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Evolving expression patterns of the homeotic gene *Scr* in insects

KARLA D. PASSALACQUA[#], STEVEN HRYCAJ[#], NAJMUS MAHFOOZ and ALEKSANDAR POPADIC*

Department of Biological Sciences, Wayne State University, Detroit, USA

ABSTRACT While the mRNA expression patterns of homeotic genes have been examined in numerous arthropod species, data on their protein accumulation is extremely limited. To address this gap, we analyzed the protein expression pattern of the hox gene *Sex combs reduced (Scr)* in six hemimetabolous insects from four divergent orders (Thysanura, Orthoptera, Dictyoptera and Hemiptera). Our comparative analysis reveals that the original domain of SCR expression was likely confined to the head and then subsequently moved into the prothorax (T1) in winged insect lineages. The data also show a trend toward the posteriorization of the anterior boundary of SCR expression in the head, which starts in the mandibles (Thysanura) and then gradually shifts to the maxillary (Orthoptera) and labial segments (Dictyoptera and Hemiptera), respectively. In *Thermobia* (firebrat) and *Oncopeltus* (milkweed bug) we also identify instances where SCR protein is not detected in regions where mRNA is expressed. This finding suggests the presence of a post-transcriptional regulatory mechanism of *Scr* in these species. Finally, we show that SCR expression in insect T1 legs is highly variable and exhibits divergent patterning even among related species. In addition, signal in the prothoracic legs of more basal insect lineages cannot be associated with any T1 specific features, indicating that the acquisition of SCR in this region preceded any apparent gain of function. Overall, our results show that *Scr* expression has diverged considerably among hemimetabolous lineages and establish a framework for subsequent analyses to determine its role in the evolution of the insect head and prothorax.

KEY WORDS: *Sex combs reduced (Scr), gene expression, hemimetabolous insects, evolution, prothorax (T1)*

Introduction

Numerous studies have shown that changes in the expression and function of homeotic (hox) genes were pivotal in the evolution of the insect bauplan (Beeman *et al.*, 1993; Beeman *et al.*, 1989; Carroll *et al.*, 2001; Hughes and Kaufman, 2000; Hughes and Kaufman, 2002; Mahfooz *et al.*, 2007; Mahfooz *et al.*, 2004; Rogers *et al.*, 1997; Rogers *et al.*, 2002; Struhl, 1982; Tomoyasu *et al.*, 2005). This insight was primarily based on mRNA expression patterns, however, data on protein accumulation are much more limited. In fact, only two hox genes (*Ubx* and *abd-A*) have been studied at such a broad level, largely due to the availability of the cross-reacting FP6.87 antibody (Kelsh *et al.*, 1994). The results obtained identified key changes in expression patterns of *Ubx* that directly correlated with changes in arthropod body plans (Abzhanov *et al.*, 1999; Angelini and Kaufman, 2005; Averof and Patel, 1997; Castelli-Gair and Akam, 1995; Damen *et al.*, 1998; Mahfooz *et al.*, 2004; Peterson *et al.*, 1999; Stern, 1998; Telford and Thomas, 1998; Zheng *et al.*, 1999). In insects, *Ubx* expression has also been linked to the differential enlargement of insect hind (T3) legs, which were subsequently confirmed by functional studies (Mahfooz *et al.*, 2007; Mahfooz *et al.*, 2004). As illustrated by these findings, the availability of cross-reacting antibodies are critical in that they provide better phylogenetic sampling by circumventing the laborious process of cloning and characterizing orthologous genes. Such broader sampling can provide a deeper understanding as to how changes of expression patterns can effect evolving morphological structures across a wider range of

Abbreviations used in this paper: dT1, dorsal prothorax; Lb, labium; Mn, mandibles; Mx, maxillae; Scr, sex combs reduced; T1, prothorax; T2, mesothorax; T3, metathorax.

***Address correspondence to:** Aleksandar Popadic. Department of Biological Sciences, Wayne State University, Detroit, MI 48202, USA. Fax: +1-313-577-6891. e-mail: apopadic@biology.biosci.wayne.edu **[#]Note:** Both authors contributed equally to this paper.

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taxa.

To address this gap, we focused on the homeotic (hox) gene *Sex combs reduced* (*Scr*). In insects, *Scr* functions in two distinct body regions, the head and the thorax. Previous insights from mRNA expression and functional studies have indicated that this gene is crucial in establishing the identity of the labial segment, suppressing wing formation on the prothorax (T1), and directing the development of T1 leg combs (Beeman *et al.*, 1993; Beeman *et al.*, 1989; Curtis *et al.*, 2001; Hughes and Kaufman, 2000; Pattatucci and Kaufman, 1991; Pattatucci *et al.*, 1991; Rogers *et al.*, 1997; Rogers *et al.*, 2002; Shippy *et al.*, 2006). The data on protein expression in two model systems (*Drosophila* and *Tribolium*) show that, in general, mRNA patterns closely coincide with the protein pattern (Curtis *et al.*, 2001; Mahaffey and Kaufman, 1987). However, a report in the terrestrial isopod *P. scaber* has described a difference between mRNA and protein expression patterns of *Scr*, revealing the existence of post-transcriptional regulation in this species (Abzhanov and Kaufman, 1999). This result highlights the importance of complementing previously published *Scr* mRNA expression patterns with data on the accumulation of SCR protein. In the present study, we utilize a previously described cross-reacting antibody (Abzhanov and Kaufman, 1999; Curtis *et al.*, 2001) to provide data on the protein accumulation of SCR in six hemimetabolous insect species. Our broad sampling encompasses four divergent insect orders (listed from early to late-branching): *Thysanura*, the firebrat *Thermobia domestica*; *Orthoptera*, the cricket *Acheta domestica* and the grasshopper *Schistocerca americana*; *Dictyoptera*, the cockroach *Periplaneta americana* and the praying mantis *Tenodera aridifolia;* and finally *Hemiptera*, the milkweed bug *Oncopeltus fasciatus*. Our analysis has revealed the following key aspects of the evolution of SCR patterning in insects: (i) the anterior border of SCR expression starts in the mandibular segment (*Thermobia*) and moves posterior to the maxillary segment in some lineages (*Acheta* and *Schistocerca*) and then shifts even more posterior to the labial segment (*Periplaneta*, *Tenodera* and *Oncopeltus*); (ii) we found two instances (*Thermobia*, *Oncopeltus*) in which *Scr* mRNA is clearly expressed in a defined region (Rogers *et al.*, 1997) but never accumulates protein, similar to the post-transcriptional regulatory situation reported in crustaceans (Abzhanov and Kaufman, 1999); (iii) SCR protein expression in T1 legs is very dynamic and can only occasionally be linked to particular morphological structures. Overall, our data suggests that SCR protein accumulation is highly labile and can be gained or lost even in closely related species.

Results

Scr expression in Thermobia domestica (firebrat), a basal insect lineage

The thysanuran *Thermobia domestica* (firebrat) is a primitively wingless species that represents a basal insect lineage. Data on SCR patterning in this species can therefore provide insight into the ancestral expression pattern of this gene. SCR protein accumulation in the firebrat is very dynamic and can be detected from ~30% - ~75% stages of development. At ~30%, when limb buds are just beginning to elongate, the anterior border of SCR expression is in a small cluster of cells in the posterior, mid-ventral portion of the mandibular segment (Fig. 1A). Signal is also detected in a narrow mid-ventral region in the maxillary segment and throughout the entire developing labial segment and its appendages (Fig. 1A). At ~35% development SCR accumulation in the mandibles slightly diminishes, whereas expression in the maxillary segment becomes stronger and expands laterally into the lobes of the developing appendages (Fig. 1B). Expression remains strong throughout the entire labial segment at this stage. At ~40% development, SCR is expressed throughout the labium while it is entirely lost in the mandibular segment (Fig. 1C). The signal is also detected in the proximal lobes of the maxillary appendages and in the posterior portion of the corresponding mid-ventral region at this stage. At ~45% development, signal is reduced in the posterior maxillary segment and is still predominantly confined to the growing labial appendages (Fig. 1D). In late developmental stages (~75%) SCR reappears in the anterior head, with strong expression in the mandibular appendages and a weaker signal in the proximal lobes of the maxillary segment (Fig. 1E). At this stage there is no longer any SCR expression in the labium. It important to note that SCR protein never accumulates in the dorsal T1 region or in the T1 leg at any time during development (Fig. 1F).

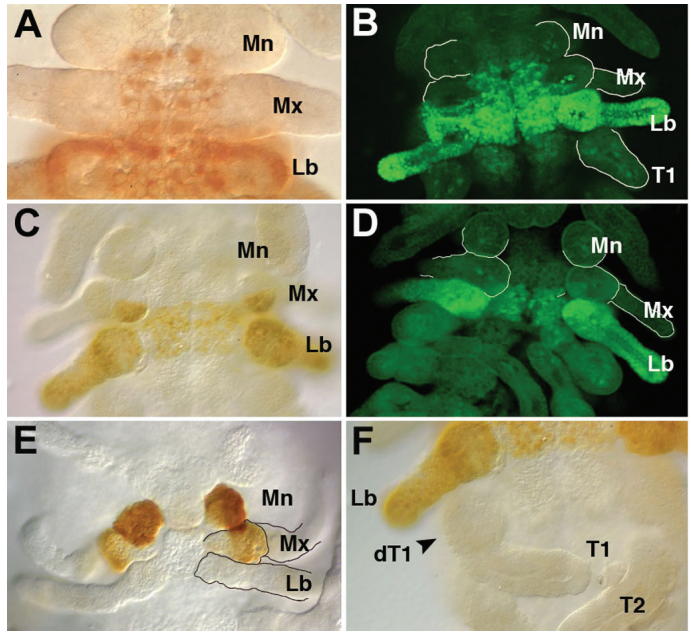

Fig. 1. SCR protein expression in *Thermobia domestica* (firebrat). (A) *At ~30%, SCR is detected in a small patch of cells in the posterior/ventral region of the mandibular segment, in the mid-ventral portion of the maxillary segment and throughout the entire labium.* **(B)** *Later in development (~35%), signal in the mandibles slightly diminishes, while in the maxillary segment it expands laterally into the base of the appendages. Expression remains throughout the labium at this stage.* **(C)** *At ~40% development, SCR is entirely lost in the mandibles, has become restricted to the posterior half of the maxillae and remains throughout the labium.* **(D)** *At ~45%, expression is primarily restricted to the labium, with only a slight signal in the posterior maxillae.* **(E)** *During late stages of development (~75%), SCR reappears in the anterior head and is strongly detected in the mandibles with a more moderate signal in the proximal lobes of the maxillary appendages. SCR is completely absent in the labium at this stage.* **(F)** *SCR protein never accumulates along the first thoracic leg or in the dorsal T1 region during development. Abbreviations: Mn, mandible; Mx, maxillae; Lb, labium; T1, first thoracic leg; dT1, dorsal T1 region.*

Expression of SCR in the insect head shows a trend of posteriorization

As shown in Fig. 2, SCR is predominantly expressed in the labial segment of all insect species examined in this report. However, the initial anterior border of head expression moves in a posterior direction in a species-specific manner. After *Thermobia*, the next two insects analyzed were orthopterans, the cricket *Acheta domestica* and the grasshopper *Schistocerca americana*. At early stages of development, cricket SCR expression is restricted to the posterior region of the maxillary segment and to the proximal lobes of its appendages (Fig. 2A). However, the signal in the maxillae of the grasshopper is much more diffuse in the mid-ventral region (Fig. 2C). In addition, SCR extends more distally in the maxillary appendages encompassing a broader expression domain compared to crickets. In both insects, SCR is still predominantly expressed throughout the labial appendage at this stage. Later in development, expression is entirely lost in the maxillary appendages and only slightly remains in the mid ventral region of this segment (Fig. 2B arrowhead, Fig. 2D). However, SCR continues to be strongly expressed throughout the labial segment and its developing appendages in both insects (Fig. 2 B,D).

The next insect lineage analyzed was *Dictyoptera*, represented by the cockroach *Periplaneta americana* and the praying mantis *Tenodera aridifolia*. At early stages of development, SCR is expressed throughout the entire labial segment but is absent in the maxillary segment in both species (Fig. 2 E,G). This result reveals a further posteriorization of the anterior border of SCR expression when compared to more basal insect lineages (*Thysanura*, *Orthoptera*). In addition, the mid-ventral region of the labial segment in the cockroach exhibits reduced SCR expression, while the mantis maintains strong signal in this area (Fig.

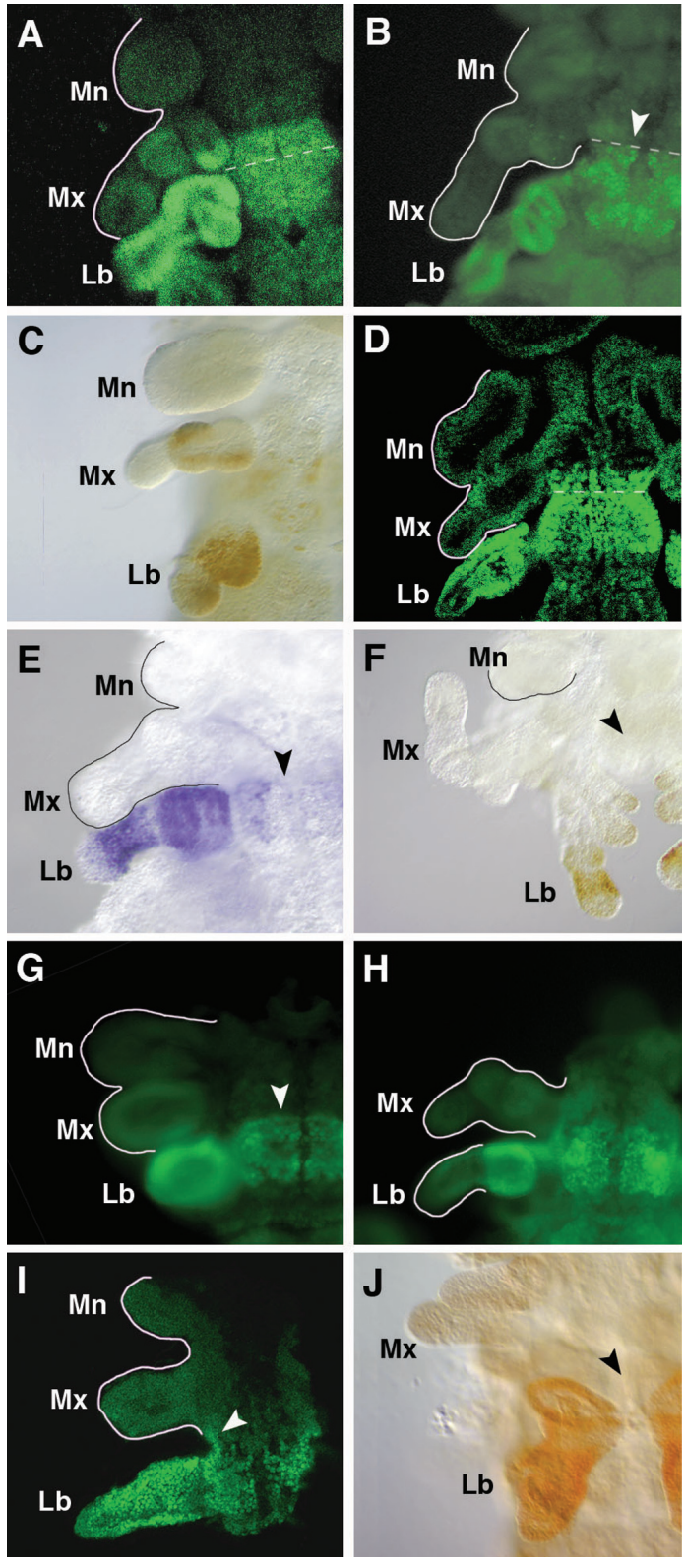

Fig. 2. SCR protein expression patterns in the head segments of five hemimetabolous species. (A,B) Acheta domestica *(cricket),* **(C,D)** Schistocerca americana *(grasshopper),* **(E,F)** Periplaneta americana *(cockroach),* **(G,H)** Tenodera aridifolia *(praying mantis) and* **(I,J)** Oncopeltus fasciatus *(milkweed bug). Left hand column* **(A,C,E,G,I)** *represents early development (25-30%). Right hand column* **(B,D,F,H,J)** *represents mid-late development (35-45%).* **(A)** *Early* Acheta *embryo showing the localization of SCR to the posterior half of the maxillary segment and the proximal region of its associated appendage. Strong expression is observed throughout the entire labial segment and its appendages.* **(B)** *At mid-development, SCR is no longer detected in the maxillary appendages and is confined to a small cluster of cells in the mid-ventral portion of this segment (arrow). SCR remains strongly expressed throughout the labium and its appendages.* **(C)** *In early* Schistocerca *embryos, SCR signal is observed in the proximal region of the maxillary and throughout the labial appendages. Ventral SCR patterning in both the maxillary and labial segments appears faint at this stage* **(D)** *Mid-staged* Schistocerca *embryo showing strong signal throughout the labium and its associated appendages. SCR is restricted to the posterior most region of the mid-ventral maxillary segment.* **(E)** *Early* Periplaneta *embryo showing strong SCR expression throughout the labial appendages and low levels of signal in the mid-ventral region of this segment.* **(F)** *Later in development, signal is restricted to the distal portions of the labial appendages, with no mid-ventral expression (arrowhead).* **(G)** *In early* Tenodera *embryos, SCR is strongly expressed throughout the labial segment and its associated appendages.* **(H)** *While strong signal persists in the ventral labium, SCR expression in the appendages is restricted to the proximal lobes.* **(I)** *Early* Oncopeltus *embryo showing strong SCR expression throughout the labial appendages with moderate signal in the mid-ventral region of this segment. Additional SCR signal can be detected at the lateral edge between the maxillary and labial segments (arrowhead).* **(J)** *At mid-development, SCR is completely lost in the mid-ventral region (arrowhead) of the labial segment but remains strongly expressed throughout its appendages. Abbreviations: Mn, mandible; Mx, maxillae; Lb, labium.*

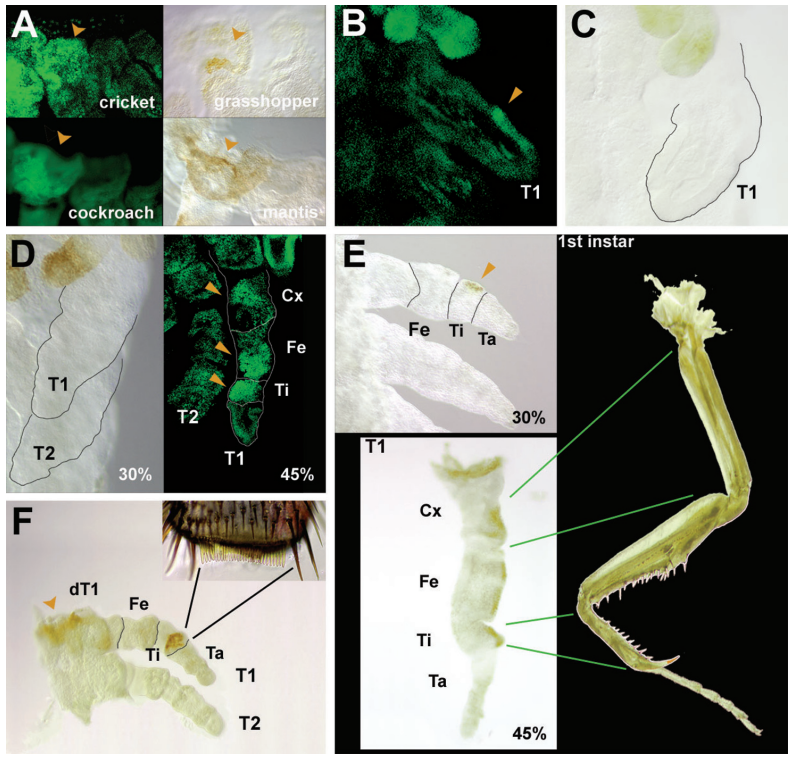

Fig. 3. SCR protein expression in the dorsal T1 (dT1) region and prothoracic (T1) leg in hemimetabolous species. (A) *An arrowhead points to SCR expression in the dorsal T1 (dT1) region of* Acheta domestica *(cricket),* Schistocerca americana *(grasshopper),* Periplaneta americana *(cockroach) and* Tenodera aridifolia *(praying mantis). In* Schistocerca, *while SCR is only detected in discrete regions of dT1, strong expression is seen throughout this region in all other insect lineages examined in this study.* **(B)** *Early* Acheta *embryo (~35% development) showing SCR expression in the anterior region of the T1 leg that corresponds to the future tibial-tarsal joint (arrowhead).* **(C)** Schistocerca *T1 leg showing a complete lack of SCR expression.* **(D)** *SCR protein expression at early and mid-developmental stages in the T1 legs of* Periplaneta. **(D, left)** *At early stages no SCR protein is expressed in the T1 legs of* Periplaneta. **(D, right)** *Later in development, strong SCR signal is now detected in the proximal coxa, distal femur and throughout most of the tibia (arrowheads).* **(E)** *SCR protein expression at early and mid-developmental stages in the T1 legs of* Tenodera. **(E, upper left)** *At early stages (~30%) SCR signal is restricted to a small patch of cells in the anterior tibia (arrowhead).* **(E, lower left)** *SCR expression expands and accumulates in the posterior ridge of the coxa, femur and tarsus at later stages.* **(E, right)** *Dissected T1 leg of* Tenodera *at the first nymphal stage. Solid lines link embryonic SCR expression patterns at mid-developmental stages to the corresponding regions of first nymph T1 legs.* **(F)** *SCR protein expression in the dT1 region and T1 leg of* Oncopeltus. **(F, lower left)** *SCR protein accumulates in the dT1 region of* Oncopeltus *(arrowhead) and in the distal tibia of the T1 leg.* **(F, upper right)** *Close up of the sex combs that appear on the distal tibia of the T1 legs of* Oncopeltus *first nymphs. Solid lines link embryonic SCR expression in the distal tibia to the corresponding structure (sex combs) on the prothoracic legs of first nymphs. Abbreviations: Cx, coxa; Fe, femur; Ti, tibia; Ta, tarsus; T1, first thoracic leg; T2, second thoracic leg; dT1, dorsal T1 region.*

2E,G, arrowheads). Later in development, cockroach SCR signal is completely absent from the mid ventral region (Fig. 2F, arrowhead) and is confined to the distal portion of the developing labial appendages only. In contrast, mantis embryos maintain strong mid-ventral expression in the labial segment. The signal in the elongating labial appendage is restricted to the proximal lobe (Fig. 2H), displaying a different pattern from the cockroach.

The hemipteran milkweed bug (*Oncopeltus fasciatus*) represents a more derived hemimetabolous lineage. In early development, SCR protein is primarily localized in the labial segment, although some faint expression can be detected at the lateral edge between the maxillary and labial segments (Fig. 2I, arrowhead). Additionally, moderate SCR signal is present in the mid ventral portion of the labial segment at this stage. This early restriction of SCR to the labium differs from previously reported mRNA expression patterns that show clear signal in the posterior region of the maxillary appendage (Rogers *et al.*, 1997). This situation is reminiscent of the previously described differences of expression in the dT1 region of *Thermobia*, and is likely due to a post-transcriptional mechanism. At later developmental stages (~35-45%), SCR protein is strictly confined to the growing labial appendages with a complete absence of signal in the mid ventral region of the labial segment (Fig. 2J, arrowhead).

The prothorax (T1): expression in the dorsal (dT1) region is highly conserved

Functional analyses in *Drosophila* and *Tribolium* have indicated that one of the primary functions of *Scr* is to suppress the formation of wings on the adult prothoracic (T1) segment (Beeman *et al.*, 1989; Carroll *et al.*, 1995; Rogers *et al.*, 1997; Struhl, 1982; Tomoyasu *et al.*, 2005). It is generally accepted that this is a conserved role due to the fact that the wingless T1 segment is a key insect feature. Hence, it is important to understand the origin(s) of SCR expression in this region as it relates to the evolution of the insect body plan. *Thermobia* (firebrats) represent an ideal starting point to assess the origins of the wing repressive function of SCR as these insects are considered to be primitively wingless. A previous analysis of *Scr* mRNA expression patterns in *Thermobia* showed clear signal in the dorsal T1 (dT1) region (Rogers *et al.*, 1997). However, as depicted in Fig. 1F, SCR protein does not accumulate in this region at any time during embryogenesis. In one orthopteran species *Acheta* (crickets), SCR is strongly expressed throughout the entire dT1 region (Fig. 3A upper left, arrowhead). At the same time, in another orthopteran *Schistocerca* (grasshoppers), the signal is observed in discrete regions of dT1 (Fig. 3A upper right, arrowhead). The remaining insect lineages examined here (*Periplaneta*, *Tenodera*, and *Oncopeltus*) all exhibit strong expression of SCR throughout the entire dT1 region similar to the pattern observed in *Acheta*. This observation is consistent with the fact that *Scr* has recently been found to repress wing formation on the prothoracic segment in hemimeatabolus species as well (Chesebro *et al.*, 2009).

SCR patterning in the T1 leg is very dynamic and characterized by a frequent gain and loss of expression domains

Insect legs exhibit a range of morphological diversity encompassing small-scale (bristle pattern, coloration) and large-scale (overall size and shape) differences. Despite such vast diversity, all

insect legs share a common modular organization and are composed of the same five basic segments: coxa, trochanter, femur, tibia, and tarsus, followed by claws. One of the distinguishable features of prothoracic legs in some species is the presence of a row of bristles (sex combs) in discrete leg segments. Functional studies in *Drosophila*, *Tribolium*, and *Oncopeltus* have all indicated that *Scr* directs the formation of this T1 leg-specific structure (Beeman *et al.*, 1989; Hughes and Kaufman, 2000; Pattatucci *et al.*, 1991). However, there are many more modifications of prothoracic legs that are distributed across insect groups. The best known example of such extreme modification is observed in praying mantids, where T1 legs are transformed into large raptorial-like appendages that use rows of unique spurs to capture and hold prey. What remains unclear is whether and to what degree *Scr* may be playing a role in the development of these highly modified structures. At the same time, other insect lineages have prothoracic legs that do not bear any unique features and are morphologically very similar to T2 legs. This leads to another important question: is SCR still expressed in the T1 legs of these species? Note that all of the insect species analyzed in this report undergo a hemimetabolous mode of development, thereby allowing a direct association between SCR expression and distinct T1 leg morphology of first nymphs.

The T1 legs of the firebrat are morphologically similar to their T2 and T3 counterparts, bearing no defining structural features such as combs or spurs. Consistent with this phenotype, neither SCR protein (Fig. 1F) nor its mRNA (Rogers *et al.*, 1997) is expressed in the T1 legs at any time during *Thermobia* development.

Despite the fact that they belong to the same group, the available expression data in three orthopteran species (*Acheta*, *Gryllus* and *Schistocerca*) reveal the presence of divergent SCR leg patterning among these lineages. At ~35% development, the T1 leg of *Acheta* accumulates both *Scr* mRNA (Rogers *et al.*, 1997) and protein (Fig. 3B, arrowhead) in a small patch of cells in the anterior region that corresponds to the future tibial-tarsal joint. In *Gryllus*, another cricket species, *Scr* mRNA has a larger domain encompassing the trochanter, femur, tiba and tarsus (Zhang *et al.*, 2005). However, in both instances there are no obvious unique T1 leg features that can be associated with these observed expression patterns. This suggests that the expansion of SCR into a novel domain (T1 leg) preceded the apparent gain of function. In contrast, grasshoppers never accumulate SCR protein in the T1 leg at any time during development (Fig. 3C). In orthopterans therefore, SCR protein patterns in the prothoracic leg are labile and cannot be linked to any specific T1 leg trait.

In the two dictyopteran species (cockroach and praying man-

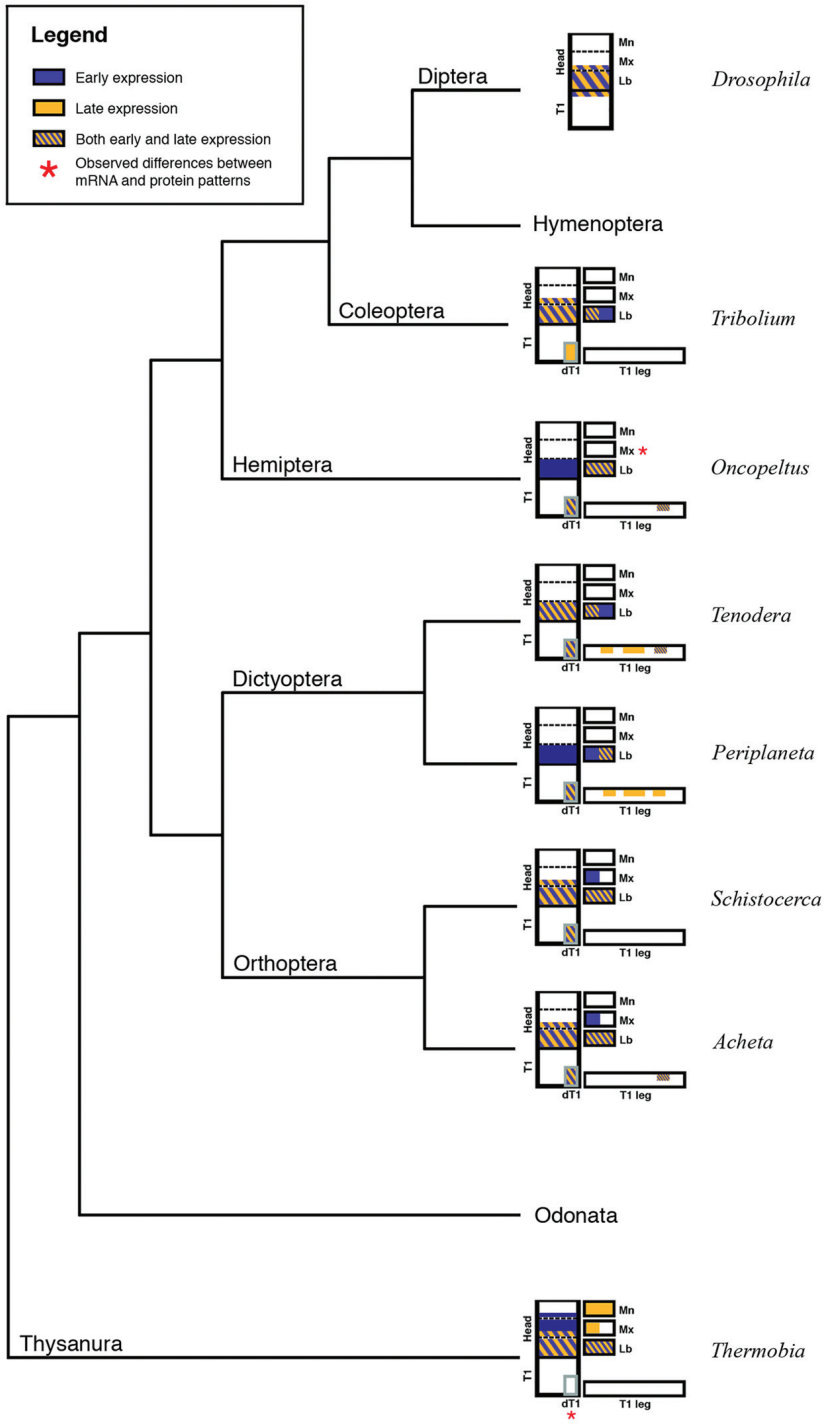

Fig. 4. Generalized summary of SCR protein expression patterns of the insect species examined in this study. *The large rectangle on the left represents the mid-ventral region of the head and first thoracic segments separated by dashed lines. The smaller rectangles on the right correspond to the associated appendages of these segments. Blue domains indicate early SCR expression and orange domains represent signal that appears only at later stages of development. Striped blue and orange domains indicate expression that occurs at both early and late developmental stages. A red asterisk denotes regions in which SCR protein accumulation differs from previously reported mRNA patterns (Rogers et. al 1997). Abbreviations: Mn, mandible; Mx, maxillae; Lb, labium; dT1 dorsal T1 region.*

tis), SCR accumulates in multiple regions along the T1 legs but only at later stages of development, around the time when leg segmentation becomes evident. As shown in Fig. 3D, no SCR protein can be detected in the T1 legs of cockroach embryos at earlier embryonic stages (~30%). At ~45% development, strong signal is detected in most of the tibia and in the distal half of the femur (Fig. 3D, right). In addition, a more moderate signal is also present in a smaller cluster of cells in the proximal region of the coxa. While the T1 legs of the cockroach exhibit spur-like outgrowths along the posterior border of these developing appendages, they are not unique structures as both T2 and T3 legs have a similar morphology. SCR accumulation in the T1 legs of *Periplaneta* therefore likely represents another variation of expression with no apparent phenotype, similar to the situation observed in orthopterans.

Compared to cockroaches, SCR expression in mantids (*Tenodera*) starts at earlier developmental stages in a small patch of cells restricted to the anterior margin of the tibia (Fig. 3E). At ~45% development, SCR patterning in the T1 leg changes and becomes confined to the opposite (posterior) edge encompassing four discrete domains: the coxa, the anterior and posterior femur separated by a narrow gap, and the tibia (Fig. 3E bottom left). As depicted in Fig. 3E (right), the prothoracic legs of mantids are highly modified appendages characterized by the presence of unique rows of spurs on the distal femoral and tibial segments. Hence, the later SCR expression patterns in these segments can be directly associated with this T1-leg specific morphology. At the same time, the SCR signal in the coxa and proximal femur do not correspond to any particular phenotype as these regions are devoid of any defining characteristic. Thus, in *Tenodera* we have a situation where the distal half of SCR expression is associated with the development of the grasping apparatus on T1, while the proximal half has no apparent function.

Finally, both mRNA expression and functional analyses in *Oncopeltus* (Hughes and Kaufman, 2000; Rogers *et al.*, 1997; Rogers *et al.*, 2002) have shown that *Scr* governs the formation of the sex combs on the distal tibia of the T1 leg (Fig. 3F inset, top). Consistent with these data, SCR protein accumulates in the same region (distal tibia) as the previously reported mRNA expression patterns (Fig. 3F). Studies in *Drosophila* and *Tribolium* have shown that the ability of SCR to direct the formation of T1 leg combs may be a shared feature in holometabolous species, as hypomorphic alleles in these lineages result in the abolition of this structure (Beeman *et al.*, 1989; Pattatucci *et al.*, 1991). Overall, these data suggest that SCR may also function in directing the formation of T1-specific structures in other insects, such as the grasping apparatus present in mantids.

Discussion

Studies utilizing cross-reacting antibodies can provide valuable insight into the evolution of expression patterns, and to a certain degree, gene function(s) in a broad range of taxa. Our global analysis of SCR protein accumulation in hemimetabolous species shows that the expression patterns of this gene are highly dynamic and species-specific. As illustrated in Fig. 4, there are five trends with regard to the evolution of the expression and putative function of this gene in insects. First, the domain of SCR is confined to the head with no expression in the dT1 or prothoracic regions in the basal insect lineage *Thermobia*. Second, in this species, the most anterior border of expression is in the mandibular segment. Rogers *et al.* (1997) originally reported that the anterior boundary of *Scr* mRNA accumulation is in the posterior maxillary region. Note, however, that the earliest embryo used in that analysis is at a mid-developmental stage (~40%), similar to the one shown in our Fig. 1C. Consistent with the mRNA data, the mandibles also lack SCR protein accumulation at this stage. However, in the present analysis we were able to obtain SCR expression patterns at both earlier and later stages of development (~30% and ~75%, respectively) that show novel accumulation in the mandibles (Fig. 1 A,E). *Thermobia* therefore displays the most anterior domain of SCR expression reported to date. Third, with the exception of the firebrats, all species analyzed in this report have expression in the dT1 region. Several studies in more derived insect species have indicated that *Scr* expression in dT1 is a conserved feature and functions in suppressing wing development on this segment (Beeman *et al.*, 1989; Carroll *et al.*, 1995; Chesebro *et al.*, 2009; Rogers *et al.*, 1997). Note however, that the firebrat represents a primitively wingless species. This observation led to the original proposition that the expression of *Scr* mRNA in the dT1 region likely predates the origin of wings (Rogers *et al.*, 1997). Our data supports this view and further suggests that the presence of mRNA in the firebrat dT1 reveals the beginning of an expansion of *Scr* into the prothorax. Fourth, while *Scr* mRNA transcript can be detected in the dT1 of firebrats and the maxillary segment of milkweed bugs (Rogers *et al.*, 1997), no eventual protein accumulates in these two regions (Fig. 4, asterisks). The observed discrepancy between mRNA and protein accumulation suggests the presence of a post-transcriptional regulatory mechanism, similar to the one previously reported in crustaceans (Abzhanov and Kaufman, 1999). Finally, SCR expression in the prothoracic legs of different insect species is highly labile and can be gained or lost easily. In addition, it is only in more late-branching lineages that domains of expression can be directly associated with T1-specific morphologies suggesting that the acquisition of SCR in the prothoracic leg may have preceded any apparent gain of function.

The availability of both protein and mRNA expression patterns provides for a much more detailed understanding of the functions of *Scr* in insects. For example, the original report of mRNA patterning in *Oncopeltus* showed that *Scr* is primarily localized in the labial segment. The signal is also confined to a few cells in the maxillary region and is never observed in the mandibles (Rogers *et al.*, 1997). Subsequent embryonic RNAi analysis confirmed that the primary influence of *Scr* is in the labial segment, indicated by the transformation of this appendage toward a leg-like identity (Hughes and Kaufman, 2000). However, phenotypic changes were also observed in the mandibular and maxillary stylets which were transformed into a mass of undifferentiated tissue. This result was inconsistent with the observed mRNA pattern leading to the suggestion that non-local indirect effects may be responsible for generating such phenotypes (Hughes and Kaufman, 2000). The present study provides an independent corroboration of this view by showing that indeed no SCR protein is present in these segments at any time during development. Hence, the function of *Scr* in the head region of *Oncopeltus* is restricted to the labial segment only, and should have no effect on the development of the more anterior mouthparts. Similarly, previous studies

in *Drosophila* and *Tribolium* showed that *Scr*/*Cx* mutations do not affect the maxillary appendages (Beeman *et al.*, 1989; Curtis *et al.*, 2001; Pattatucci *et al.*, 1991; Shippy *et al.*, 2006; Wakimoto and Kaufman, 1981), suggesting that restriction of its function to the labium preceded the divergence between hemimetabolous and holometabolous insect lineages. As illustrated by Fig. 4, among hemimetabolous species, the orthopterans have SCR expression in both the maxillary and labial appendages and represent an ideal choice for future extension of functional studies. Such studies will be able to delineate *Scr* function in both of these segments, thus providing a more complete understanding of this gene's role in the evolution of insect mouthparts.

The current study also reveals a trend toward the continuous posteriorization of the anterior border of SCR expression in the insect head consistent with the divergence of insect lineages from early to late-branching. While the basal insect species *Thermobia* shows clear signal in the mandibles at early and late developmental stages, the most anterior expression of SCR is in the maxillary segment in the orthopterans (cricket and grasshopper). An identical mRNA pattern has been reported in another cricket species (*Gryllus bimaculatas*), suggesting that the maxillary border or *Scr* expression may be a conserved feature within the orthopterans (Zhang *et al.*, 2005). Finally, dictyopterans and hemipterans (cockroach, mantis, and milkweed bug) all have an anterior border of SCR expression in the labial segment. Due to the fact that *Thermobia* represents an early-branching insect group, it is tempting to speculate that SCR patterning in this lineage represents the ancestral anterior border of expression in the insect head. Alternatively, it is possible that *Thermobia* acquired a derived character state that is species-specific. In order to distinguish between these two alternatives it is necessary to extend SCR expression analyses to encompass other more early-branching lineages (such as Odonata) in order to truly delineate what is the ancestral patterning in the head region. It is equally important to note that studies of SCR protein accumulation in the highly derived holometabolous insects *Drosophila* and *Tribolium* show that the anterior expression boundary reverts back to the posterior portion of the maxillary segment (Carroll *et al.*, 1988; Curtis *et al.*, 2001; Riley *et al.*, 1987; Shippy *et al.*, 2006). This result suggests that while SCR protein expression was lost in the maxillary segment of late-branching hemimetabolous lineages, it was subsequently regained prior to the divergence of the Holometabola.

Classical studies in *Drosophila* and *Tribolium* have indicated that one of the primary functions of *Scr* is to provide identity to the prothoracic (T1) segment and that this role is likely conserved in all insects (Beeman *et al.*, 1989; Curtis *et al.*, 2001; Pattatucci *et al.*, 1991; Shippy *et al.*, 2006; Wakimoto and Kaufman, 1981). Furthermore, recent insight from *Oncopeltus* shows that in hemimetabolous species, the role of *Scr* in directing T1 morphology is restricted to post-embryonic development (Chesebro *et al.*, 2009). This finding highlights the importance of determining the role(s) of *Scr* in the prothorax of other insect lineages such as orthopterans and dictyopterans. In addition, many hemimetabolous species display extreme morphological modifications of their T1 segments that are established during post-embryogenesis. Except for *Oncopeltus*, virtually no data is currently available regarding the potential role *Scr* may play in the divergence of the prothorax during this stage of development. Addressing these questions will be necessary for determining the full extent of *Scr* involvement in the evolution of T1 morphology in hemimetabolous insect species.

Materials and Methods

The laboratory cultures of firebrats (*Thermobia domestica*), crickets (*Acheta domesticus*) and milkweed bugs (*Oncopeltus fasciatus*) were reared under conditions previously described in Rogers *et al.,*1997) and Peterson *et al.,*1999). The egg cases of the cockroach (*Periplaneta americana*) and the praying mantis (*Tenodera aridifolia*) were purchased from Carolina Biological Supply Company and were used to establish laboratory cultures. The grasshopper (*Schistocerca americana*) embryos were gifts from Markus Friedrich (Wayne State University).

Dissection and fixation of embryos was performed as previously described in Mahfooz *et al.* (2004). Expression was detected by using a rat polyclonal antibody generated against a C-terminal fragment of *Drosophila* SCR and kindly donated by D.J. Andrew and M.P. Scott (unpublished). This antibody was subsequently described and was found to cross-react to SCR in crustaceans (Abzhanov and Kaufman, 1999) and *Tribolium* (Curtis *et al.*, 2001). In this study, we confirmed that the protein patterns produced by this antibody closely mirror signal observed in parallel *in situ* hybridization experiments in *Periplaneta* and *Oncopeltus* (Supp. Fig. 1). The staining was performed as described in Mahfooz *et al.* (2004). The antibody was detected by using a secondary anti-rat antibody that was conjugated to FITC, horseradish peroxidase, or alkaline phosphatase (The Jackson Laboratory). Detailed protocols on maintaining insect cultures, collection of embryos and antibody staining are available upon request.

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