing discs of winged castes sampled for mean brk AP ratio during early larval, mid-to-late larval, and prepupal development is 9, 11,

and 6, respectively. The number of wing discs sampled for the mean compartment AP ratio during early larval, mid-to-late larval, and prepupal development is 3, 4, and 1, respectively. The number of soldier forewing discs sampled for mean compartment AP ratio during late last larval development is 2, and during beginning-to-mid prepupal development is 4.

expression of *brk* will be upregulated throughout the soldier forewing disc to reduce growth and induce apoptosis. Surprisingly, even though *dpp* is absent (compare Fig. 5A' with A), *brk* is not upregulated, but is also absent (compare Fig. 5B' with B). Interestingly, when compared with winged castes, the absence of *brk* expression remains correlated to the size differences between the anterior and posterior compartments (Fig. 4). En expression shows that the posterior compartment is slightly larger in size than that of the anterior (Figs. 5D' and 4).

The absence of *dpp* expression is consistent with the interruption of its downstream target gene sal in the soldier forewing disc. We found previously that relative to winged castes, the expression of *sal* is conserved in the hinge but absent in the pouch (Abouheif and Wray 2002). By examining a larger number of samples in this study, however, we found that sal expression is significantly downregulated in the pouch, and forms a weak and wide patch of expression that is displaced toward the anterior (Fig. 5C'). This result is consistent with Drosophila because the loss of both dpp and brk signaling does not result in the complete loss of sal. Instead, sal is weakly expressed because it receives direct input from other activators (del Alamo Rodriguez et al. 2004; Winter and Campbell 2004). As in the wing disc of winged castes (Fig. 5E), there is no significant apoptosis in the vestigial soldier forewing disc (Fig. 5E').

# In the vestigial soldier forewing disc, dynamic changes in *dpp* and *sal* expression are spatiotemporally correlated with the induction of apoptosis during prepupal development

During the transition from larval-to-prepupal development of the soldier forewing disc, we discovered that dpp and sal expression change dynamically, whereas *brk* expression remains absent (Fig. 5B"). Even though dpp expression is absent during the last larval instar (Fig. 5A'), it suddenly appears at the beginning of prepupal development as a circle domain of expression that is displaced toward the anterior side of the disc (Fig. 5A" and Fig. S1). As the disc increases in size, the domain of dpp becomes displaced even further toward the anterior (inset in Fig. 5A''). The sudden appearance of *dpp* expression also coincides with a change in the expression of sal. sal expression in the wing pouch is now stronger and well defined (Fig. 5C'') as compared with its expression during the last larval instar (Fig. 5C'). It forms an oblong circle that is displaced toward the anterior. In the Drosophila wing disc, the Dpp-signaling pathway directly activates sal (de Celis et al. 1996, 1999), and thus, in the soldier forewing disc, the appearance of *dpp* during the beginning of prepupal development may be responsible for the further activation of sal expression in the pouch.

Surprisingly, we discovered that dynamic changes in *dpp* and *sal* expression are spatially and temporally correlated

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with the induction apoptosis, even though we initially predicted that apoptosis would be induced through the upregulation of *brk*. Expression of *dpp* correlates with apoptosis in the wing pouch, where dpp and apoptosis overlap in a circle domain that is displaced to the anterior side (compare Fig. 5A" with E"), whereas *sal* expression correlates with the



induction of apoptosis in both the hinge and pouch (compare Fig. 5C" with E"). As disc development progresses during this stage, apoptosis diffuses further toward the anterior side of the pouch (inset in Fig. 5E"). En expression shows that the posterior compartment is slightly larger in size than the anterior (Figs. 5D" and 4).

Finally, at the end of prepupal development, the soldier forewing disc undergoes dramatic shape changes and becomes reduced in size (Fig. 6). Both *dpp* (Fig. 5A''') and *sal* (Fig. 5C''') expression domains significantly increase in size and are now expressed throughout the middle of the soldier forewing disc. Apoptosis begins to spread throughout the disc (Fig. 5E'''), and when the disc becomes greatly reduced in size, apoptosis spreads throughout the entire disc (inset in Fig. 5E'''). These patterns of apoptosis are consistent with those found in another *Pheidole* species (Sameshima et al. 2004). *brk* expression at this stage remains absent (Fig. 5B'''), whereas En expression remains confined to the posterior compartment (Fig. 5D''').

#### DISCUSSION

The network underlying wing polyphenism in ants is interrupted at different points in the wingless castes of different species, even though wing polyphenism evolved only once and is a universal feature of all ants (Abouheif and Wray 2002). Here, we tested whether or not the evolution of interruption points is mediated through a key node in the network. We predicted that brk is a key gene that would be upregulated throughout the vestigial soldier forewing disc to reduce growth and induce apoptosis. In the ant P. morrisi, we show that expression of brk, dpp, and sal is conserved in winged castes, but the expression of these genes is interrupted in the wingless soldier caste. Surprisingly, we found that in the vestigial soldier forewing disc: (1) brk is not upregulated but is instead absent throughout development; (2) interruption of dpp and sal expression change dynamically during the transition between larval and prepupal development; and (3) these dynamic changes in *dpp* and *sal* are spatially and temporally correlated to the induction of apoptosis.

#### *brk* may be interrupted independently of its upstream regulator *dpp* and its absence may be due to the absence of an unidentified activator

Our results suggest that the expression of the Dpp/Brk inverse gradients may be conserved in the wing disc of winged castes, whereas in the soldier forewing disc brk expression remains absent even though dpp expression changes dynamically during development. During the last larval instar, brk expression is not upregulated in the absence of *dpp*, and remains absent throughout development even though dpp expression suddenly appears and changes during the transition between larval and prepupal development. This raises the possibility that brk is being interrupted independently of dpp. Furthermore, in Drosophila, the presence of an unidentified activator(s) can maintain the ubiquitous expression of brk throughout the wing disc in the absence of *dpp* expression (Muller et al. 2003; Yao et al. 2008). Therefore, one possible explanation for the independent interruption of brk may be the absence of this unidentified activator(s) in the soldier forewing disc. Alternatively, the DNA binding sites of brk may be epigenetically modified to prevent its expression.

## Absence of *brk* expression may displace the A--P boundary toward the anterior in the soldier forewing disc

In the third instar *Drosophila* wing disc, *brk* functions to repress cell proliferation in the lateral regions to even out the rate of cell proliferation throughout the disc (Schwank et al. 2008). When *brk* is absent in the *Drosophila* wing disc, cell proliferation in the lateral regions significantly increases relative to the medial region (Schwank et al. 2008), and the overall size of the wing disc increases along it is A–P axis (Campbell and Tomlinson 1999; Martin et al. 2004; Schwank et al. 2008). In the wing disc of winged castes, the reduction in the size differences in the anterior and posterior domains of *brk* expression is correlated to the reduction in the size differences between the anterior and posterior compartments. This suggests that *brk* may play a conserved role in repressing growth such that the reduction or expansion of *brk* expression

**Fig. 5.** Differential expression patterns of *dpp*, *brk*, *sal*, En, and detection of apoptosis between the wing disc of winged castes and the vestigial soldier forewing disc in *Pheidole morrisi*. (A–E) Wing discs of winged castes during last larval development showing (A) *dpp*, (B) *brk*, (C) *sal* mRNA expression (n = 9 wing discs analyzed), (D) En protein expression, and (E) TUNEL assay to detect apoptosis (n = 5 wing discs). (A'–E''') Vestigial wing discs of soldiers. (A'–A''') *dpp* mRNA expression in last larval (A', n = 10 wing discs), beginning-to-mid prepupal (A'', n = 4 wing discs), and late prepupal (A''', n = 2 wing discs) development. (B'–B''') *brk* mRNA expression in last larval (B', n = 13 wing discs), beginning-to-mid prepupal (B'', n = 10 wing discs), beginning-to-mid prepupal (B'', n = 3 wing discs), beginning-to-mid prepupal (C'', n = 3 wing discs), beginning-to-mid prepupal (C'', n = 5 wing discs), and late prepupal (C'', n = 3 wing discs), and late prepupal (C'', n = 5 wing discs), and late prepupal (C'', n = 5 wing discs), and late prepupal (C'', n = 5 wing discs), beginning-to-mid prepupal (D'', n = 5 wing discs), beginning-to-mid prepupal (D'', n = 5 wing discs), beginning-to-mid prepupal (D'', n = 5 wing discs), and late prepupal (D'', n = 5 wing discs), beginning-to-mid prepupal (D'', n = 6 wing discs), beginning-to-mid prepupal (C'', n = 6 wing discs), and late prepupal (E'', n = 4 wing discs) development. Insets represent the progress of wing disc development at the corresponding stage. The magnification of all images is  $20 \times$ , and thus, the size differences between all discs are respected, with the exception of the insets, which are  $40 \times$ . All discs are oriented so that the hinge is to the top and posterior is to the right.

domains leads to size differences between the anterior and posterior compartments.

Therefore, we determined whether or not the absence of brk expression may affect the overall growth of the vestigial soldier forewing disc, as well as affect the size differences between the anterior and posterior compartments of this disc. If brk plays a conserved role in repressing growth in the soldier forewing disc, then the absence of brk should lead the soldier forewing disc to grow at a faster rate relative to the wing disc of winged castes, and would also lead to an expansion in the size of its posterior compartment relative to the wing disc in winged castes. Indeed, the soldier forewing disc has previously been shown to grow at a faster rate than the wing disc of winged castes (Wheeler and Nijhout 1981). However, the final size of the soldier forewing disc is smaller because it only starts to grow during the last larval instar, whereas the wing disc of winged castes start to grow early in larval life (Wheeler and Nijhout 1981). Furthermore, our results show that in late larval and beginning-to-mid prepupal soldier forewing disc the posterior compartment (as marked by En expression) becomes slightly larger in size than that of the anterior.

We then performed morphometric analyses to find comparable size ranges between soldier forewing discs and wing discs of winged castes. These analyses show that the size range



Fig. 6. The size range of early wing discs of winged castes is comparable to the size range of large soldier forewing discs. The surface area (mm<sup>2</sup>) of forewing discs of winged castes (black squares) was measured from the beginning of larval life to the end of the prepupal stage, whereas the surface area of forewing discs of soldiers (gray diamonds) was measured from the beginning of the last larval to the end of the prepupal stage. The average surface area  $(mm^2)$  of forewing discs of winged castes and soldiers is plotted as a function of body length (mm). Body length is significantly correlated to the size of wing discs of winged castes, (r = 0.647, P = 0.000), and to the size of soldier forewing discs (r = 0.483, P = 0.003). The area of overlap between the sizes of wing discs of winged castes and the sizes of soldier forewing discs is indicated by an oval. The wing discs of both queens and males were used to construct the curve for winged castes because our goal was to identify comparable ranges of wing disc size, and not to construct growth trajectories of queens or males relative to that of soldiers.

of the early larval wing discs of winged castes is comparable to the size range of large soldier forewing discs during last larval and beginning-to-mid prepupal development (Fig. 6). In the early wing disc of winged castes, where brk is expressed in a significantly larger domain in the posterior than in the anterior, the relative size of the posterior compartment is smaller than that of the anterior (Fig. 4). In contrast, the posterior is slightly larger in size than the anterior in the large soldier forewing discs during last larval and beginning-to-mid prepupal stage (Fig. 4). Therefore, our results suggest that the absence of brk may disrupt normal growth of the soldier forewing disc, such that its posterior compartment becomes larger in size relative to that of the early wing disc of winged castes. One possible outcome of this disruption in the soldier forewing disc may be that the A-P boundary is displaced toward the anterior, and may consequently explain the anterior displacement of *dpp* and *sal* expression as well as apoptosis. Although the absence of brk may disrupt overall growth and produce size differences of the anterior and posterior compartments of the soldier forewing disc, it remains unclear whether or not this disruption functions in the induction of apoptosis or in the suppression of wing development in wingless castes.

## Interruption of *dpp* and *sal* expression changes dynamically during the larval-to-prepupal transition

We used previously a single time point (during the end of the last larval stage) to identify interruption points in vestigial wing primordia in the wingless castes of different ant species (Abouheif and Wray 2002; Bowsher et al. 2007). We had therefore assumed that interruption points were static, and once genes in the network were interrupted they would remain so throughout development. Although the expression of brk remains absent throughout development, and fits our assumptions about the static nature of interruption points, we were surprised to discover that the expression of *dpp* and *sal* changes dynamically during development. These dynamic expression changes, which occur during the transition from larval-to-prepupal development, are associated with the small surge of ecdysone observed in holometabolous insects (Nijhout 1994; Truman and Riddiford 2002). In the absence of JH, this small surge of ecdysone is known to be responsible for reprogramming epidermal cells and imaginal discs, as well as terminating larval feeding and promoting premetamorphic behaviors like cocoon spinning (Nijhout 1994; Truman and Riddiford 2002). Therefore, in the soldier forewing disc of P. morrisi the small surge of ecdysone may play a role in dynamically changing *dpp* and *sal* expression. The dynamic changes of these genes should be considered as interruption points because they are more than just a delayed onset of their expression relative to the wing disc of winged castes. In the

soldier forewing disc, when the *dpp* expression domain suddenly appears during the prepupal stage it is shifted toward the anterior, which is unlike its expression domain in the middle of the wing disc of winged castes. Furthermore, the expression domain of *sal* in the wing pouch, unlike its expression in the wing pouch of winged castes, forms a light patch of expression that is displaced toward the anterior of the disc during the last larval stage. During the prepupal stage, at approximately the same time when *dpp* suddenly appears, *sal* expression becomes stronger and well defined and remains displaced anteriorly. Therefore, there is a change in both the timing and spatial pattern of *dpp* and *sal* expression relative to the wing disc in winged castes. The dynamic nature of interruption points may be a general feature of gene network architecture underlying polyphenism.

### The dynamic changes in *dpp* and *sal* expression may induce apoptosis in the soldier forewing disc

The spatial and temporal correlation between dpp|sal and apoptosis is striking and suggests that *dpp/sal* may play a role in the induction of apoptosis. It remains unclear, however, how *dpp/sal* would induce apoptosis in the soldier forewing disc. Several lines of evidence from Drosophila genetics indicate that disrupting the normal expression of *dpp* and *sal* can trigger apoptosis in the wing disc: first, in a process known as "morphogenetic apoptosis," discontinuities in the reception of dpp or wg signals activate the JNK pathway, and therefore apoptosis in cells on either side of the discontinuity (Adachi-Yamada and O'Connor 2002); second, in a process known as "extrusion," cells deficient for the *dpp* signal extrude from the cell layer as viable cysts and show abnormalities in cell shape and cytoskeletal organization (Gibson and Perrimon 2005; Shen and Dahmann 2005). These abnormalities are thought to generate mechanical stress between cells, which activates the JNK pathway and induce apoptosis (Gibson and Perrimon 2005; Shen and Dahmann 2005); and finally, the mispositioning of *sal* in the wing disc can negatively regulate two cell surface proteins called Tartan and Capricious. This disrupts cell-cell communication and induces apoptosis (Milan et al. 2002).

These processes may have evolved in *P. morrisi* such that dpp/sal induces apoptosis in the soldier forewing disc but not in the wing disc of the winged castes. The induction of apoptosis in the soldier forewing disc may be due to changes in the timing, level, or spatial pattern of dpp/sal expression. Changes in the timing of dpp/sal expression in the soldier forewing disc may be an important parameter for the induction of apoptosis, in which apoptosis is induced by the sudden appearance of dpp and further activation of sal during the beginning of the prepupal stage. Another possibility is that it is not necessarily the sudden appearance of dpp that induces apoptosis, but rather, it is the changes in the levels of sal expression induced by the

sudden appearance of dpp that induces apoptosis. Alternatively, the changes in the spatial pattern of dpp/sal expression may also be important. Apoptosis may be induced primarily by the displacement of dpp/sal expression domains toward the anterior when dpp suddenly appears and *sal* is further activated. If this is the case, then the absence of *brk* may play a role in the induction of apoptosis by displacing the A–P boundary, and consequently, the dpp/sal expression domains toward the anterior. It is also possible that dpp/sal induces apoptosis through a combination of any of the possible mechanisms above. In the absence of functional analyses of *brk*, dpp, and *sal*, however, we cannot distinguish between these or other possibilities. For example, apoptosis may be induced independently of dpp/sal expression either through ecdysone or through the presence or absence of a soldier specific factor.

#### CONCLUSIONS

Understanding the developmental and evolutionary mechanisms that drive the evolution of interruption points will take us one step closer to understanding the complex relationship between genotype, phenotype, and environment. Here, our results do not support the key node model (Nahmad et al. 2008), in which the evolution of interruption points are mediated through a key gene in the network. Two alternative models may better explain the evolution of different interruption points in the wingless castes of different ant species (Abouheif 2004; Nahmad et al. 2008): (1) the "neutral interruption points" model, in which interruption points may evolve neutrally through genetic drift. Under this model the interruption of genes in the network do not play any functional roles in the suppression of wings in wingless castes, and other independent pathways are activated to eliminate the vestigial wing discs through apoptosis; and (2) the "interruption through multiple nodes" model, in which multiple genes are interrupted and each play a role in suppressing wings. Under this model, each of these interruption points is under selection, and they all cumulatively maintain the suppression of wings in wingless castes. Although it remains unclear which of these models best fits our results, we favor the interruption through multiple nodes model because of the strong spatiotemporal correlation we discovered between the dynamic expression of *dpp/sal* and apoptosis. However, future studies will require comparative gene expression and functional data to distinguish between these models.

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#### SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

**Fig. S1.** Morphological and molecular markers for determining the orientation of wing discs and location of gene expression domains within wing discs. Before removing the wings discs from the cuticle of a specimen stained for the gene of interest (A), we align the wing and leg discs of this specimen

to the wing and leg discs of a specimen that has been stained with En (B). Once the position of the legs in these two specimens are aligned, we can then match the orientation of the wing disc stained for the gene of interest (A) with the orientation of the wing disc that has been stained with En (B). We can then infer the position of the posterior compartment in the wing disc that has been stained with the gene of interest (A), and determine the location of the expression domain of the gene of interest relative to the location of the posterior compartment. By comparing (A) and (B) we can determine where the posterior compartment lies relative to the expression domain of the gene of interest, which in our case is the domain of *dpp* expression in (A). The inset in (B) shows En expression in a vestigial soldier forewing disc that is at the same stage as the disc showing the dpp expression domain in (A). By comparing the position of the black dotted line in (A) with the position of white dotted line in the inset of (B), our results suggest that the dpp expression domain is displaced anteriorly in the vestigial soldier forewing disc.

**Table S1.** Degenerate and gene specific primers to amplify fragments from *P. morrisi dpp* and *brk* genes.

**Table S2.** PCR conditions to amplify fragments from*P. morrisi dpp* and *brk* genes.

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