Differential expression patterns of the hox gene are associated with differential growth of insect hind legs

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Diversification of leg appendages is one of the hallmarks of morphological evolution in insects. In particular, insect hind (T3) legs exhibit a whole spectrum of morphological diversification, ranging from uniform to extremely modified. To elucidate the developmental basis of T3 leg evolution, we have examined the expression patterns of two homeotic genes, *Ultrabithorax* and *abdominal-A* (collectively referred to as *UbdA*), in a broad range of species. First, our results show that *UbdA* expression in hemimetabolous insects is localized only in specific T3 leg segments undergoing differential growth (compared to their foreleg counterparts). In contrast, in basal hexapod and insect lineages, the absence of the *UbdA* signal coincides with uniform leg morphology. The same situation exists in first instar larvae of holometabolous insects, in which absence of *UbdA* expression in the embryonic T3 legs is associated with the lack of larval T3 leg diversification. Second, there is a clear difference in the timing of expression between species with greatly enlarged T3 leg, such as crickets and grasshoppers, and species that exhibit more moderate enlargement of hind legs, such as mantids and cockroaches. In the former, the *UbdA* expression starts much earlier, coinciding with the elongation of T3 limb buds. In the latter, however, the *UbdA* expression starts at much later stages of development, coinciding with the establishment of distinct leg segments. These results suggest that diversification of insect hind legs was influenced by changes in both the *spatial* and *temporal* regulation of the *UbdA* expression.

As a result of advancements in the past two decades, the establishment of basic features of animal morphology can largely be understood as the product of an elaborate molecularly encoded developmental program. The emerging view indicates that many of the principal genes of this program are conserved even among organisms as distantly related as insects and mammals. These findings further suggest that morphological evolution is not governed by completely different developmental pathways that evolved independently in each lineage. Instead, changes in body forms likely resulted from changes in the common developmental processes and mechanisms (1, 2). At present, we are just beginning to elucidate exactly how and to what degree variation at the developmental level affects variation at the morphological level (3).

The combination of extreme morphological diversity and a strongly conserved bauplan, which is organized in repetitive modules such as segments, makes insects a particularly well suited group in which to study the evolution of development (4). Hexapoda, the taxonomic term for insects, implies the possession of three pairs of legs, which is one of the unifying characters of this group. One pair of legs originates from each of the prothoracic (T1), mesothoracic (T2), and metathoracic (T3) segment. The conserved ground plan of the insect leg includes six segments, which in proximal–distal direction are identified as coxa, trochanter, femur, tibia, tarsus, and claws. Whereas the number and arrangement of these segments is highly conserved, their relative size and functional morphology is diverse, reflecting different adaptive responses. Among the most striking cases of leg diversification are the jumping legs of orthopteran insects such as crickets and grasshoppers, which originate from the T3 segment and can be two times longer than the anterior legs. However, basal insect lineages such as collembolans and thysanurans exhibit a uniform size and structure of all three leg pairs. Also, the larvae of higher holometabolous insects have reduced thoracic leg appendages that are morphologically very similar to each other. Thus, insect hind legs exhibit the whole spectrum of morphological diversification, ranging from uniform to extremely modified. This leads to the intriguing question of what are the actual molecular mechanisms that govern the morphological evolution of insect hind legs.

During animal development, the homeotic selector genes play a major and conserved role in regulating the assignment of different identities to cells along the anteroposterior axis. Because of their potential to affect morphology of an organism, hox genes have been used as molecular markers for studying evolution of animal body plans, especially in arthropods (5–8). In *Drosophila*, the homeotic gene *Ubx* has two main functions. Whereas early *Ubx* expression initiates abdominal development, at later stages *Ubx* is also required for proper development of the T3 segment including the leg and wing appendages (9–11). In addition, a recent study of related *Drosophila* species shows that differential *Ubx* expression is directly regulating the fine-scale morphological differences (such as bristle pattern) between T3 and T2 legs (12). Interestingly, the putative role of *Ubx* in the evolution of grasshopper hind legs has also been postulated on the basis of its expression pattern in this species (13). These results prompted us to hypothesize that instead of being restricted to only a couple of species, *Ubx* may actually play a role in diversification of insect hinds legs in general. If correct, our hypothesis would predict that *Ubx* expression should be consistently associated with T3 leg modifications.

To detect *Ubx* expression in the hind leg, we use the cross-reacting mouse monoclonal antibody FP6.87 (13). This antibody recognizes both the *Ubx* protein and that of the second abdominal homeotic regulator *abdominal-A* (*abd-A*) in a wide range of arthropod species (13–17). Thus, the observed expression is a composite pattern that includes both the *Ubx* and *abd-A* genes (collectively referred to as *UbdA*). Because *abd-A* expression in insects does not extend beyond the first abdominal segment, it is generally accepted that the thoracic expression detected with this antibody is solely *Ubx* expression (9, 13, 18–22). Here, we examine the *UbdA* pattern in a broad range of insects, each of which exhibits a distinct, species-specific hind leg-specific morphology. In each instance, we find that *UbdA* expression is localized specifically in the T3 leg segments that are morphologically different from their T1 and T2 leg counterparts. These results suggest that changes in *UbdA* expression may indeed be a general mechanism governing the evolution of insect hind legs.

Materials and Methods

The laboratory cultures of firebrats (*Thermobia domestica*) and crickets (*Acheta domesticus* and *Gryllus firmus*) were reared

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under conditions previously established by Rogers *et al.* (23) and Peterson *et al.* (19). The egg cases of the cockroach (*Periplaneta americana*), praying mantis (*Tenodera aridifolia*), and green lacewing (*Chrysoperla carnea*) were purchased from Carolina Biological Supply and Buglogical Control Systems (Tucson, AZ) and were used to establish and replenish the laboratory cultures. The grasshopper (*Schistocerca americana*) and field cricket (*Gryllus firmus*) embryos were gifts from Markus Friedrich (Wayne State University) and Anthony Zera (University of Nebraska, Lincoln), respectively. For all species, a portion of the embryos was separated and allowed to hatch into first nymph (for hemimetabolous insects) or first instar (for holometabolous insects) stages. Legs of these first nymphs and first instars were then dissected and photographed by using a Leica MZ 12.5 microscope.

Embryos were dissected from their chorions and extraembryonic membranes, fixed in 4% formaldehyde in PBT for 30 min, and stored in MeOH at −20°C. The only exception was the early limb bud stage embryos, which were fixed for 20 min only. *UbdA* expression was detected by using the mouse monoclonal antibody FP 6.87 (13), generously provided by R. White (University of Oxford); the antibody was detected by using a secondary (anti-mouse) antibody conjugated to FITC or horseradish peroxidase (The Jackson Laboratory). Immunohistochemical staining was performed according to the protocols described in refs. 13 and 24. In the cases when FITC-conjugated anti-mouse antibody was used, embryos were labeled with propidium iodide (PI) at a concentration of 5 μg/ml in PBT for 20 min. Imaging was carried out on Leica TCS SP2 confocal and Zeiss Axiophot microscopes. Detailed protocols on maintaining insect cultures, collection of embryos, and antibody staining and immunohistochemistry are available on request.

Results and Discussion

***UbdA* Expression Patterns in Insects with Greatly Enlarged Hind Legs.** Firebrat *T. domestica* belongs to a primitively wingless lineage (Thysanura), which is also considered a basal insect group (25). Adult firebrats have very similar legs, providing this species with excellent running abilities. We also dissected the legs of just hatched animals, which is equivalent of first nymph stages in hemimetabolous insects (firebrats are ametabolous species; they hatch as miniature adults). Focusing on just hatched animals allows us to establish the direct link between a resulting leg morphology and embryonic gene expression pattern. As illustrated in Fig. 1*A*, the morphology and overall size of hind legs is almost the same as that of T2 legs. In the early firebrat embryo, the anterior expression of *Ubx* is restricted to several cells at the base of the T3 and T2 limb buds, but not in the buds themselves (data not shown; ref. 19). As these limb buds begin to elongate (Fig. 1*B*), they exhibit no *UbdA* expression. This pattern continues throughout mid and late embryogenesis (Fig. 1 *C–D*). From early to late stages, then, there is a complete absence of *UbdA* expression in the developing T3 legs. A similar pattern was also observed in springtails, another wingless group, which are considered an outgroup to true insects (26). Thus, the high similarity between T3 legs and T1/T2 legs in basal insect lineages is associated with the absence of *UbdA* expression in hind legs.

In contrast to firebrats, orthopterans (grasshoppers and crickets) represent a prime example of species with differentially enlarged hind legs. For example, in the first nymphs of grasshopper *S. americana*, the hind legs are more than twice the size of their foreleg counterparts (Fig. 1*E*). Note that this increase is due to the differential enlargement of only two T3 leg segments, femur and tibia. Interestingly, this extensive morphological modification of the T3 tibia and femur is tightly associated with strong *Ubx* expression throughout development (Fig. 1 *F–H*) (13). At early germ band stages, when legs just begin to elongate, *UbdA* expression is localized in the large region of the distal half of T3 legs (Fig. 1*F*). However, *UbdA* expression is absent from the distalmost region of hind legs. As legs continue to elongate, the *UbdA* pattern expands proximally (Fig. 1*G*) and now encompasses the whole distal half of T3 legs (except the distal most region). As development progresses, distinct leg segments can now be recognized, and this is when *UbdA* expression becomes localized to T3 femur and tibia (Fig. 1*H*). Note the difference between a strong *UbdA* expression in enlarged tibia and complete absence of its expression in adjacent normal size tarsus (Fig. 1*H*). Thus, compared to firebrats, the grasshopper *UbdA* expression pattern exhibits two key differences. First, the specific T3 leg expression starts early (at the limb bud stage) and continues to expand through mid and late development. Second, as leg segments begin to differentiate, the expression becomes localized to greatly enlarged T3 femur and tibia.

To test whether the grasshopper pattern is unique to *Schistocerca* or whether it is representative of orthopterans in general, we also examined *UbdA* expression in a cricket *A. domesticus*. The legs of first nymphs in crickets exhibit a typical orthopteran trend toward greatly enlarged T3 leg femoral and tibial segments (Fig. 1*I*). However, in *Acheta* the tarsal segment is also enlarged. Therefore, the differential growth of T3 legs in *Acheta* displays both the similarities and a distinct difference, when compared to grasshoppers (Fig. 1 *E* and *I*). At the early germ band stage of cricket development, the *UbdA* is expressed as a wide ring in the middle of the T3 limb bud (Fig. 1*J*). As limb buds begin to elongate, the expression also expands in both proximal and distal direction (Fig. 1*K*). Finally, as leg segments become visible, the *UbdA* expression becomes localized to femoral and tibial T3 leg segments (Fig. 1*L*). In addition, the expression is also present in the proximal portion of the T3 tarsal segment. Note the similarities and differences between *Acheta* and *Schistocerca* at this stage (Fig. 1 *L* vs. *H*). In both species, the *UbdA* expression is localized specifically in T3 femur and tibia. However, the cricket expression also expands in the proximal region of the T3 tarsus. This enlargement of T3 tarsus in *Acheta* first nymphs is a result of increased size of only the first (proximal) tarsal subsegment (Ta1), whereas the second (Ta2) and third (Ta3) subsegments are similar in length to their T2 counterparts (Fig. 1*M*). Coincidentally, at the very late stages of development, the *UbdA* expression becomes localized in the elongated Ta1 subsegment only (Fig. 1*M Lower*). In the other two tarsal subsegments, the *UbdA* signal is either barely visible (as in Ta2; yellow arrowhead, Fig. 1*M*) or completely absent (as in Ta3; open arrowhead, Fig. 1*M*).

To further examine the observed association between the *UbdA* expression and differential enlargement of hind legs in orthopterans, we also included a related cricket species, *G. firmus*, in our study. The leg morphology of *Gryllus* first nymphs is similar to the situation observed in other crickets, characterized by a greatly enlarged T3 femur, tibia, and tarsus (Fig. 1*N*). The *UbdA* expression is also reminiscent of the previously observed patterns, with an early expression in the distal half of the T3 legs and subsequent expansion in proximal direction (Fig. 1 *O* and *P*). As leg segments begin to differentiate, the *UbdA* expression becomes localized to femoral, tibial, and tarsal T3 leg segments only (Fig. 1*Q*). Note, however, that in addition to their similarities, the two crickets also exhibit a key morphological difference. Whereas only the most proximal T3 tarsal subsegment (Ta1) is elongated in *Acheta* (Fig. 1*M Upper*), all three T3 tarsal subsegments (Ta1, Ta2, and Ta3) are enlarged in *Gryllus* (Fig. 1*R Upper*). Coincidentally, the *UbdA* expression in *Gryllus* also expands into Ta2 and Ta3 subsegments of hind legs (Fig. 1*R*, yellow and open arrowheads). This expression is very strong (compare the strength of the signal in Ta2 and Ta3 subsegments between *Gryllus* and *Acheta* in Figs. 1 *M* and *R*) and is directly associated with the elongation of these additional tarsal subsegments in *Gryllus*. Overall, these results show that *UbdA* expres-

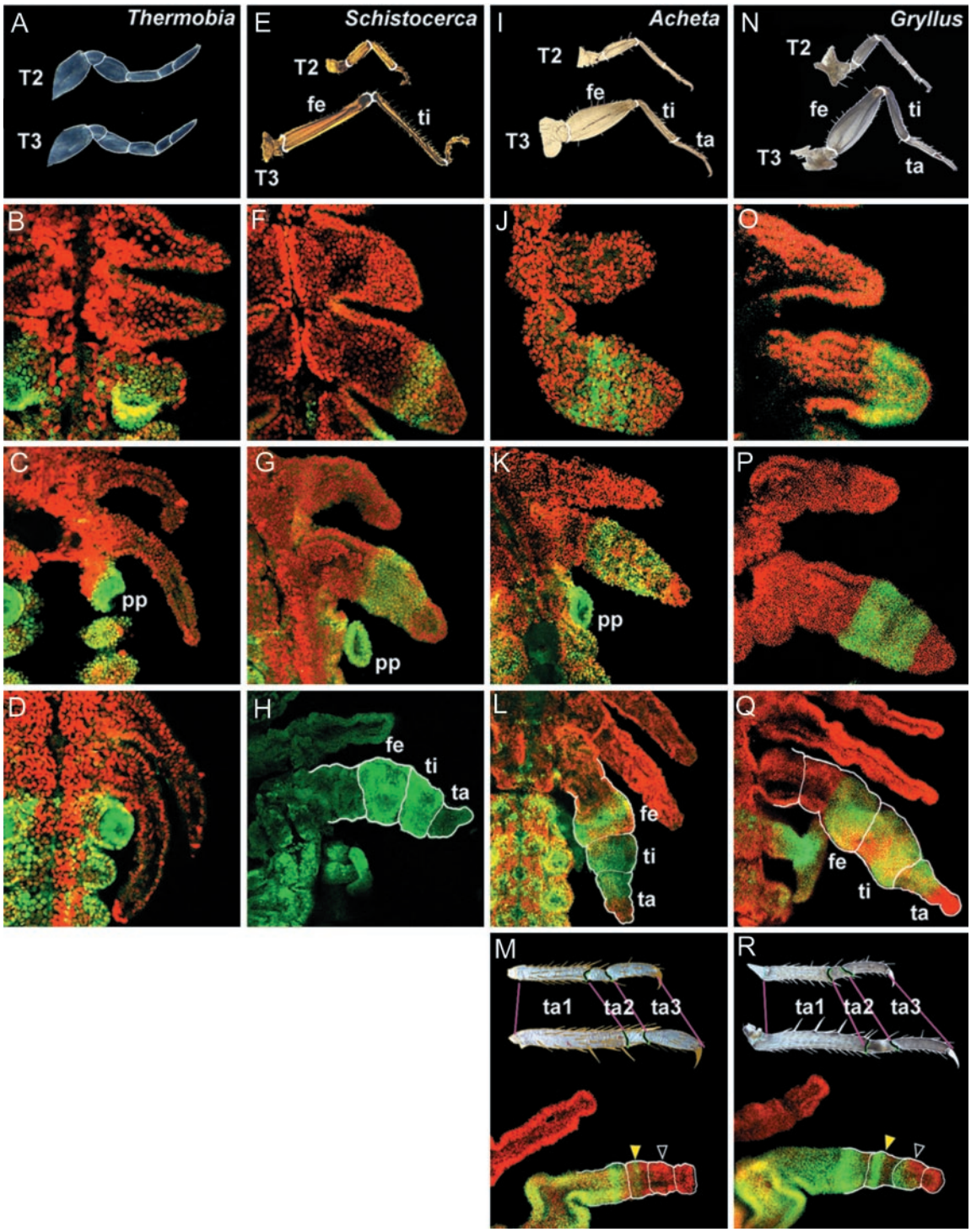

Fig. 1. Early onset of differential *UbdA* expression in orthopterans is associated with the great enlargement of hind legs. (*A–D*) Firebrat, *T. domestica*. (*E–H*) Grasshopper, *S. americana*. (*I–M*) House cricket, *A. domesticus*. (*N–R*) Field cricket, *G. firmus*. For each species, the dissected whole T2 and T3 legs of corresponding first nymph stages are shown at the top of each column (*A*, *E*, *I*, and *N*). This is followed by a progression of embryonic stages, from early to mid to late development (*B–D*, firebrat; *F–H*, grasshopper; *J–M*, house cricket; *O–R*, filed cricket). In addition, *M* and *R* display the magnified dissected tarsal segments (*Upper*) and corresponding embryonic tarsal *UbdA* expression (*Lower*) in the two cricket species. Herein, the yellow arrowhead points to tarsal subsegment 2, and the open arrowhead points to tarsal subsegment 3. Embryos were stained with FP6.87 antibody (green) against UBX and ABD-A proteins and propidium iodide (red). T2–3, legs of the second and third thoracic segment; A1, first abdominal segment; fe, femur; ti, tibia; ta, tarsus; ta1–3, tarsal subsegments 1–3; pp, pauropodia (appendages of the first abdominal segment).

sion in differentially enlarged T3 leg segments exhibits a species-specific pattern, supporting the general role of *Ubx* in evolution of hind legs in orthopterans.

***UbdA* Expression in Insects with Moderately Enlarged Hind Legs.** Results of our study of grasshoppers and crickets raise the question as to whether changes in the *UbdA* patterns may play

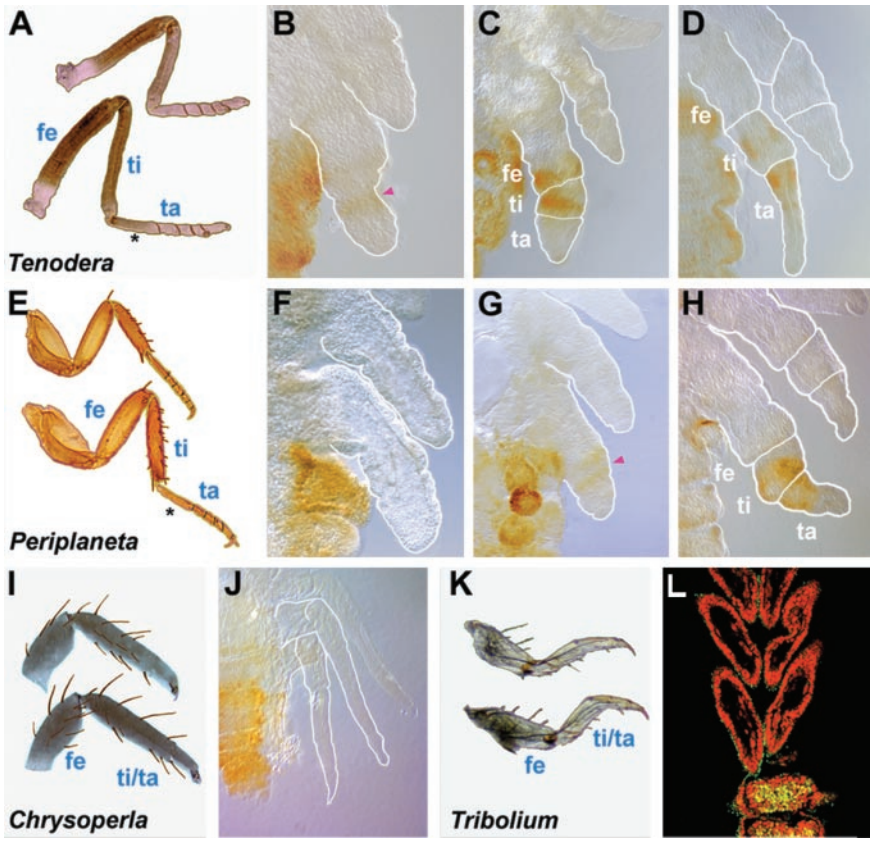

Fig. 2. Late start of *UbdA* expression is associated with moderately enlarged hind legs, whereas absence of the *UbdA* expression coincides with the uniform leg morphology. (*A–D*) Mantis, *T. aridifolia*. (*E–H*) Cockroach, *P. americana*. (*I–J*) Green lacewing, *C. carnea*. (*K–L*) Flour beetle, *Tribolium castaneum*. T3 legs are shown at the bottom. For hemimetabolous species (mantids and cockroaches), dissected T2 and T3 legs of first nymph stages are displayed in *A* and *E*. This is followed by a series of embryonic stages, from mid to late development (*B–D*, mantis; *F–H*, cockroach). For holometabolous species (green lacewings and flour beetles), the dissected T2 and T3 legs of first instar larvae are shown in *I* and *K*, followed by corresponding late embryonic stages in *J* and *L*, respectively. Embryos were stained with FP6.87 antibody (brown). In *L* only, the embryo is double stained with both FP6.87 (green) and propidium iodide (red). Red arrowhead points to early expression, which precedes leg segmentation. An asterisk in *A* and *E* denotes the first tarsal subsegment. T2–3, second and third thoracic leg; fe, femur; ti, tibia; ta; tarsus; ti/ta, fused tibial and tarsal segment in first instars of holometabolous insects.

a general role in the evolution of insect hind legs. To examine further this possibility, we focused on *T. aridifolia* (praying mantis) and *P. americana* (cockroach), two hemimetabolous species that exhibit distinctively different leg morphology from that observed in orthopterans. More specifically, in *Tenodera* and *Periplaneta*, the degree of the enlargement is lesser, ≈25–30% (compared to 60–70% in orthopterans). In addition, mainly tibia and tarsus are enlarged in cockroaches and mantids, compared with the femur and tibia enlargement found in orthopterans.

As shown in Fig. 2*A*, the principal T3 leg segments that are elongated in *Tenodera* first nymphs are tibia and tarsus. However, of the four tarsal subsegments, only the first one (Ta1) is actually enlarged. In addition, the T3 femur is also slightly longer than its corresponding T2 counterpart. We found that, as limb buds begin to elongate through the mid stages of development, there is a complete absence of *UbdA* expression in T3 legs. It is only toward the end of mid stage of development that we can observe a very faint *UbdA* expression in the middle part of an appendage (arrowhead, Fig. 2*B*). Note, however, that at this stage the T3 and T2 legs are the same in size. It is only later, after the legs become segmented, that we can observe a strong *UbdA* signal in the distal region of T3 femur and throughout T3 tibia (Fig. 2*C*). Then, as development proceeds, the *UbdA* expression in the femur disappears (Fig. 2*D*). At the same time, however, there is a continuous expression in the tibia as well as an expression in the proximal tarsus (corresponding to first tarsal subsegment, Ta1). Therefore, the expression patterns of *UbdA* in *Tenodera* are also directly associated with differentially enlarged T3 leg segments.

The T3 leg morphology of first nymphs in *P. americana* (cockroach) is similar to that observed in praying mantis, characterized by enlarged tibia and first tarsal subsegment (Fig. 2*E*). The main difference between the two species is that the T3 femur in *Periplaneta* is of approximately the same size as its foreleg counterparts. Thus, in this situation we would expect corresponding similarities and differences in the *UbdA* expression pattern (compared to the situation observed in mantids). During early to mid stages of development, there is a complete absence of *UbdA* signal in hind legs (Fig. 2*F*). Then, while all legs are still unsegmented and identical in size, two faint bands of *UbdA* appear in hind legs (arrowhead, Fig. 2*G*). It is only after completion of leg segmentation that *UbdA* becomes localized to the T3 tibia and proximal tarsus (Fig. 2*H*). Note the absence of the signal in T3 femur, which is very similar in size to its foreleg counterpart. Thus, cockroaches, too, exhibit a species-specific *UbdA* expression that is localized strictly to differentially enlarged T3 leg segments.

In contrast to the hind leg divergence observed in first nymphs of hemimetabolus insects, the larvae of holometabolous species generally exhibit a simplified, more uniform leg morphology. Consequently, as illustrated by the first instars of the neurop-

teran *C. carnea* (green lacewing), legs in all three thoracic segments are highly similar (Fig. 2*I*). Our analysis shows that this uniformity in leg morphology is associated with a complete absence of the *UbdA* signal during embryonic development (Fig. 2*J*). Similar results were obtained for the first instars of additional holometabolous insects such as beetle *Tribolium* (Fig. 2 *G* and *H*) (27) and species of lepidopterans (22, 28). These data show that absence of *UbdA* expression in the embryonic T3 legs correlates with the lack of larval T3 leg diversification. This is reminiscent of the situation observed in firebrats, which are also characterized by the highly uniform thoracic legs (Fig. 1*A*). Thus, both in the basal lineage such as firebrat and in the derived holometabolous insects, the leg uniformity is associated with the absence of *UbdA* expression. At the same time, T3 leg segment-specific *UbdA* patterns observed in hemimetabolous insects are directly associated with segment enlargement. Together, these findings suggest that enlistment of differential *UbdA* expression may represent a general mechanism for generating leg diversity in insects.

Evolution of *UbdA* Expression in Insect Hind Legs. In this study, we used insect hind legs as a paradigm for understanding morphological change in nature. Although all insects share the same modular leg organization, there is an extraordinary divergence in the size and function of these appendages. Hind legs in particular exhibit a wide range of morphologies, ranging from uniform to greatly enlarged (compared to their foreleg counterparts). As illustrated in Fig. 3, we found that this diversification of hind legs is tightly associated with the pattern of the *UbdA* expression. First, the *UbdA* expression in hemimetabolous insects is localized only in the specific T3 leg segments undergoing differential growth (compared to their foreleg counterparts). In contrast, in basal lineages such as collembolans (*Folsomia*) and firebrats (*Thermobia*), the absence of the *UbdA* signal coincides with the uniform leg morphology. This result also indicates that the observed embryonic differential *UbdA* expression in hemimetabolous insects represents a novel acquisition, which was then subsequently lost in holometabolous groups. Second, there is a striking difference in the timing of expression between species with greatly enlarged T3 leg, such as orthopterans, and species that exhibit a more moderate enlargement of hind legs, such as mantids and cockroaches. In the former, the *UbdA* expression starts much earlier in development, coinciding with the elongation of T3 limb buds and continuing through later stages (dark blue, Fig. 3). In the latter, the *UbdA* expression starts at much later stages of development, coinciding with the establishment of distinct leg segments (light blue, Fig. 3). Although they are based on a limited number of taxa, these results suggest that diversification of insect hind legs is influenced by changes in both the spatial and temporal regulation of the *UbdA* expression. Furthermore, this seems to be an evolutionarily plastic process, as the gain and loss of expression domains occur frequently. For example, there is an acquisition of *UbdA* in tarsus in crickets that can encompass one (*Acheta*) or all three tarsal subsegments (*Gryllus*). At the same time, in cockroaches and mantids there is a trend toward the loss of expression in the femur. More extensive studies of additional groups will be required to determine the actual degrees of plasticity in the regulation of *UbdA* in insects in general.

As illustrated in Fig. 3, the changes in the *UbdA* expression likely played an important role in the evolution of insect hind legs. We propose that in the first step, hemimetabolous insects acquired a new expression domain in the T3 legs. This expression was then co-opted to play a role in regulating differential tissue growth. During divergence of hemimetabolous lineages, there was a refinement of *UbdA* expression leading to the establishments of specific patterns (Fig. 3) that are tightly linked with lineage-specific enlargement of hind legs. In contrast, holometabolous insects exhibit the general absence of the embryonic *UbdA* expression in T3 legs resulting in the uniform larval legs. However, these larval appendages are not homologous to hemimetabolous legs. In holometabolus insects, the adult legs are formed from entirely different cells (imaginal disks) during pupal stage. As such, the previous observation that *Ubx* is expressed in the *Drosophila* pupal T3 femur where it regulates its size (29) is consistent with our hypothesis of evolution of *Ubx* expression and function in insects in general.

Recent studies also show that *Ubx* had a more general role in morphological evolution of arthropod appendages and specific body regions (14, 30, 31). At the protein level, specific amino acid changes at its carboxyl terminus can lead to either conditional (crustaceans) or constitutive (insects) repression of limb devel-

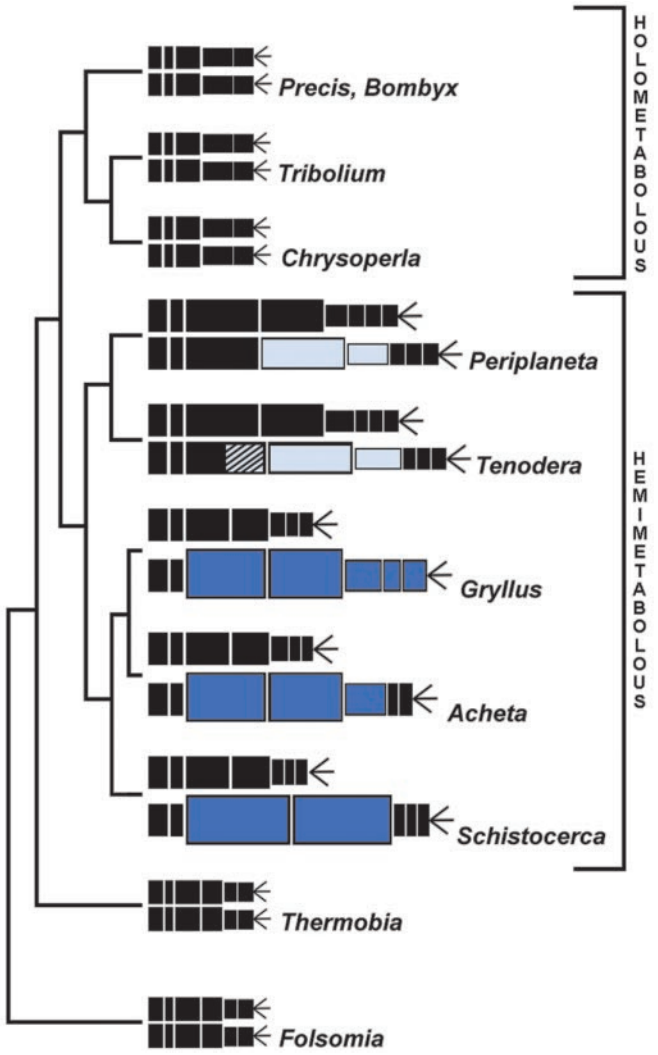

Fig. 3. Evolution of *UbdA* expression in insect hind legs. The early start of expression (dark blue) is associated with the great enlargement of hind leg segments, whereas late expression (light blue) coincides with a moderate T3 leg segment increase. Absence of *UbdA* expression (black) is correlated with the uniform leg morphology. In *Tenodera*, the stripped labeling indicates the transient *UbdA* expression in femur. The relationships between different species were based on generally accepted insect phylogeny (34). For each leg pair, T2 leg is above and T3 leg is below. Each rectangle represents a scaled leg segment, in the following order (left to right): coxa, trochanter, femur, tibia, and tarsus. If present, tarsal subsegments are indicated by a narrow line. Holometabolous species (*Chrysoperla*, *Tribolium*, *Precis*, and *Bombyx*) have fused tibial and tarsal segments, which are represented with a single rectangle. The figure is based on data from this and previous studies (22, 26–28).

opment. As a consequence, in crustaceans *Ubx* does not repress appendage formation, resulting in the presence of limbs on most of their segments (31). However, in insects, *Ubx* has a key function in the differentiation of the third body region, the limbless abdomen (30, 32). At the regulatory level, significant changes in the *UbdA* expression were observed in mallacostracan crustaceans (14). In this group, the *UbdA* expression was independently lost in anterior thoracic appendages of several species. In each case, the loss of *UbdA* is linked with the transformation from the leg-like to mouthpart-like identity of affected appendages. Our present study shows that equally significant changes in the *UbdA* regulation occurred in insects. Furthermore, these regulatory changes are tightly linked with the differential enlargement of insect T3 legs. In addition, *Ubx* also played a role in the divergence of T3 dorsal appendages (wings and halteres) in flies and butterflies (22, 33). The emerging evidence suggests that a surprising portion of morphological evolution in insects and crustaceans may be contributed to the regulatory and structural evolution of a single hox gene and its downstream targets.

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