Growth and Patterning are Evolutionarily Dissociated in the Vestigial Wing Discs of Workers of the Red Imported Fire Ant, *Solenopsis Invicta*

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ABSTRACT

Over the last decade, it has become clear that organismal form is largely determined by developmental and evolutionary changes in the growth and pattern formation of tissues. Yet, there is little known about how these two integrated processes respond to environmental cues or how they evolve relative to one another. Here, we present the discovery of vestigial wing imaginal discs in worker larvae of the red imported fire ant, *Solenopsis invicta*. These vestigial wing discs are present in all worker larvae, which is uncommon for a species with a large worker size distribution. Furthermore, the growth trajectory of these vestigial discs is distinct from all of the ant species examined to date because they grow at a rate slower than the leg discs. We predicted that the growth trajectory of the vestigial wing discs would be mirrored by evolutionary changes in their patterning. We tested this prediction by examining the expression of three patterning genes, *extradenticle*, *ultrabithorax*, and *engrailed*, known to underlie the wing polyphenism in ants. Surprisingly, the expression patterns of these three genes in the vestigial wing discs was the same as those found in ant species with different worker size distributions and wing disc growth than fire ants. We conclude that growth and patterning are evolutionarily dissociated in the vestigial wing discs of *S. invicta* because patterning in these discs is conserved, whereas their growth trajectories are not. The evolutionary dissociation of growth and patterning may be an important feature of gene networks that underlie polyphenic traits.


of juvenile hormone, which controls both queen determination (Robeau and Vinson, '76; Passera and Suzzone, '79) and worker caste in ants (Wheeler and Nijhout, '81b; Ono, '82; Wheeler, '90).

A striking characteristic of ant caste polyphenism is the absence of wings in worker ants and their presence in reproductives. The evolution of winglessness in the worker caste is one of the main factors contributing to the ecological and evolutionary success of ants in that it allowed a eusocial insect to colonize the soil and rotting vegetation in the ground (Holldobler and Wilson, '90). In contrast, the winged reproductive caste's main role is mating and dispersal. In the case of Solenopsis invicta, females can fly up to 1,000 ft in the air and as far as 12 miles before founding a nest (Vinson and Sorensen, '86; Taber, 2000). Such long flights have undoubtedly contributed to their efficiency as an invasive pest (Taber, 2000). Although these flights play a crucial role in the dispersal of S. invicta, virtually nothing is known about the development of wings in this pest species.

As in other holometabolous insects, wings in adult queen and male ants develop from imaginal discs present in the larvae. Although workers are wingless as adults, the larvae of all species examined to date grow vestigial wing imaginal discs that disappear before pupation (Dewitz, 1878; Wheeler and Nijhout, '81a; Sameshima et al., 2004). The shape and size of these vestigial wing imaginal discs are diverse. For example, vestigial wing discs in the genus Crematogaster appear as two pairs of small and elongated epidermal invaginations (Wheeler and Nijhout, '81a), whereas in the genus Pheidole, they appear as two large forewing discs in soldiers, and are small or completely absent in the minor workers (Wheeler and Nijhout, '81a; Abouheif and Wray, 2002; Sameshima et al., 2004). Interestingly, previous studies had failed to find worker vestigial wing discs in S. invicta (Petralia and Vinson, '80).

Variation in the size and shape of vestigial wing discs in the workers of different ant species is matched by the variation in the expression of the gene network underlying the wing polyphenism in ants. Abouheif and Wray (2002) characterized the expression of six ant homologs of Drosophila genes involved in wing-patterning, Ultrabithorax, extradenticle, engrailed, wingless, scalloped, and spalt, during imaginal disc development in the winged and wingless castes of four ant species. They discovered that this wing-patterning network is conserved in the winged queens and males of these species, but is interrupted at different points in the wingless castes. An “interruption point” is defined by the absence of gene expression in vestigial wing primordia. For example, a striking difference was found between the wingless castes of different ant species in the expression of engrailed, a gene that defines the posterior compartment in the wing imaginal disc (Cohen, '93). Engrailed is normally expressed in the vestigial wing discs of the ant Pheidole morrisi, but is absent from the vestigial wing discs of the ant Crematogaster lineolata and Formica nitidiventris (Abouheif and Wray, 2002). Discovering that the interruption points differ in the wingless worker castes of different ant species was unexpected because the wing polyphenism in ants evolved just once (Wilson, '87; Holldobler and Wilson, '90).

One possible explanation for this unexpected divergence of interruption points between worker castes of different ant species is that interruption points evolve when the worker caste demography evolves. Growth and patterning are considered related processes because a change in the size of a worker ant and its wing discs will likely result in changes in the patterning of those discs. In the ant species C. lineolata, the size distribution of workers is monomorphic with a narrow size range, meaning that all the workers within the colony are all of the same proportion and differ only slightly in size. In contrast, in the ant P. morrisi, the size distribution is completely dimorphic with two distinct castes, “soldiers” and “minor workers.” These two distinct castes show a striking pattern of allometry, meaning that the soldiers are disproportionately larger than the minor workers (Wheeler and Nijhout, '81a). Interestingly, Abouheif and Wray (2002) found that the two monomorphic species (C. lineolata and F. nitidiventris) with a narrow size distribution shared the same interruption point, whereas the dimorphic species (P. morrisi) was interrupted further downstream in the wing-patterning network. If the distribution of the worker caste plays an important role in the evolution of the shape and size of vestigial wing discs and interruption points, then examining a species with a worker caste distribution that is intermediate between the monomorphic and completely dimorphic species examined in Abouheif and Wray (2002), could reveal a novel relationship between the growth and patterning of vestigial wing discs.

S. invicta workers have a very broad size distribution with only the slightest sign of allometry between the largest and smallest workers.
(Wilson, '78; Wood and Tschinkel, '81). This broad range of worker size in S. invicta represents an intermediate type of worker caste demography. Thus, we predicted that growth and patterning of the vestigial wing discs in this species should be different from the growth and patterning of vestigial wing discs in monomorphic and completely dimorphic species.

In this study, we present the discovery and characterization of vestigial wing discs in worker S. invicta and compare their growth and patterning to that found in other ant species (Skellam et al., '89; Abouheif and Wray, '81a; Wheeler and Nijhout, '81). Vestigial wing discs in S. invicta were missed by previous studies most likely because of their consistently small size throughout development. We characterized both the growth and patterning of these discs to determine if there is an association between their growth trajectories and the points of interruption within the wing-patterning network.

**MATERIALS AND METHODS**

**Wing disc growth study**

We collected larvae from a single colony of S. invicta in April 2001 from the Croatan National Forest in Eastern North Carolina. We fixed the larvae according to Patel ('94) and stored them at 4°C in 100% MeOH. We randomly sampled 37 larvae from the colony, and measured their lengths and widths using Image 1 software (Universal Imaging Corporation; MDS Analytical Technologies, Toronto, Canada). We defined length as the longest distance from anterior to posterior, and width as the widest girth. Both measurements on individual larvae were taken while they were resting on their backs.

To better visualize the imaginal tissue, we stained the larvae with hematoxylin. We hydrated larvae in phosphate buffered saline (PBS) with 10% TritonX (PBS, 0.1% Triton X-100 (PBT)), and dissected them so that the imaginal discs were visible. We stained the larvae in 100% Harris modified hematoxylin for 30 sec. Excess stain was washed away with PBT. We cleared the larvae in 70% glycerol for at least 3 days and then mounted them on glass slides. Slides were photographed with a Nikon digital camera (Melville, NY) mounted on a dissecting scope. We measured the length and width of leg and wing imaginal discs using SigmaScan Pro Image Analysis 5.0.0 software (SPSS Inc., Chicago, IL), and determined the instar of each larva based on morphological characters described in Petralia and Vinson ('79).

Larvae in the third and fourth instars were represented in the sample. By the third instar, reproductives are easily differentiated from workers by their discs. The reproductive larvae have much smaller discs for their overall body size than workers.

Statistical analyses were carried out using SYSTAT (Systat Software Inc., San Jose, CA). We performed a “homogeneity of slopes” or “parallelism” test to determine whether growth rates of wing and leg discs were statistically equal between workers and reproductives (Sokal and Rohlf, '95).

**Colony surveys**

We overturned roadside anthills and randomly sampled larvae from four different colonies of S. invicta in November 2001 at the Croatan National Forest, NC. From these samples we randomly selected a subsample of at least 10 fourth instar worker larvae, and fixed and stored them in 100% MeOH at 4°C (Patel, '94). We rehydrated the larvae in ddH2O and dissected them for staining. We stained the larvae in 100% Harris modified hematoxylin for 30 sec. We removed excess stain with washes of ddH2O, and cleared the larvae in 70% glycerol. We scored each larva for the presence or absence of vestigial imaginal discs.

**Immunohistochemistry**

We investigated the expression patterns of three proteins: Extradenticle (Exd), Ultrabithorax/abdominal-A (Ubx/abd-A), and Engrailed (En), using antibodies for the protein products. We chose Exd, Ubx, and En because they have different developmental roles and occupy different positions within the wing-patterning pathway. Exd patterns the hinge region in the Drosophila wing and is expressed in the region of the wing discs that meets the cuticle wall (Gonzalez-Crespo and Morata, '95), whereas Ubx functions in haltere specification, and is expressed in the entire hindwing disc (Weatherbee et al., '98). En specifies the posterior compartment of the wing (Brook et al., '96). These antibodies have been used successfully in other insects (Cohen, '93; Carroll et al., '94; Weatherbee et al., '98, '99) as well as in other ant species (Abouheif and Wray, 2002).

We fixed and stained larvae according to the protocol of Patel ('94). We stored the fixed larvae in 100% MeOH at 4°C. We used the primary antibodies in the following concentrations: Exd (B11 M) 1:4 (Aspland and White, '97), Ubx/abd-A (Fp6.87) 1:10 (Kelsh et al., '94), and En (4D9) 1:4 (Patel et al., '89). The secondary antibody, goat anti-mouse immunoglobulin G conjugated to horseradish

peroxidase (Jackson ImmunoResearch Laboratories), was diluted to a final concentration of 1:300. Following incubation with the secondary for 2 hr at room temperature sections were washed 3 x 10 min in the PBT. Sections were incubated for 10 min a solution of DAB (3,3'-diaminobenzidine, Sigma, St. Louis, MO) with NiCl₂, and the stain was visualized by adding 3 μL of 0.3% H₂O₂. After staining was apparent, the sections were washed three times quickly with PBT, and mounted in 70% glycerol.

We used three types of control to confirm that any observed absence of expression is real and not artifact. First, other imaginal structures within the same animal served as internal positive controls. Second, we overdeveloped the staining reaction to ensure there was no weak signal present in the vestigial discs. Third, we stained reproductive larvae within the same tube as worker larvae as a control for expression pattern in the wing, as all three genes are expressed in wing discs of reproductives of other ant species (Abouheif and Wray, 2002).

RESULTS

Wing disc growth study

Wing discs in both workers and reproductives were visible at the beginning of the third larval instar and persisted until the end of the fourth. Wing discs in reproductives were significantly larger than those in workers, which were reduced to small pads of tissue (Fig. 1). We did not observe any discs in the second instar or late fourth instar larvae that were preparing to molt into the pupal stage.

Growth rates between leg discs of reproductive and worker castes were compared using the homogeneity of slopes tests (Sokal and Rohlf, '95) in SYSTAT (Systat Software Inc). The null hypothesis that slopes (growth rates) of leg discs from both castes are equal could not be statistically rejected (Fig. 2A; P = 0.220). Therefore, we concluded that leg discs of workers and reproducitives grow at comparable rates. In contrast, the wing discs of workers and reproductives grow at significantly different rates (Fig. 2). The null hypothesis that the slope of disc growth plotted against leg disc growth of workers (R² = 0.4725) is equal to that of reproductives (R² = 0.9175) was rejected (Fig. 2B; P > 0.0000).

Finally, the slope of the line representing the relative growth rate of vestigial worker wing
discs was significantly different from 0 (Fig. 2; 
$$P = 0.04$$). This indicates that vestigial worker 
wing discs do grow once they appear, but at a 
rate slower than the leg discs. This result is 
striking because in all other ant species examined 
to date, wing disc growth exceeds or keeps pace 
with leg disc growth in workers and reproductives 
(Skellam et al., '59; Wheeler and Nijhout, '81a).

**Colony surveys**

Four colonies were sampled to determine the 
proportion of worker larvae with vestigial wing 
discs. From these surveys we concluded that all 
worker larvae in the third and fourth instars 
possess vestigial wing discs.

**Immunohistochemistry**

Both reproductive and worker larvae were 
stained with antibodies for Exd, Ubx, and En 
proteins (Fig. 3). Exd, Ubx, and En expression in 
reproductive wing discs was consistent with the 
expression patterns in *Drosophila* and other ant 
species (Abouheif and Wray, 2002). Exd staining 
appeared in the hinge region of the wing imaginal 
disc (Fig. 3C and D), although Ubx was expressed 
in the hindwing discs in both worker and 
reproductive larvae (Fig. 3D and F). En was 
expressed in the posterior half of the reproductive 
wing discs (Fig. 3G).

In vestigial wing discs, expression patterns of Exd 
and Ubx were consistent with those of the repro-
ductive and the expectations from *Drosophila* (Fig. 
3D and F). However, En expression was absent in 
the vestigial wing discs of worker larvae (Fig. 3H). 
The absence of staining was not an artifact as En 
expression did appear in the leg discs and nerve 
ganglia of workers and reproductives.

**DISCUSSION**

We provide evidence for the existence of 
vestigial wing imaginal discs in worker larvae of 
*S. invicta*. Growth and patterning in these discs is 
characterized by four features: (1) vestigial wing 
discs are present in all worker larvae, and appear 
in the third instar and persist through the four 
until pupation; (2) although these vestigial wing 
discs do grow once they appear, they grow at a 
significantly slower rate than worker leg discs;

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**Fig. 3.** Gene expression in worker and reproductive leg and wing discs. (A) Adult reproductive ants have fully developed 
wings. Antibody staining in fourth instar reproductive ant larvae shows that expression patterns for (C) Extradenticle, (E) 
Ultrabithorax/abdominal-A, and (G) Engrailed are the same as those found in reproductives of other ant species (see Abouheif 
and Wray, 2002). (B) Worker larvae have vestigial wing discs, but workers are wingless as adults. In worker fourth instar larvae, 
expression patterns of (D) Extradenticle, and (F) Ultrabithorax/abdominal-A are the same in wing and leg discs as those 
patterns found in reproductive larvae (HW is missing in D). Expression differs in workers for (H) Engrailed in that Engrailed is 
not expressed in the wing discs. FW, forewing disc; HW, hindwing disc; L1, prothoracic leg disc; L2, mesothoracic leg disc; L3, 
metathoracic leg disc.
(3) gene expression in reproductive wing discs of *S. invicta* is conserved relative to reproductives from other ant species; and (4) the interruption of patterning in worker wing discs is similar to that found in ant species that possess a monomorphic and narrow worker size distribution.

Vestigial wing discs are present in all workers of the third and fourth instars. This timing of appearance is consistent with observations from *Formica pallidefulva*, *Pogonomyrmex badius*, and *Camponotus pensylvanicus* (Wheeler and Nijhout, ’81a). We observed that worker discs, both wing and leg, are already distinct from those of reproductives by the third larval instar, indicating that caste determination must occur before this point. This observation is important given that little is known about the timing of caste determination in *S. invicta*.

*S. invicta* is distinctive among the ants investigated to date because worker wing discs grow at a slower rate than the leg discs. Growth trajectories for the imaginal discs of *Pheidole bicornata* (Wheeler and Nijhout, ’81a) and *Myrmica rubra* (Skellam et al.,’59) show that growth between wing and leg discs is synchronized. Reproductive wing and leg disc growth in *S. invicta* conforms to the synchronized pattern found in other species. In workers, however, wing disc growth is stunted compared to the leg discs. Therefore, some aspect of the caste determination process in *S. invicta* dissociates worker vestigial wing discs growth from that of other imaginal discs. It is possible that a similar pattern of vestigial wing disc growth might be found in other species with similarly small wing discs, such as in the genus *Crematogaster*, but disc growth in such species has yet to be examined.

We predicted that the broad distribution of the worker caste and stunted growth trajectory of worker vestigial wing discs would cause an alteration in the interruption point of wing disc patterning, as controls of growth and pattern are usually closely integrated. However, the expression patterns of three protein products, Exd, Ubx, and En in worker wing discs indicate that, at least in this case, patterning and growth have been evolutionarily dissociated. In *S. invicta*, expression of the wing-patterning network in workers’ vestigial wing discs differs from that found in the wing discs of reproductives. On the basis of the expression patterns of Exd, Ubx, and En in reproductives’ wing discs, *Drosophila* and *S. invicta* reproductives show evidence of conserved wing patterning (Fig. 3C, E, G), which has been found also in reproductives of other ant species (Abouheif and Wray, 2002). The expression of Exd and Ubx in vestigial wing discs in worker larvae conformed to the expression patterns in functional reproductive wing discs (Fig. 3D and F). Because these two genes have the same expression pattern in worker vestigial discs as in reproductive wing discs, the wing-patterning pathway is at least partially activated in vestigial discs. However, the expression pattern of En differed between reproductive and worker wing discs. En was not expressed in worker vestigial wing discs, indicating the patterning pathway is interrupted. Interruption of *en grailed* expression has also been observed in *F. nitidiventris* and *C. lineolata* (Abouheif and Wray, 2002) indicating that the same interruption point is shared among different ant species with different caste distributions in workers. For example, *S. invicta* shows a unimodal caste distribution with a large size range of workers, whereas both *F. nitidiventris* and *C. lineolata* are unimodal, but show a small size distribution. We expected that this difference in the size range of the worker caste and growth rate of the vestigial discs in *S. invicta* would result in a unique interruption point for wing patterning. However, vestigial wing discs in all three species share the same interruption point.

This evolutionary dissociation in the growth and patterning of vestigial discs indicates that the “extrinsic” control of organismal size, caste distribution, and wing disc growth are decoupled from the “intrinsic” control of wing disc growth (Truman et al., 2006). Extrinsic growth is controlled largely by nutritional (Nijhout, 2003) and hormonal (Robeau and Vinson, ’76; Wheeler, ’90) signals, whereas intrinsic growth is controlled largely by the expression of transcription factors or signaling molecules, such as *decapentaplegic*, a wing-patterning gene that affects organ size (Crickmore and Mann, 2006). A loose association between the factors controlling extrinsic and intrinsic growth of worker size and vestigial wing disc growth may explain why an evolutionary change in caste distribution did not result in an evolutionary change in the interruption of the wing-patterning network in the wingless worker caste in *S. invicta*.

Future studies documenting the growth and patterning of vestigial discs in several ant species will be needed to understand the nature of this dissociation. It will also be important that future studies focus their attention on the proximate pathways that may connect extrinsic and intrinsic
controls of growth of imaginal discs, such as the insulin signaling pathway (Emlen and Allen, 2004; Emlen et al., 2006). This will bring us one step closer to understanding the complex relationship between the environment, development, and evolution.

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