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Functional analysis of Scr during embryonic and post-embryonic development in the cockroach, Periplaneta americana

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Abstract

The cockroach, *Periplaneta americana* represents a basal insect lineage that undergoes the ancestral hemimetabolous mode of development. Here, we examine the embryonic and post-embryonic functions of the hox gene *Scr* in *Periplaneta* as a way of better understanding the roles of this gene in the evolution of insect body plans. During embryogenesis, *Scr* function is strictly limited to the head with no role in the prothorax. This indicates that the ancestral embryonic function of *Scr* was likely restricted to the head, and that the posterior expansion of expression in the T1 legs may have preceded any apparent gain of function during evolution. In addition, *Scr* plays a pivotal role in the formation of the dorsal ridge, a structure that separates the head and thorax in all insects. This is evidenced by the presence of a supernumerary segment that occurs between the labial and T1 segments of RNAi*Scr* first nymphs and is attributed to an alteration in *engrailed* (*en*) expression. The fact that similar *Scr* phenotypes are observed in *Tribolium* but not in *Drosophila* or *Oncopeltus* reveals the presence of lineage-specific variation in the genetic architecture that controls the formation of the dorsal ridge. In direct contrast to the embryonic roles, *Scr* has no function in the head region during post-embryogenesis in *Periplaneta*, and instead, strictly acts to provide identity to the T1 segment. Furthermore, the strongest *Periplaneta* RNAi*Scr* phenotypes develop ectopic wing-like tissue that originates from the posterior region of the prothoracic segment. This finding provides a novel insight into the current debate on the morphological origin of insect wings.

Introduction

The tri-partite division of the insect bauplan into a head, thorax and limbless abdomen is a highly conserved, class-defining feature that differentiates this group from other arthropods. Due to this high conservation, it would seem likely that the developmental networks controlling the establishment of these three regions would also be highly conserved. However, what is only now becoming evident is that there may be an extensive amount of lineage-specific variation in these networks. For example, a recent functional analysis of the paired-domain gene *nubbin* (*nub*) in the milkweed bug *Oncopeltus fasciatus* has shown that the limbless abdomen is established differently as compared to *Drosophila* (Hrycaj et al., 2008). In a similar fashion, functional studies of the homeotic gene *Cephalothorax* (*Cx*), an

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ortholog of *Sex combs reduced* (*Scr*), have shown that in the absence of this gene, an extra supernumerary segment forms between the labial and T1 segments in *Tribolium* (Shippy et al., 2006). Interestingly, this phenotype has never been reported for similar *Scr* functional analyses conducted in either *Oncopeltus* or *Drosophila* (Chesebro et al., 2009; Hughes and Kaufman, 2000; Pattatucci et al., 1991; Struhl, 1982), and suggests that *Tribolium* has evolved a variation in the developmental program that acts to properly maintain the separation of the head and thorax that differs from the latter two species. However, what still remains unclear is whether this function of *Scr* is specific to *Tribolium* or if it is an ancestral function that has been subsequently lost in both *Oncopeltus* and *Drosophila*.

While there is an extensive amount of data available on *Scr* function during insect development, these studies have been primarily performed in holometabolous species such as *Drosophila* and *Tribolium* in which the larval and pupal stages are phenotypically different from the eventual adult morphology (Beeman et al., 1993; Beeman et al., 1989; Curtis et al., 2001; DeCamillis et al., 2001; Pattatucci et al., 1991; Shippy et al., 2006; Struhl, 1982). In contrast, the majority of insect lineages undergo a hemimetabolous mode of development in which the embryo hatches into a nymph that is phenotypically similar to the adult. While there is data available on the roles Hox genes play in the embryonic development of such species (Angelini et al., 2005; Chesebro et al., 2009; Hughes and Kaufman, 2000; Hughes and Kaufman, 2002; Mahfooz et al., 2007), we are only now beginning to understand exactly what roles these genes play during post-embryonic development. In fact, Chesebro et al. (2009) represents the only such study and has shown that the embryonic functions of *Scr* differ from those observed during post-embryonic development in the hemimetabolous species *Oncopeltus* (milkweed bug).

In this study, we chose to perform a detailed expression and functional analysis of *Scr* at both the embryonic and post-embryonic level in the cockroach *Periplaneta americana* due to the fact that this species represents a phylogenetically older insect lineage as compared to *Oncopeltus*. Results from our embryonic analysis indicate that, similar to *Oncopeltus*, the primary functions of *Scr* are restricted to the head region during embryogenesis with little to no effect on the prothoracic segment. However, in contrast to *Oncopeltus*, the T1 legs of *Periplaneta* RNAi-*Scr* first nymphs are wild type despite distinct embryonic expression of *Scr* at both the mRNA and protein level in these appendages (Passalacqua et al., 2009). In addition, *Periplaneta* RNAi-*Scr* first nymphs also develop an ectopic supernumerary segment between the labial and prothoracic segments reminiscent of the one previously described in *Tribolium Cx* mutants (Shippy et al., 2006). Similar to what was previously reported in *Oncopeltus* (Chesebro et al., 2009), the primary effect of *Scr* during post-embryonic development is to direct the proper growth and development of the prothoracic segment in *Periplaneta*. In addition, the abolition of *Scr* during later nymphal stages results in the growth and development of ectopic T1 wings that originate from the paranotal region of the T1 segment.

Materials and Methods

Periplaneta cultures

Original colonies of *Periplaneta* adults and nymphs were purchased from Carolina Biological Supplies Company and were used to establish laboratory cultures. Both adults and nymphs were reared at 25°C in plastic terrariums with a thick layer of petroleum jelly around the top perimeter to prevent them from escaping and were fed a diet of apples, cat food, and tap water.

Similar to *Oncopeltus*, *Periplaneta* also exhibits the hemimetabolous mode of development in which nymphs that hatch from eggs resemble miniature adults except that they lack wings

and are sexually immature. Unlike *Oncopeltus* however, the number of molts between the first instar and the final adult varies quite considerably, ranging anywhere between 6 to 14 times (Bell and Adiyodi, 1982). This variation exists even in controlled environments in which temperature, size of container, and the numbers of individuals reared together are closely monitored (Bell and Adiyodi, 1982). In our rearing conditions, the average number of nymphal molts was approximately 7, with at least one month of time between each nymphal stage. Overall, it takes approximately one year for *Periplaneta* to develop from egg to adult under our laboratory conditions.

Cloning of Periplaneta americana Scr (Pa-Scr) fragment

Mixed stages of *Periplaneta* embryos were used for total RNA extraction using Trizol (GibcoBRL/Life Technologies) following the manufacturers protocol. cDNA synthesis, RT-PCR, and cloning were performed as described in Li and Popadic, (2004). Two degenerate primers targeted to the conserved amino acid motifs PQIYPWM (5' CCR CAR ATH TAY CCR TGG ATG 3') and WFQNRR (5' GCT CTA GAC GIC GRT TTT GRA ACC A 3') were used to generate a 225 bp fragment of *Scr* that contains the highly conserved homeodomain region. Ten clones were isolated and sequenced. The resulting nucleotide sequences were then compared to each other and previously published *Scr* data in GenBank and were determined to be a *Periplaneta Scr* ortholog. In order to obtain a larger fragment of *Periplaneta Scr*, we used the above sequence as a template to design unique primers for 3' RACE amplification using the FirstChoice RLM-RACE Kit (Ambion). By using this approach we were able to obtain an additional 420bp of sequence including the stop codon and the 3' untranslated region (GenBank sequence accession number XXXXXX). Our analysis showed that whereas both fragments yielded comparable results, the larger 3' RACE fragment produced less phenotypic variation in our RNAi experiments and slightly stronger and more specific signal in our *in situ* analyses. To address the possibility of non-specific effects, we injected a previously cloned 710 bp fragment of the jellyfish Green Fluorescent Protein (GFP) (Chesebro et al., 2009) into the abdomens of either fertilized *Periplaneta* adult females or later staged nymphs. All resulting first nymph progeny or emerged adults were indistinguishable from wild type. In addition, *Scr in situ* analyses performed on random embryos collected from *Scr* dsRNA injected females from clutch 3 and on showed no staining (Fig. 1E). Together, these results suggest that the phenotypes observed from ds*Scr* injections can be attributed to the specific loss of *Scr* function.

Immunohistochemistry

Various stages of *Periplaneta* embryos were hand dissected from their oothecae (egg cases) and fixed for either *in situ* hybridization (as according to Li and Popadic, 2004) or antibody staining (as according to Mahfooz et al., 2004). Riboprobe synthesis for both *Periplaneta Sex combs reduced* (*Scr*) and *Engrailed* (*En*) as well as the *in situ* hybridization procedure were performed as described in Li and Popadic, (2004). The clone used to generate the *Periplaneta En* riboprobe was generously provided by J.P. Couso (University of Sussex, U.K.). Expression of SCR protein was detected using a rat polyclonal antibody generated against a C-terminal fragment of *Drosophila SCR* kindly donated by D.J. Andrew and M.P. Scott (unpublished). This antibody has been effectively proven to cross react in several hemimetabolous insect species (Passalacqua et al., 2009), *Tribolium* (Curtis et al., 2001) and in crustaceans (Abzhanov and Kaufman, 1999). The staining was performed as previously described in (Passalacqua et al., 2009). The antibody was detected by using a secondary anti-rat antibody that was conjugated to FITC (The Jackson Laboratory). Detailed protocols on maintaining *Periplaneta* cultures, collection/fixation of embryos and immunohistochemistry are available upon request.

Preparation of Pa-Scr dsRNA

The cloned cDNA fragments of *Periplaneta Scr* were linearized with the Not-I and Pme-I restriction enzymes and were subsequently used as templates to generate sense and anti-sense single stranded RNA transcripts using the T3/T7 MEGAscript kit (Ambion). Following ethanol precipitation, the concentration of each transcript was determined using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific). The two strands were annealed by mixing equimolar amounts of the sense and anti-sense transcripts and running a PCR-type reaction that initiated at 85°C, and then slowly cooled as follows: 55°C for 20 minutes; 40°C for 10 minutes; 30°C for 5 minutes.

RNA-interference (RNAi)

Approximately 4uL of a 3ug/uL concentration of *Periplaneta Scr* dsRNA was prepared and injected into a single side of the abdomen of adult *Periplaneta* females using a Hamilton syringe with a 32-gauge needle. Three hours following the original injection, an additional 4µ1 of *Scr* dsRNA of the same concentration was injected into the other side of the abdomen. Injected females were then placed in cages with one male per female. Typically between five and eight pairs of males and females were kept in a single container. Oothecae, each of which contain 12–18 eggs, were collected several times per week and placed in Petri dishes with a moist paper towel and sealed with parafilm. Embryos were allowed to mature at 30°C until hatching, which usually occurred in approximately 30 days, upon which their phenotypes were analyzed. Typically, the first 2–3 clutches were wild type. Subsequent clutches showed phenotypic alterations that persisted for at least 20 clutches before reverting back to wild type.

Maternal RNAi experiments were repeated three times with a total of 22 adult females injected. In short, a total of 820 first nymphs were examined with 432 displaying a wild type phenotype and 388 displaying an RNAi*Scr* phenotype. In addition, a total of 346 *Scr*-depleted embryos were fixed for subsequent molecular analysis.

On average, under our laboratory conditions, there are approximately seven nymphal stages of *Periplaneta* post-embryonic development. To perform our nymphal RNAi experiments, we injected approximately 4uL of a 3ug/uL *Scr* dsRNA solution into the abdomens of *Periplaneta* nymphs at various stages (3^{rd}-4^{th}, 5^{th}-6^{th}, and 7^{th}) using a Hamilton syringe with a pulled glass capillary needle. Briefly, a total of 21 nymphs at stages 3–4, 18 nymphs at stages 5–6 and 19 nymphs at stage 7 were injected. All injections performed at stages 3–6 were boosted with an additional 4uL of *Scr* dsRNA every three weeks until the nymph either matured to adult or died during post-embryogenesis. In addition, 19 7^{th} staged nymphs were injected only once in order to assess the contributions that *Scr* solely has during the final nymphal stage. On average, injections at early stages of development (stages 3–4) were generally lethal (80%) with few surviving individuals displaying more moderate phenotypes. These data indicate that there may be a functional requirement for *Scr* during early stages of *Periplaneta* of post-embryogenesis and that the complete abolition of *Scr* transcript during these stages is lethal. A similar situation has been recently reported in *Oncopeltus*, in which the abolition of *Scr* during early post-embryonic stages was lethal (Chesebro et al., 2009). In contrast, injections at the 5^{th}–6^{th} nymphal stages generally resulted in moderate to strong phenotypes (61%) with small percentages of weak phenotypes (6%) and lethality (33%). Finally all surviving 7^{th} staged nymphal injections resulted in weak phenotypes only, suggesting that *Scr* function may be continuously required throughout post-embryogenesis in *Periplaneta*.

RT-PCR analysis

Periplaneta sixth nymphs were injected with 4μl of a 3μg/μl concentration of *Scr* dsRNA and allowed to molt into seventh nymphs. At this stage, the T1 plates from three RNAi*Scr* 7th staged nymphs were dissected and the total RNA was extracted in three independent reactions using Trizol (GibcoBRL/Life Technologies). This RNA was subsequently used as a template to generate cDNA utilizing a poly-T primer (Promega). For comparison, total RNA and cDNA was generated from wild type T1 plates in an identical manner. Equal concentrations of cDNA of both wild type and RNAi*Scr* seventh nymphs were subsequently used as templates in individual PCR reactions to assess the amount of *Scr* transcript that was abolished in injected individuals. Unique *Scr* primers were designed according to the shorter *Periplaneta Scr* fragment described in this study. As a positive control, primers were also designed to the *Periplaneta* 18S ribosomal subunit sequence originally published in Giribet et al., (2001) and were used in both wild type and *Scr* injected seventh nymphs. The PCR conditions were as follows: 94°C for 3 min; 30 cycles of 94°C for 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds; one cycle of 72°C for 7 minutes.

Results

I. Embryonic functions of *Scr* in *Periplaneta americana*

Embryonic expression patterns of Scr in Periplaneta americana—A 420bp partial cDNA fragment specific to the 3' end of *Periplaneta americana Scr* (*Pa-Scr*) was used to study the patterns of mRNA accumulation throughout embryonic development. At ≈10% development, *Scr* is broadly expressed in the mid-posterior region of the embryo with two strong bands of expression that correspond to the future Lb/T1 segments (Fig. 1A). At approximately 20% development, strong *Scr* signal localizes to the labial segment only (Fig 1B). Slightly later in development, when limb buds are fully formed (≈25%), *Scr* signal remains localized to the labial segment, with no expression in the maxillary or T1 segments (Fig. 1C). As described in Passalacqua et al. (2009), during dorsal closure *Scr* signal is maintained in the labial appendages and fades away in the mid-ventral region of the segment. At later stages (≈65%), *Scr* expands into the T1 segment, with clear signal in the dorsal T1 region (Fig. 1D, orange arrowhead) and in two discrete clusters of cells in the T1 legs (Fig. 1D, white arrowheads). Overall, both mRNA and protein expression data (Passalacqua et al., 2009) show that *Scr* signal is initially confined to the labial segment at early stages of development and does not expand into the T1 segment and its associated appendages until much later. These data suggest that the primary embryonic functions of *Scr* should be to control the proper development of the labial segment and its appendages, with a secondary role in the T1 segment. In order to test this hypothesis, we employed a parental RNAi (pRNAi) approach to assess the embryonic functions of *Scr* in *Periplaneta. Scr in situ* analyses performed on random embryos collected from clutch 3 and on showed no staining, indicating that our pRNAi application completely abolishes its expression (Fig. 1E).

Role of Scr in Periplaneta labial development—*Periplaneta* exhibits the ancestral mandibulate or "chewing" mouthparts where the labial segment is fused at the base with each side composed of three "branches" called the glossa (innermost), the paraglossa (middle), and the labial palp (outermost) (Fig. 2A). The labial palp is articulated and composed of three sub-segments that, for ease of description, will be referred to as S1 (most proximal), S2 and S3 (most distal). In moderate RNAi*Scr* phenotypes, the S2 sub-segment of the labial palp develops several large, sclerotized bristles (arrowheads) that are reminiscent of those found on the femur of thoracic legs (Fig. 2B). The S3 sub-segment is also modified and develops numerous bristles and long hairs (Fig. 2B, asterisk) that appear to have a mixed leg/antennal identity. Strong RNAi*Scr* first nymph phenotypes (Fig. 2C) are characterized by a complete transformation in which S2 assumes the identity of the T1

femur complete with a row of organized bristles (arrowheads) along the ventral margin. In addition, a large spur develops at the distal end of S2 (arrow), which is a distinct feature of the femoral segment of wild type thoracic legs. The third sub-segment is also transformed, becoming partially segmented with a mixed leg-antennal identity. Note that the distal-most portion of S3 (Fig. 2C, asterisk) exhibits hairs that are reminiscent of ones found on antennae (Fig. 2C1). At the same time, the morphologies of the glossa and paraglossa remain unaltered even in the strongest observed RNAi phenotypes and suggest that *Scr* does not play a role in the establishment of these structures. These results indicate that the role of *Scr* during embryonic development in *Periplaneta* is restricted to controlling the identity of the labial palps, but not the entire labial appendage.

Role of Scr in Periplaneta T1 leg development—Unlike the situation observed in *Drosophila, Tribolium* and *Oncopeltus*, the T1 legs of *Periplaneta* first nymphs only slightly differ from their T2 counterparts. Specifically, the femurs of both T1 and T2 legs contain an organized row of bristles along the ventral side, with approximately 17 bristles on T1 legs that are closely spaced together and only approximately 10 bristles on the T2 femur that are spaced further apart (compare Fig. 2D1 to Fig. 2F1). Notably, the T1 femoral bristle pattern in the prothoracic legs of *Periplaneta* RNAi*Scr* first nymphs is unaffected and the appendages appear wild type (compare Fig. 2D1 and Fig. 2E1). These data show that despite clear T1 leg expression at later stages of development (Fig. 1D; (Passalacqua et al., 2009), *Scr* has no obvious role in defining the external morphology of this appendage.

Role of Scr in dorsal ridge development—The dorsal ridge is a multipartite structure that forms a division between the head and thoracic region in insects (Rogers and Kaufman, 1996; Shippy et al., 2006). As originally proposed by Rogers and Kaufman, (1996), the dorsal ridge is composed of both gnathal and pregnathal segments and can be divided into two parts, Dr-I and Dr-II. Dr-I is the segmental like entity that forms from the dorsolateral extension of the labial and maxillary segments during dorsal closure, while Dr-II is derived from the dorsal-most portions of the maxillary, mandibular, intercalary and antennal segments (Rogers and Kaufman, 1996). As shown in Fig. 3C–D1, strong RNAi*Scr* *Periplaneta* first nymphs develop an ectopic supernumerary segment between the head and prothorax that disrupts the ancient boundary that is normally defined by the dorsal ridge. This result indicates that *Scr* plays a pivotal role in directing the proper establishment of this highly conserved division in *Periplaneta*.

Previous studies in *Drosophila* and *Tribolium* have shown that *Scr* expression co-localizes with that of the segment polarity gene *engrailed* (*en*) in the posterior portion of Dr-I and suggests that both *Scr* an *en* may play important roles in the proper formation of the labial and maxillary derived portions of the dorsal ridge (Rogers and Kaufman, 1996; Shippy et al., 2006). More specifically, the first evidence of dorsal ridge development is the connection of stripes of *en* expression in the posterior compartments of the maxillary and labial segments by a second stripe of *en* expression along the lateral edge of the anterior compartment of the labial segment (Rogers and Kaufman, 1996; Rogers et al., 1997; Shippy et al., 2006). This lateral connection of the labial and maxillary stripes of *en* expression has been reported for all insects studied to date, including *Periplaneta* (Marie and Bacon, 2000; Patel et al., 1989; Peterson et al., 1998; Rogers and Kaufman, 1996; Rogers et al., 1997; Shippy et al., 2006). Once connected, this single band of *en* expression subsequently extends dorsally and ultimately forms the posterior portion of the dorsal ridge (Rogers and Kaufman, 1996; Rogers et al., 1997; Shippy et al., 2006). As depicted in Fig. 4A–A1, this process is conserved in *Periplaneta*, as a single band of combined maxillary and labial expression extends dorsally to encircle the developing embryo during dorsal closure. However, in RNAi*Scr* embryos, this single band of expression bifurcates during dorsal extension, resulting in the formation of a *de novo* band of *en* expression anterior to the labial stripe

(Fig. 4B, green arrowheads). The presence of two stripes of en expression correlates with the presence of the two additional segmental grooves that appear on either side of the ectopic supernumerary dorsal segment in strong RNAi*Scr Periplaneta* first nymphs (Fig. 3D1).

II. Post-embryonic functions of *Scr* in *Periplaneta americana*

Unfortunately, first nymphs that hatch from maternal RNAi*Scr* injections never successfully complete post-embryonic development and usually die by the third nymphal stage. This result indicates that there may be a critical functional requirement of *Scr* during the early stages of *Periplaneta* post-embryogenesis, similar to that what was reported in *Oncopeltus* (Chesebro et al., 2009). To circumvent this induced lethality, we focused our post-embryonic analysis of *Scr* function on the final three stages of development. In general, RNAi*Scr* performed at the last nymphal stage resulted in weak to moderate phenotypes, while treatments 2–3 stages prior to adulthood resulted in much stronger phenotypes. These data suggest that the functional requirement of *Scr* during post-embryonic development in *Periplaneta* may be cumulative and is reminiscent of the situation observed in *Oncopeltus* (Chesebro et al., 2009). In order to determine the effectiveness of our RNAi methodology, injected sixth stage nymphs were allowed to molt into the next stage upon which their T1 plates were dissected and evaluated for *Scr* mRNA. As shown by our RT-PCR analysis in Fig. 6E, the expression of *Scr* mRNA is abolished in T1 of RNAi*Scr* nymphs and indicates that the observed adult phenotypes can be attributed to the depletion of *Scr*.

Scr abolition does not affect structures previously established during embryogenesis—Due to their hemimetabolous mode of development, the final morphology of the labial appendage in *Periplaneta* is established during embryogenesis and only increases in size during post-embryogenesis. Despite the wide range of severity of RNAi*Scr* phenotypes, the labial appendage is never affected and appears wild type (Fig. 5A–B). In addition, there is no change in the morphology of the T1 legs regardless of the stage at which the RNAi treatment was performed (Fig 5C–D). This is consistent with our observation in first nymphs (Fig. 2D–F), suggesting that *Scr* does not play a role in establishing the external morphology of these appendages during either embryonic or post-embryonic development. This is consistent with results from *Oncopeltus* (Chesebro et al., 2009), indicating that the post-embryonic abolishment of *Scr* has little to no effect on structures that are previously established during embryogenesis in hemimetabolous insect species.

The role of Scr in the prothorax—In contrast to appendages such as mouthparts and legs, the prothoracic (T1) plate itself displays major morphological alterations with regard to its size and shape throughout post-embryogenesis. As shown in Fig. 6A, the wild type adult pronotum is a greatly enlarged, oval-shaped structure that is morphologically distinct from T2, particularly in the posterior margins. More specifically, the T1 segment has a rounded, smooth morphology while the posterior margin of T2 exhibits a thickening of the cuticle that forms a ridge-like structure (Fig. 6A, white arrowheads). A second unique feature of T2 is the presence of a longitudinal invagination at the point where the left and right plates meet at the midline (Fig. 6B, lower green arrowhead). In strong RNAi*Scr* phenotypes, the T1 segment is transformed toward a T2-like identity as evidenced by the appearance of an ectopic thickening of the cuticle of the posterior margin of this segment (Fig. 6C1–C3). In addition, an ectopic groove appears along the midline of the T1 segment that is normally only seen on T2 (Fig. 6B, green arrowheads). These data mirror what was recently reported in *Oncopeltus* (Chesebro et al., 2009), and provide independent corroboration that the input of *Scr* is critical for the proper growth and development of the T1 segment during post-embryonic development in hemimetabolous insect species.

Scr suppresses wing development on the prothorax during post-embryonic development in Periplaneta—The most noticeable post-embryonic phenotype is the presence of ectopic wings that clearly originate from the paranotal tissue of the prothorax (Fig. 6D–D1). Normally in wild type, wing pads begin to form on the meso- and metathorax (T2 and T3, respectively) two to three nymphal stages preceding adulthood and easily distinguish these two segments from T1, which lacks these structures. By the last nymphal stage, the wing pads on T2 increase in size and point toward the posterior, while T1 remains devoid of wing pads (Fig. 7A). In addition, under indirect lighting conditions wing veins (trachea) become clearly evident in the lateral margins of T2 wing pads (Figs. 7B2) while T1 lacks these structures even at high magnification (Figs. 7B1). Our data show that RNAi*Scr* treatments administered at the last nymphal stage generally result in weak phenotypes. However, RNAi treatments two to three stages before adulthood generally result in much stronger phenotypes as evidenced by the formation of an ectopic wing pad on the prothoracic segment at the last nymphal stage. As shown in Fig. 7C, the ectopic T1wing pad exhibits a posterior-lateral extension of tissue that is reminiscent of the morphology of the mesothoracic wing pad. In addition, this ectopic structure also features the formation of trachea that is normally only found within the lateral regions of the T2 wing pads (Fig. 7D–D1). The fact that earlier RNAi*Scr* treatments generally result in stronger phenotypes, complete with an ectopic wing pad, suggests that the suppression of the wing developmental program on T1 by *Scr* may be cumulative, and that the input of this gene may be required throughout post-embryogenesis to completely suppress wing growth on this segment. These data may at least partially explain why fully developed wings can never be recapitulated when *Scr* is depleted at later nymphal stages in both *Periplaneta* (this study) or in *Oncopeltus* (Chesebro et al.,2009).

Discussion

Studies in *Drosophila, Tribolium*, and *Oncopeltus* have shown that *Scr* functions in two distinct body regions (head and thorax), playing critical roles in establishing identity to the labial segment, suppressing wing growth on the prothoracic (T1) segment and directing the formation of T1 leg combs (Beeman et al., 1993; Beeman et al., 1989; Chesebro et al., 2009; Curtis et al., 2001; DeCamillis et al., 2001; Hughes and Kaufman, 2000; Pattatucci et al., 1991; Shippy et al., 2006; Struhl, 1982; Wakimoto et al., 1984). *Scr* expression analyses have also been performed in a wide range of insect species ranging from (listed early to late-branching): Zygentoma, Orthoptera, Dictyoptera, Hemiptera, Coleoptera, and Diptera (Curtis et al., 2001; Mahaffey and Kaufman, 1987; Passalacqua et al., 2009; Rogers et al., 1997; Zhang et al., 2005). As a result, a rather large, comprehensive data set of *Scr* expression is available and has been used to gain an insight into how the roles of this gene may have changed throughout insect evolution. However, it is necessary to provide support for hypotheses drawn from such studies with functional data. The present study imparts novel insights into this very issue as *Periplaneta* represents the most basal insect lineage in which a detailed functional analysis of *Scr* has been performed.

Role of Scr in labial development

As shown in Fig. 2C, strong *Periplaneta* RNAi*Scr* phenotypes result in a labial appendage with a mixed leg/antennal identity. More specifically, the middle (S2) sub-segment of the labial palp is clearly transformed into a femur while the distal most sub-segment of this appendage (S3) is more reminiscent of an antennae based its on morphology, bristle patterning and the lack of claws. Note, however, that the inner-most portions of the labium (glossa and paraglossa) are unaffected even in the strongest RNAi*Scr* phenotypes. This result is intriguing, as *Scr* is expressed throughout the labial segment at both early and mid embryonic stages of development (Fig. 1B–C, Passalacqua et al., 2009). It is only at late stages of development that *Scr* signal fades from the proximal portions of the labial

appendage and localizes in the distal-most portions that will eventually form the elongated palps (Fig. 1D, Passalacqua et al., 2009). These data suggest that *Scr* expression at later stages of development is critical for the proper formation of the labial palps and that the earlier segmental expression in the labium has no function in the development of the glossa and paraglossa. It therefore appears that additional genes have to be involved in directing the formation of these two proximal structures during *Periplaneta* embryogenesis.

A second intriguing aspect of the labial *Pa-Scr* phenotype is the clear morphological distinction between the middle (S2) sub-segment of the palp that is leg-like and the distal (S3) one that has an antennal identity. The commonly accepted paradigm is that insect appendages assume an antennal identity only in a hox-free state (Percival-Smith et al., 1997; Struhl, 1981). Previous functional analyses of hox gene function in the labial segments of *Drosophila*, *Tribolium*, and *Oncopeltus* have all shown that this paradigm is conserved within these lineages (Brown et al., 2000; Curtis et al., 2001; DeCamillis et al., 2001; Hughes and Kaufman, 2000; Percival-Smith et al., 1997; Stuart et al., 1991). More specifically, in *Drosophila* and *Oncopeltus*, both *Scr* and another hox gene *proboscipedia* (*pb*) are co-expressed in the labium during embryonic development (Pattatucci et al., 1991; Percival-Smith et al., 1997; Rogers et al., 1997; Rogers et al., 2002; Struhl, 1982). Accordingly, it is only when both *Scr* and *pb* are simultaneously depleted that the labial appendage assumes an antennal identity in these species (Hughes and Kaufman, 2000; Percival-Smith et al., 1997). Similarly, the *Tribolium* orthologs of *Scr* (*Cx*) and *pb* (*maxillopedia*, *mxp*) are also co-expressed in the labium during embryonic development (Curtis et al., 2001; DeCamillis et al., 2001; Shippy et al., 2000a; Shippy et al., 2000b; Shippy et al., 2006). In this species, it has been determined that *Cx* positively regulates *mxp* in this segment (DeCamillis et al., 2001). As a consequence, the single depletion of *Cx* causes the labium to develop in a hox-free state and assumes an antennal identity. Based on the fact that the distal most sub-segment of the labial palp of RNAi*Scr Periplaneta* first nymphs develops as antennae (Fig. 2C–C1), it is tempting to speculate that a similar *Scr/pb* regulatory relationship may also exist in this species as it does in *Tribolium*. However, such a putative *Scr-pb* regulatory relationship does not account for the fact that the middle (S2) portion of the labial palp is transformed into a femur in RNAi*Scr Periplaneta* first nymphs (Fig. 2C). The fact that at later stages of embryogenesis *Scr* is only expressed in the S3 region of the labial palps (Fig. 1D) suggests that the S2 sub-segment of this appendage would likely retain *pb* expression and function. Studies in *Drosophila* have shown that the ectopic expression of *pb* results in the transformation of legs into maxillary or labial palps (Aplin and Kaufman, 1997) and that the sole expression of *pb* leads to maxillary palp identity (Percival-Smith et al., 1997). According to these data, the proposed residual *pb* expression in the S2 sub-segment of the labial palp of RNAi*Scr Periplaneta* embryos should cause this region to develop as a mouthpart and not a femur. This result suggests that additional genes are required to establish labial identity in the cockroach as compared to flies.

Scr does not play a role in the development of T1 legs in Periplaneta americana

In the insect lineages *Drosophila*, *Tribolium* and *Oncopeltus*, *Scr* directs the formation of a T1-specific structure (combs) that clearly differentiates them from their T2 counterparts (Beeman et al., 1989; Chesebro et al., 2009; Hughes and Kaufman, 2000; Pattatucci et al., 1991). However, the majority of insect lineages do not bear any unique features on their T1 legs and as a result, are morphologically very similar to their T2 legs. One such example is the cricket species *Acheta domestica*. Interestingly, *Scr* is clearly expressed in the prothoracic legs at both the mRNA (Rogers et al., 1997) and the protein (Passalacqua et al., 2009) level in this species despite the fact that they are morphologically indistinguishable from their T2 counterparts. Similarly, *Scr* is also expressed in the T1 legs of *Periplaneta*

(Fig. 1D; Passalacqua et al., 2009) which are morphologically very similar to those that appear on T2. These observations led to the proposition that the posterior expansion of *Scr* into the T1 legs of more basal insect lineages may have preceded any apparent gain of function during evolution (Passalacqua et al., 2009; Rogers et al., 1997). Consistent with this scenario, the depletion of *Scr* has absolutely no effect on defining the external morphology of T1 legs of *Periplaneta* first nymphs or adults (Fig. 2E, Fig. 5D). However, it is possible that *Scr* may play some role other than defining external morphology of T1 legs, such as in sensory organ differentiation and/or the positioning of PNS neurons.

Role of Scr in Dorsal Ridge Development

The dorsal ridge is a multipartite structure that forms a distinct boundary between the head and thorax of most, if not all insect species (Rogers and Kaufman, 1996; Shippy et al., 2006). Studies in several insect groups including *Drosophila* and *Triboilum* have shown that two genes, *Scr* and the segment polarity gene *engrailed* (*en*) are important in the formation of this structure during embryogenesis (Peterson et al., 1998; Rogers and Kaufman, 1996; Rogers et al., 1997; Shippy et al., 2006). The present study reveals that these same two genes play a critical role in the normal growth and development of the dorsal ridge in *Periplaneta* as well. More specifically, the embryonic abolition of *Scr* results in the formation of an ectopic supernumerary segment between the head and prothoracic (T1) segments (Fig. 3C–D1). Consistent with this phenotype, the connected stripes of maxillary and labial *en* expression bifurcate during dorsal extension in RNAi*Scr Periplaneta* embryos (Fig. 4B, green arrowheads), and ultimately form the *de novo* boundaries of the ectopic supernumerary segment. Interestingly, while functional analyses of the *Tribolium Scr* ortholog *Cx* result in an identical phenotype (Shippy et al., 2006), analogous studies in both *Drosophila* and *Oncopeltus* do not (Chesebro et al., 2009; Hughes and Kaufman, 2000; Pattatucci et al., 1991; Struhl, 1982). These data reveal the presence of lineage-specific variation in the genetic mechanisms that controls the formation of the dorsal ridge. In a similar fashion, a recent report on the functional role of paired domain gene *nubbin* (*nub*) in *Oncopeltus* has shown that this gene has a novel role in the establishment of the limbless abdomen by up-regulating the homeotic gene *abdominal-A* (*abd-A*) in this species (Hrycaj et al., 2008). Identical experiments performed in *Drosophila* indicate that no such regulatory relationship between *nub* and *abd-A* exist in this species (Hrycaj et al., 2008). These results therefore provide a second instance in which variation exists in the regulation in the development of a key insect trait. Future analyses of both *Scr* and *nub* in other more basal insect lineages will therefore be able to shed light onto the ancestral genetic ground state that governs the formation of such ancient features.

Insect wing origins

One of the most important innovations in the evolution of the insect body plan was the appearance of articulated, fully functional wings. A remaining fundamental question is to determine the origin and development of articulated wings. While it is generally accepted that insect wings originated only once (i.e. are monophyletic), the morphological origins of these structures remain an unresolved, highly contested debate that has been ongoing for over a century. There are two main theories regarding the evolution of these structures. The paranotal theory suggests that insect wings evolved from fixed extensions of the thoracic terga originally used for gliding from tall terrestrial vegetation (Grimaldi and Engel, 2005; Hamilton, 1971; Quartau, 1986; Snodgrass, 1935). In contrast, the exite or gill theory proposes that wings are derived from the dorsal structures of multibranched ancestral appendages that probably functioned as gills in aquatic environments (Grimaldi and Engel, 2005; Kukalova-Peck, 1991; Wigglesworth, 1973). While both theories have gained an equal amount of support over the past several decades, the use of traditional anatomical, histological and embryological approaches has been unable to provide a definitive answer to

the question of insect wing origins (Grimaldi and Engel, 2005; Hamilton, 1971; Kukalova-Peck, 1978; Kukalova-Peck, 1991; Quartau, 1986; Ross, 1964; Snodgrass, 1935; Wigglesworth, 1973).

More recently, modern molecular techniques have been employed in an attempt to distinguish between the two hypotheses. One such study showed that two known *Drosophila* wing genes are expressed in the dorsal lobe (distal epipodite) of the multibrached limbs of crustaceans and therefore, is consistent with the exite theory of wing origins (Averof and Cohen, 1997). The caveat of this study however, is that the inferences obtained are indirect since no extant insect species possesses multibranched appendages. Hence, such indirect comparative analyses of gene expression lack the means to definitively prove true homologies of divergent structures due to the fact that individual genes can acquire different roles in different developmental contexts (Averof and Cohen, 1997; Choe and Brown, 2007; Choe et al., 2006; de Jong et al., 1989; Hrycaj et al., 2008; Liu and Kaufman, 2005; Patel et al., 1992; Schroder, 2003; Stuart et al., 1991).

An alternative approach is to study the histological and genetic origins of insect wings in a system in which the ancestral form can be recapitulated. According to fossil evidence, extinct pterygotes exhibited wings on every thoracic and abdominal segment (Carroll et al., 1995; Kukalova-Peck, 1978; Tanaka and Ito, 1997). Expression and functional analyses have since established that the subsequent acquisition of *Scr* in the prothorax of modern winged insect lineages gained a novel role in suppressing the ancestral wing developmental program on this segment (Beeman et al., 1989; Carroll et al., 1995; Chesebro et al., 2009; Rogers et al., 1997). *Scr* analyses in two hemimetabolous lineages, *Oncopeltus* (Chesebro et al., 2009), *Periplaneta* (this study), and in the holometabolous species *Tribolium* (Beeman et al., 1989; Tomoyasu et al., 2005) and *Drosophila* (Rogers and Kaufman, 1997) therefore recreate an ancestral condition by relaxing the normally suppressed ancient wing developmental program on the prothorax. As shown Fig. 6D–D1, the abolishment of *Scr* results in the growth of ectopic T1 wings that originate from the posterior lateral terga of the prothoracic plate. In addition, SCR protein is expressed in the dorsal lateral region of the prothorax of all modern winged species at late stages of embryogenesis (Passalacqua et al., 2009). This finding pinpoints the exact area where *Scr* is acting to suppress the ancestral wing developmental program on T1. Hence, by using *Scr* signal as a proxy, we can show that wing primordia are localized to the dorsal lateral region of the prothorax.

In a strict sense, these combined data swing the pendulum back in support of the paranotal theory. However, it is important to note that while these data are consistent with this hypothesis, they do not effectively disprove the exite theory. In essence, while the present results unambiguously show that ectopic wings arise from the dorsal lateral portion of the pronotum, what remains to be determined is the cellular origin of the tissue itself. Based on its position, it is tempting to postulate that the ectopic T1 wing tissue is of paranotal origin. And yet, it is conceivable to imagine a scenario where the exopodite tissue in crustaceans was reabsorbed and migrated dorsally in the ancestor of modern winged insects to its current position on the pronotum. Future studies should therefore focus on performing critical hypothesis-testing experiments that can provide support for a single theory. For example, determining that crustacean epipod-specific genes are expressed in regions outside of the observed embryonic *Scr* pronotal domain would identify tissues that are homologous to exites. The distinction of such tissue from the pronotal domain would provide direct evidence against the exite theory. Such experiments, coupled with studies analyzing the histological origins of ectopic wing tissue in RNAi*Scr Oncopeltus* or *Periplaneta* individuals can provide a direct manner for investigating the evolution of insect wings.

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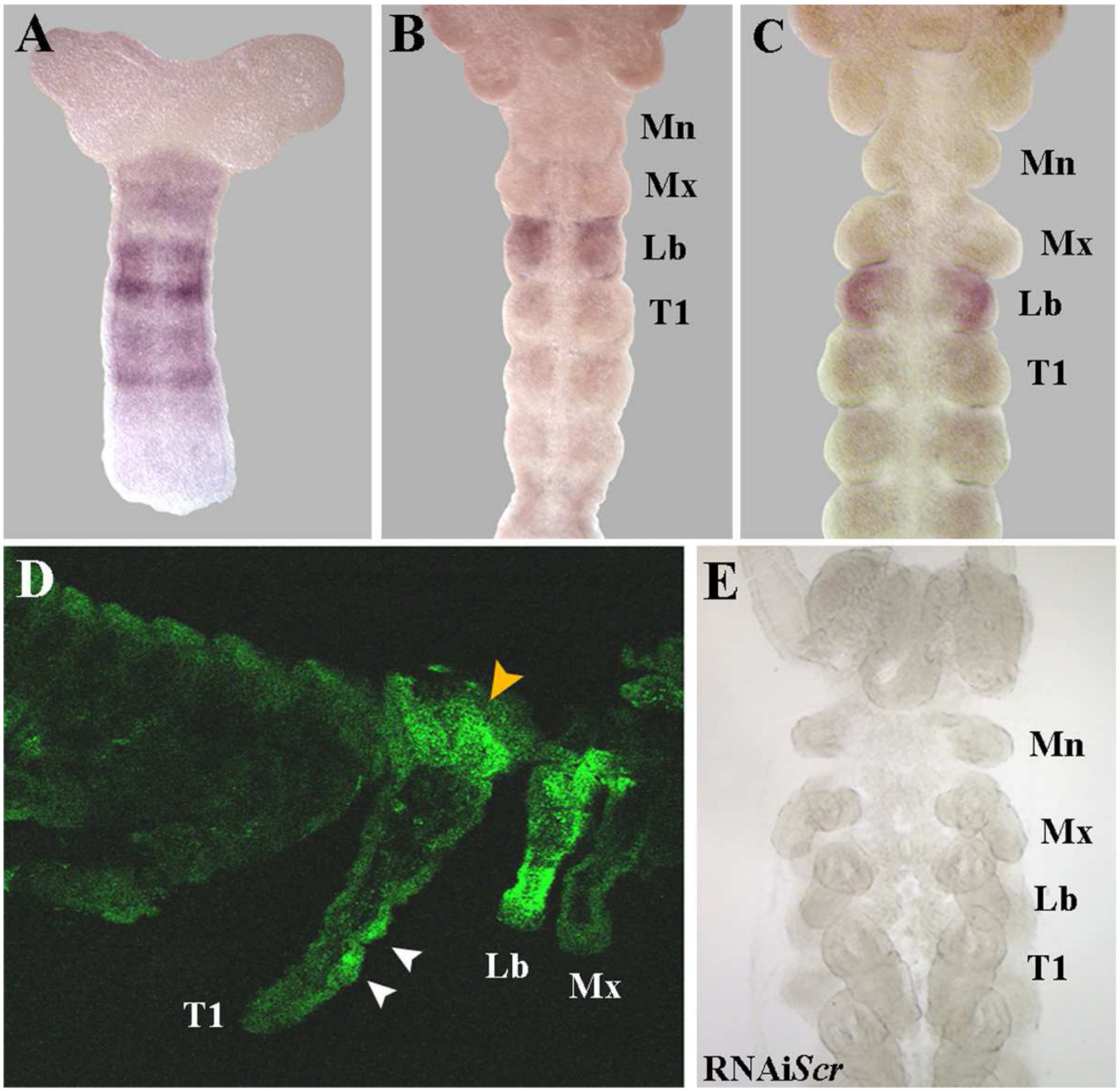

Fig. 1.

Embryonic expression patterns of *Scr* in *Periplaneta americana*. (A) At ≈10% development, *Scr* mRNA is broadly expressed in the mid-posterior region with two strong bands that correspond to the future Lb/T1 region. (B) At ≈20% development, strong *Scr* mRNA localizes to the labial segment. (C) Slightly later at ≈25%, *Scr* continues to be solely expressed in the labium with no signal in the Mx or T1 segments. (D) SCR protein accumulation in an embryo at ≈75% development. While strong expression remains in the labial palps, SCR has now expanded into the T1 segment with clear signal in the dorsal T1 region (orange arrowhead) and in two discrete clusters of cells in the T1 leg (white arrowheads). (E) RNAi*Scr* embryo that has been stained for *Scr* mRNA accumulation. The lack of signal indicates the complete depletion of *Scr*.

Abbreviations: Mn = Mandibles, Mx = Maxillary, Lb =Labial, T1 = First thoracic segment

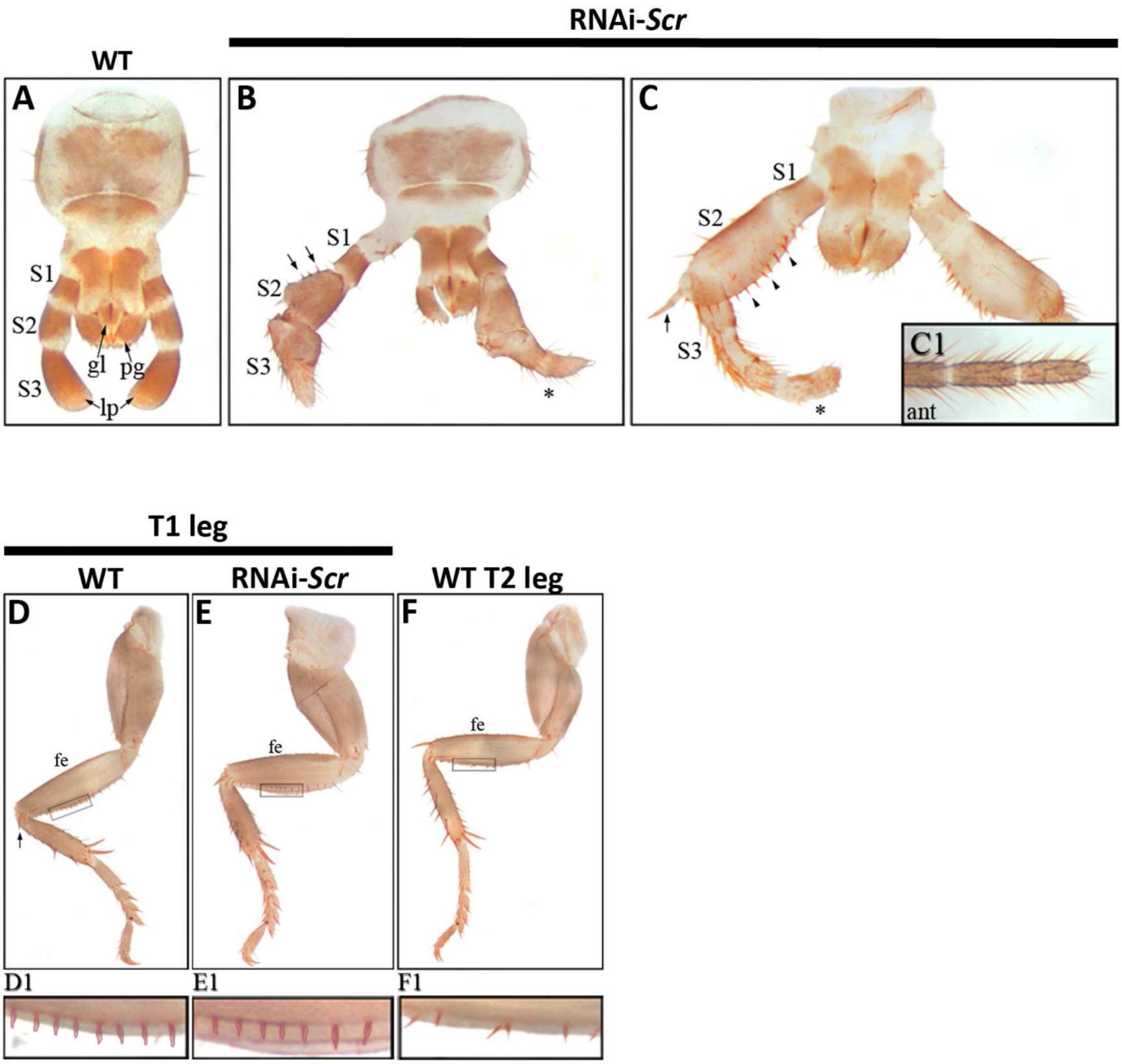

Fig. 2.

Embryonic RNAi*Scr* phenotypes in the labial palps and T1 legs of *Periplaneta*. (A–C) Wild type and RNAi*Scr* labial phenotypes. (A) Wild type labium of first nymph. The articulated labial palps are composed of three sub-segments: S1 (proximal), S2 (middle), and S3 (distal). (B) The labial palps of a moderate RNAi*Scr* first nymph. The phenotypic effects are most noticeable in S2 and S3 where large thoracic leg-like ectopic bristles form (black arrowheads). The distal half of S3 develops numerous hairs (*) resembling those covering the antennae; compare to (C1). (C) Strong RNAi*Scr* first nymph showing a transformation of the labial palp into a mixed leg-antennal identity. S2 shows a complete transformation, assuming the identity of the T1 femur. Note the row of bristles (black arrowheads) and the large spur at its distal end (black arrow; compare to 1D, black arrow). S3 transforms into a

mixed leg (proximal) and antennal (distal) identity. (C1) Distal tip of a wild type antenna; compare with distal half of transformed S3 in B and C (*). (D–F) Wild type and RNAi*Scr* T1 and T2 leg phenotypes. (D) Wild type T1 leg. (E) T1 leg of RNAi*Scr* first nymph. The femur is characterized by a row of approximately seventeen small, closely organized bristles along the entire length of the ventral side. These features remain unaffected in *Scr*-depleted nymphs, compare to wild type T1 femur in D. (F) Wild type T2 leg showing similar morphology to T1 legs. The only observable difference is the row of bristles along the ventral margin, which are fewer in number (approximately 10) and more spaced out. (D1–F1) Magnified view of boxes in D–F showing that the T1 leg of RNAi*Scr* first nymphs retains its identity.

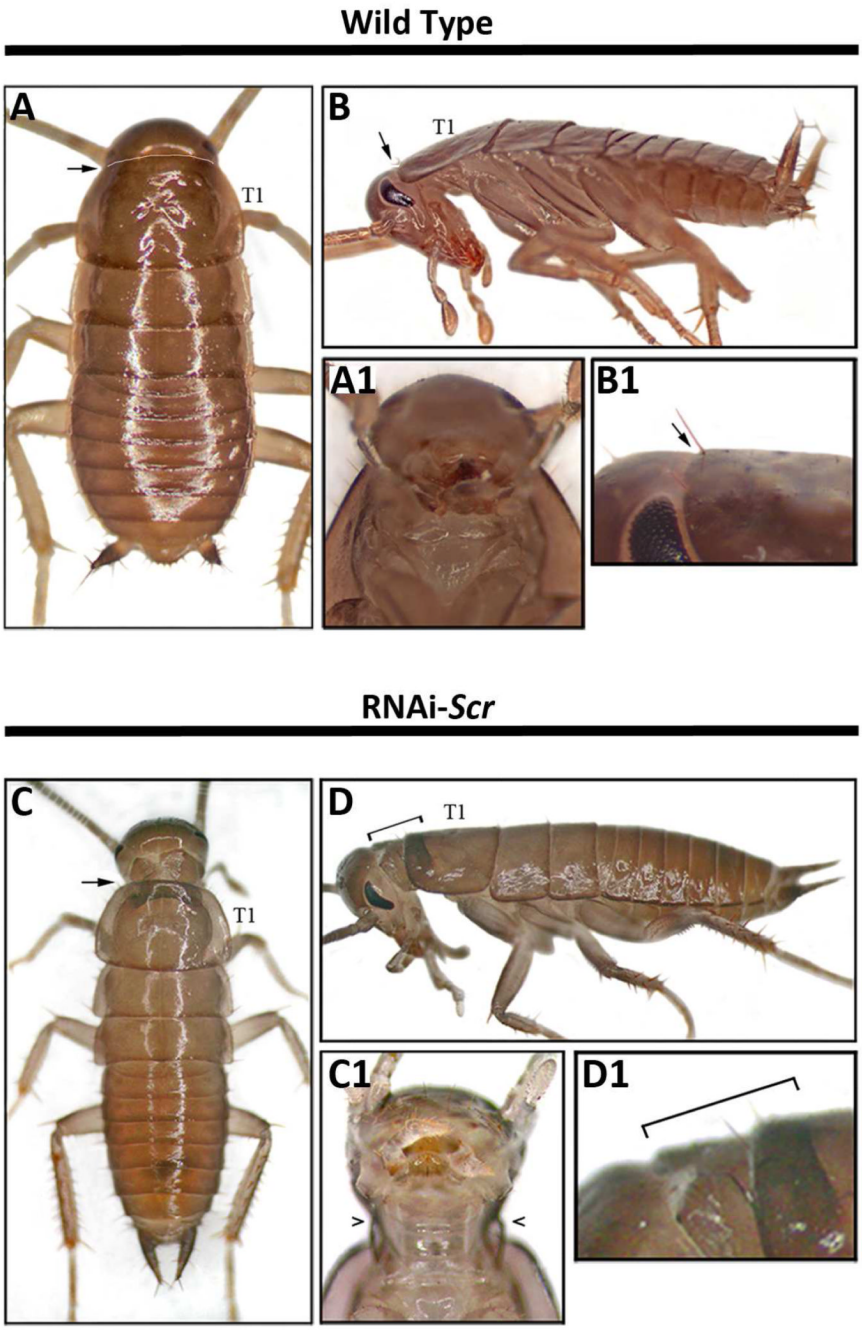

Fig. 3.

Dorsal ridge phenotypes of RNAi*Scr Periplaneta americana* first nymphs. (A) Wild type first nymph showing the characteristically large pronotum that conceals most of the head. (A1) Ventral view of the head and thoracic boundary. (B) Lateral view of wild type first nymph. (B1) Magnified view of lateral head and T1 of wild type first nymph shown in (B). Black arrows in A, B, and B1 point to the dorsal ridge (C) Strong RNAi*Scr* nymph phenotype showing the development of a supernumerary segment between the head and the prothorax. (C1) Extra segment viewed from the ventral side (open black arrowheads point to the ectopic segment). (D) Lateral view of RNAi*Scr* first nymph. (D1) Close up of supernumerary segment shown in (D). Black brackets depict the length of the ectopic

segment in (D–D1). Abbreviations: ant = antenna; fe = femur; gl = glossa; pg = paraglossa; lp = labial palp; S1 = sub-segment 1 of lp; S2 = sub-segment 2 of lp; S3 = sub-segment 3 of lp.

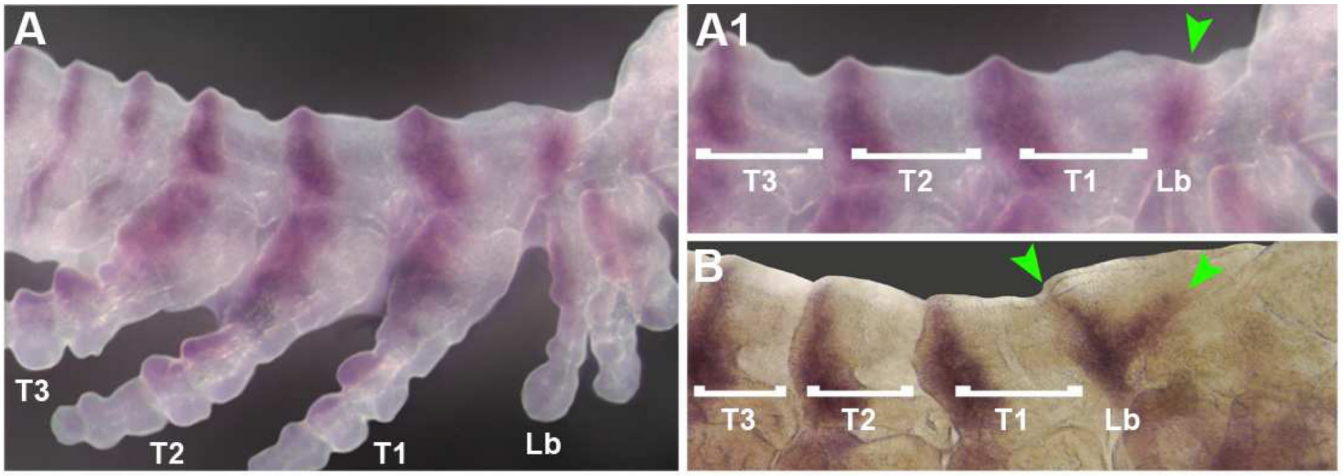

Fig. 4.

Engrailed (*en*) mRNA accumulation in wild type and RNAi*Scr Periplaneta americana* embryos. (A) Wild type embryo showing a combined Mx/Lb stripe of engrailed expression that circumvents the embryo. (A1) Close up of the embryo shown in (A). Green arrowhead points to the single Mx/Lb stripe of *en* expression. (B) Similarly staged RNAi*Scr* embryo stained for *en* mRNA accumulation. The single band of Mx/Lb expression bifurcates (green arrowhead) and results in the formation of a *de novo* band of *en* expression anterior to the Lb stripe. Abbreviations: Mx = Maxillary, Lb = Labial

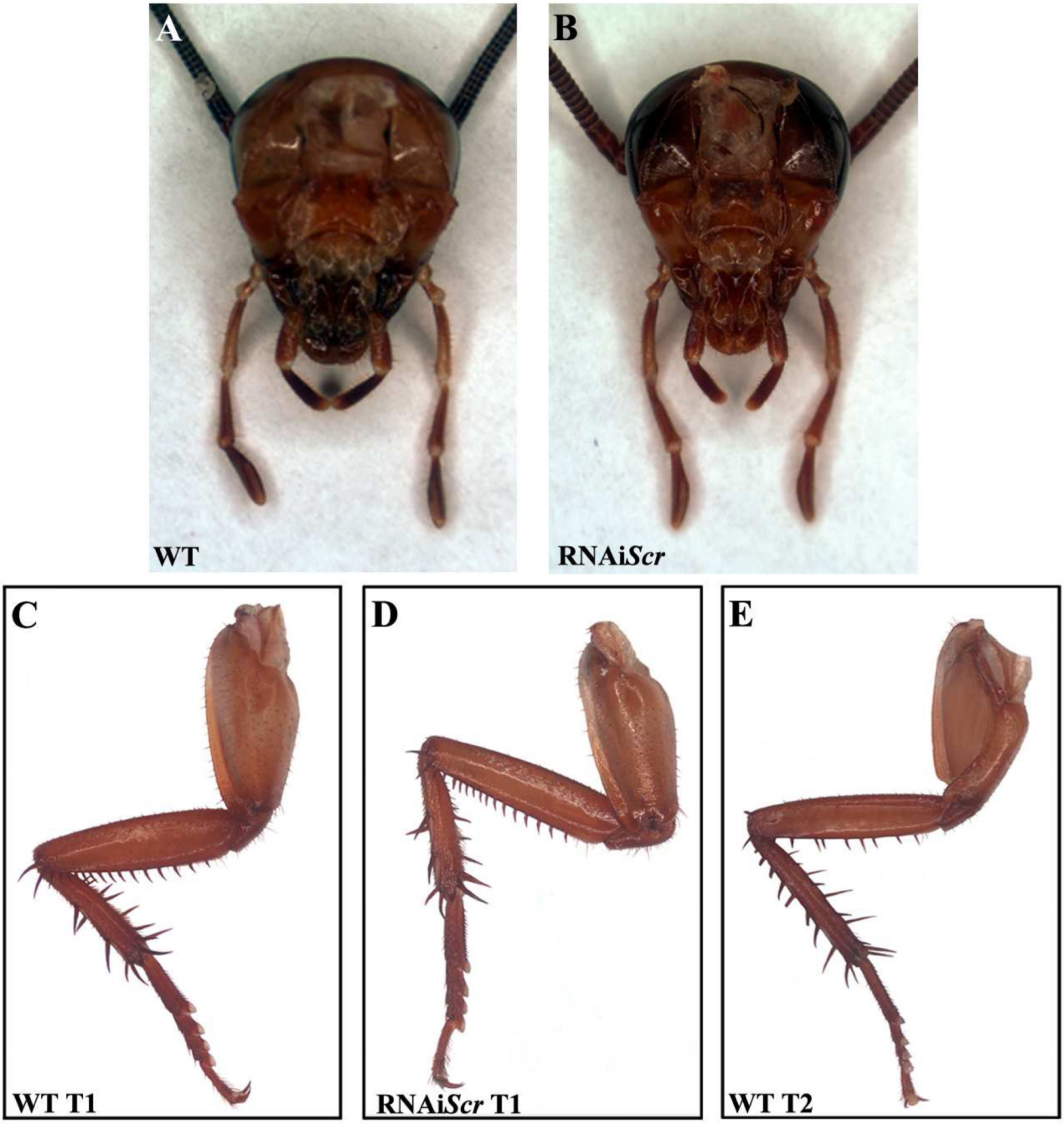

Fig. 5.

Comparison of the labial appendages and the thoracic legs of wild type and RNAi*Scr* *Periplaneta americana* adults. (A) Dissected head of a wild type adult. (B) Dissected head of an RNAi*Scr* adult. Note that the labial appendages are unaffected and appear wild type. (C) Wild type adult T1 leg. (D) T1 leg of an RNAi*Scr* adult. The legs remain unaltered and do not take on a T2 identity. (E) Wild type adult T2 leg.

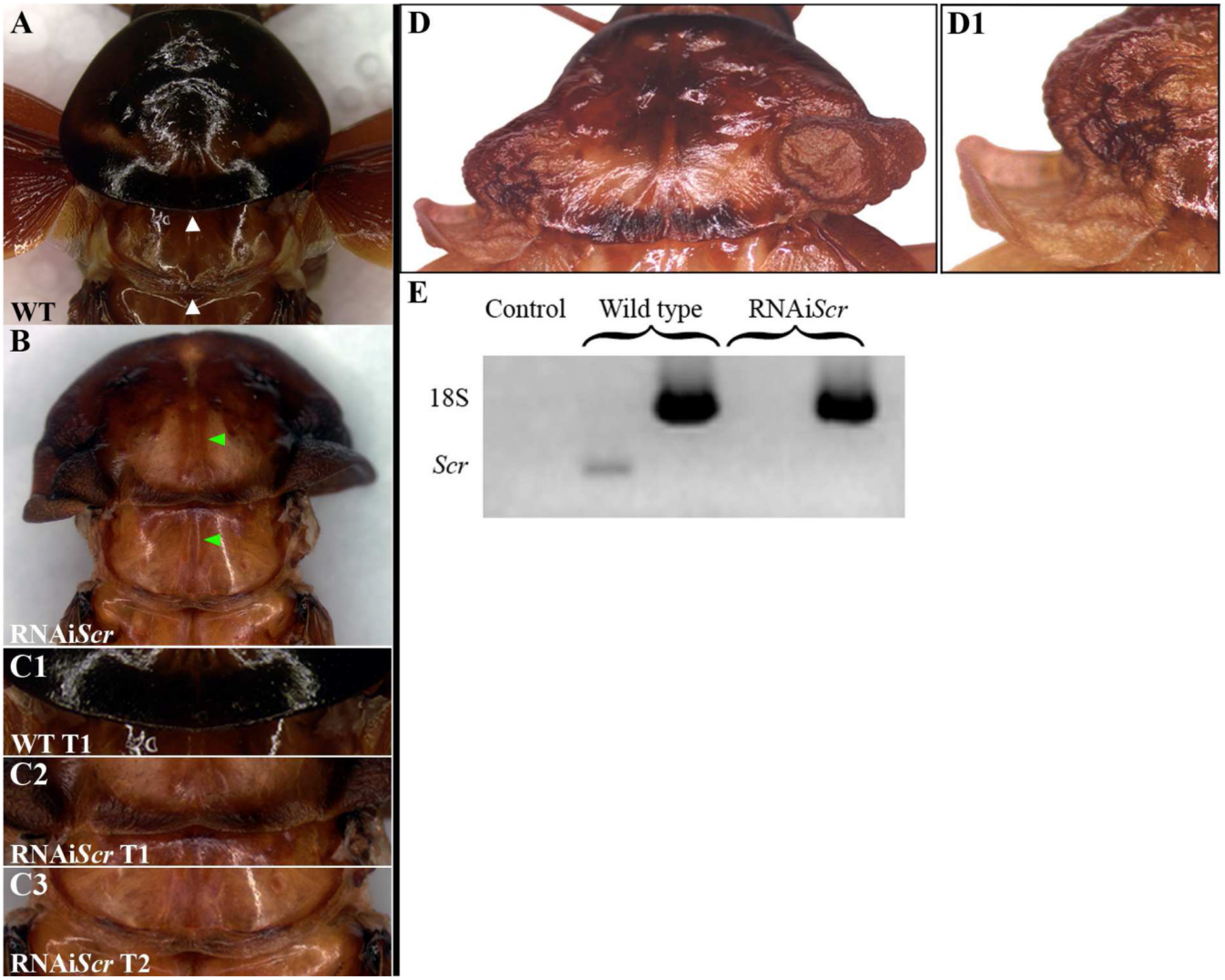

Fig. 6.

Adult RNAi*Scr* phenotypes of the prothoracic (T1) segment in *Periplaneta americana*. (A) Dorsal view of the prothoracic (T1) and mesothoracic (T2) segments of a wild type adult. The posterior margin of T1 has a rounded, smooth morphology while T2 exhibits a thickening of the cuticle that forms a ridge-like structure (white arrowheads). (B) Dorsal view of the prothoracic (T1) and mesothoracic (T2) segments of a RNAi*Scr* adult. The posterior margin of T1 now exhibits a thickening of the cuticle and starts to assume a T2-like identity. In addition, an ectopic groove appears along the midline of the T1 segment that is normally only found on T2 (green arrowheads). (C1) Close up of the posterior portion of the T1 segment of the wild type adult shown in (A). (C2) Close up of the posterior portion of the T1 segment of the RNAi*Scr* adult shown in (B). Note that the cuticle exhibits a thickening and appears like the ridge-like structure present on T2 (compare to (C3)). (C3) Close up of the posterior portion of the T2 segment of the RNAi*Scr* adult shown in (B). This segment is unaffected and appears wild type (compare to (A)). (D) Strong RNAi*Scr* adult phenotype. Ectopic wing-like tissue develops from the posterior lateral portion of T1. (D1) Close up view of the left ectopic wing-like tissue of the strong RNAi*Scr* adult shown in (D). (E) RT-PCR analysis of *Scr* mRNA in the prothoacic plates of seventh nymphs. RNAi*Scr* nymphs show a complete depletion of *Scr* mRNA in T1 as compared to wild type.

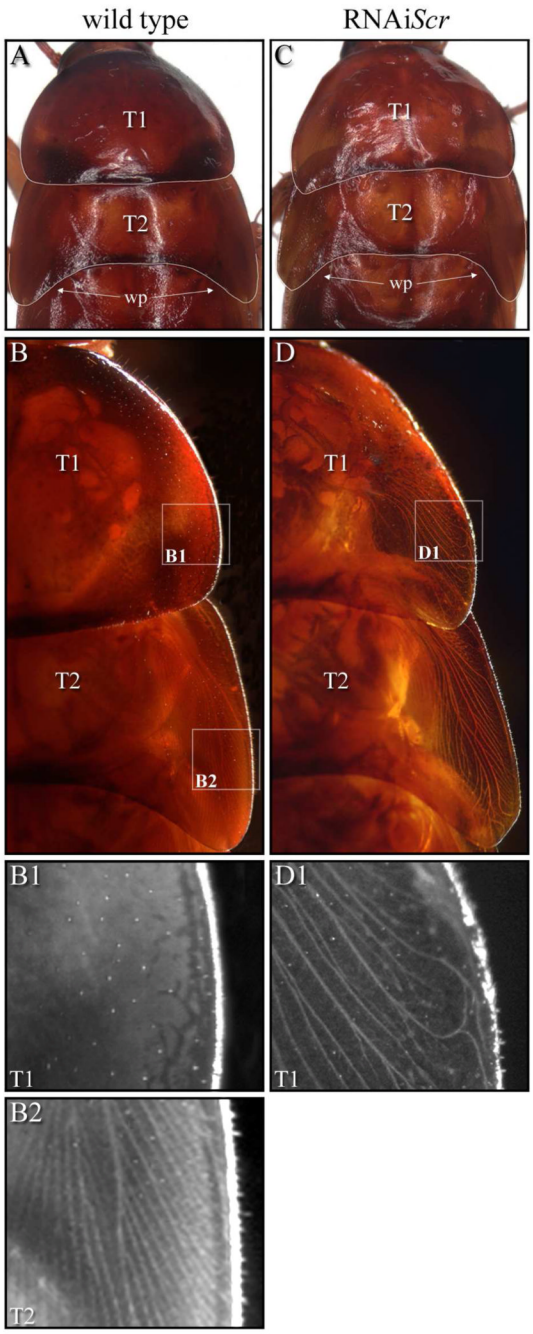

Fig. 7.

Morphology of *Periplaneta* wild type and RNAi*Scr* seventh instars. (A–B2) Wild type. (C–D1) RNAi*Scr*. (A) Wild type seventh nymph pronotum (T1) and mesonotum (T2). Note the large wing pads (arrows) on the lateral margins of T2 making this segment morphologically distinct from T1. (B) Dorsal image of wild type T1 and T2 illuminated by indirect light. The branching wing veins (trachea) along the lateral margins of T2 are quite evident, while trachea are not observed in the pronotum. (B1) Magnified image of lateral margin of T1 (upper box in B1). Note the absence of wing veins. (B2) Magnified image of lateral margin of T2 (lower box in B). Branched veins are clearly present in the developing wing pad. (C) T1 and T2 of an *Scr*-RNAi seventh nymph. Compared to wild type, the morphology of T1 is

altered due to the development of ectopic wing pads at the lateral margins of this segment. (D) Close-up view of lateral margins of T1 and T2 illuminated with indirect light showing the development of trachea in the ectopic wing pads. (D1) The development of veins in T1 is unmistakable (compare to B1). Note, however, that the developing trachea are not identical to those in wild type T2, suggesting an incomplete transformation of T1 toward T2. Legend: T1 = prothorax; T2 = mesothorax; wp = wing pad.