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Evolution of Developmental Control Mechanisms

Giant, *Krüppel*, and *caudal* act as gap genes with extensive roles in patterning the honeybee embryo

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ABSTRACT

In *Drosophila*, gap genes translate positional information from gradients of maternal coordinate activity and act to position the periodic patterns of pair-rule gene stripes across broad domains of the embryo. In holometabolous insects, maternal coordinate genes are fast-evolving, the domains that gap genes specify often differ from their orthologues in *Drosophila* while the expression of pair-rule genes is more conserved. This implies that gap genes may buffer the fast-evolving maternal coordinate genes to give a more conserved pair-rule output. To test this idea, we have examined the function and expression of three honeybee orthologues of gap genes, *Krüppel*, *caudal*, and *giant*. In honeybees, where many *Drosophila* maternal coordinate genes are missing, these three gap genes have more extensive domains of expression and activity than in other insects. Unusually, honeybee *caudal* mRNA is initially localized to the anterior of the oocyte and embryo, yet it has no discernible function in that domain. We have also examined the influence of these three genes on the expression of honeybee *even-skipped* and a honeybee orthologue of *engrailed* and show that the way that these genes influence segmental patterning differs from *Drosophila*. We conclude that while the fundamental function of these gap genes is conserved in the honeybee, shifts in their expression and function have occurred, perhaps due to the apparently different maternal patterning systems in this insect.

Introduction

Evidence implies that the initial events in a development pathway may evolve more rapidly than later ones and that the earliest acting genes in a cascade may be the most recently evolved (Davidson, 2006; Levine and Davidson, 2005; Wilkins, 2002). While this bottom-up mode of evolution is understandable when describing the development of particular structures in an organism, it is perhaps counter-intuitive when applied to the earliest events in development, such as axis formation, since all later events depend on this foundation.

This bottom-up mode of evolution is supported, however, by studies of segmentation genes in insects. In *Drosophila*, segmentation genes are separated into 4 classes based on their mutant phenotypes (Nusslein-Volhard and Wieschaus, 1980): maternal coordinate genes, gap genes, pair-rule genes, and segment polarity genes. During segmentation, these genes act sequentially, with maternal coordinate genes acting earliest to establish axes. Studies of the orthologues of these genes in other insects demonstrate that much of the variability in the expression of segmentation gene orthologues lies early in the pathway, particularly within the gap genes (Bucher and Klingler, 2004; Huang et al., in press; Liu and Kaufman, 2004; Mito et al., 2006; Patel et al., 2001) and that the maternal coordinate genes are rapidly evolving and even missing from the genomes of some insects (Dearden et al., 2006). A remarkable example is the anterior determinant in *Drosophila*, *bicoid* (*bcd*), which is an invention of the Diptera (Stauber et al., 2000). These findings raise the question; how do fast-evolving early events in segmentation integrate with more conserved events later in the pathway?

Three gap genes, *giant* (*gt*), *caudal* (*cad*), and *krüppel* (*kr*), have been extensively studied outside of *Drosophila*. *Giant* encodes a leucine zipper transcriptional repressor expressed in domains at the anterior and posterior ends of the *Drosophila* embryo (Kraut and Levine, 1991b) regulating both pair-rule genes and the Hox gene *antennapedia* (Langeland et al., 1994; Reinitz and Levine, 1990; Small et al., 1991; Wu et al., 1998) as well as restricting expression of other gap gene products (Kraut and Levine, 1991a). *Dm-gt* mutants have defects in head and abdominal segments (A5–A7) (Mohler et al., 1989; Petschek et al., 1987). In *Tribolium castaneum*, a short germ band insect, *Tc-giant* is expressed in the head, trunk, and posterior and knockdown of *Tc-gt* results in multiple segmentation defects (Bucher and Klingler, 2004). In *Nasonia vitripennis*, a long germ band hymenopteran, *Nv-gt* is initially expressed maternally with transcript localized to the anterior pole in oocytes. RNAi knockdown of *Nv-gt*

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results in loss of head and thoracic segments and fusion of A6 and A7 segments (Brent et al., 2007).

The abdominal gap gene *Krüppel* has a more conserved role in insect segmentation. In *Drosophila*, *Kr* is expressed in a central domain and *Kr* mutants lack T2–T3 and first five abdominal segments (A1–A5) (Gaul et al., 1987; Wieschaus et al., 1984). In *Nasonia*, *Nv-Kr* is expressed in a similar central domain (Olesnicky et al., 2006), and RNAi knockdown results in the loss of T3 and A1–A4 segments (Brent et al., 2007). In *Tribolium*, expression begins at the posterior pole of the blastoderm corresponding to T3/A1 regions (Cerny et al., 2005). Later, this central domain expression is in the thoracic segments of the embryo and in the developing CNS and gut. In *Tc-Kr* embryos, the head is normal, but there is both deletion and homeotic transformation of segments (Cerny et al., 2005).

Caudal is a key player in patterning the posterior of many embryos including the posterior abdominal segments in insects. In *Drosophila*, cad protein accumulates in a gradient across the anterior–posterior axis in the syncytial blastoderm (Mlodzik and Gehring, 1987) and is required for posterior development (Wu and Lengyel, 1998). In *Nasonia*, *Nv-cad* maternal mRNA is localized to the oosome, in the posterior, and RNAi causes defects in posterior development (Olesnicky et al., 2006). *Tribolium cad* is detected in the posterior of the elongating embryo, and RNAi knockdown results in embryos lacking posterior segments (Copf et al., 2004; Schulz et al., 1998).

These gap genes provide important information for positioning pair-rule gene expression domains along the AP axis in *Drosophila*. Pair-rule genes are expressed as stripes across the embryo and are the first indication of the placement of parasegment boundaries. The gap genes and maternal coordinate genes direct expression of pair-rule genes via stripe-specific enhancers (Peel et al., 2005). Studies have indicated that many pair-rule gene orthologues in holometabolous insects are expressed in a stripe pattern (Binner and Sander, 1997; Brown et al., 1994; Choe et al., 2006; Eckert et al., 2004; Osborne and Dearden, 2005a; Patel et al., 1992b, 1994; Rohr et al., 1999), indicating that the contribution of the pair-rule genes to segmentation is conserved or is evolving more slowly compared with the maternal coordinate and gap genes. There are, however, some differences, particularly with regards to timing; in contrast to *Drosophila*, in which all stripes of *eve* appear simultaneously, the expression of *eve* stripes in many insects (Patel et al., 1992a, 1994), including the honeybee (Binner and Sander, 1997), progressively appears in an anterior-to-posterior succession. How is this relatively conserved pair-rule expression achieved with significant changes in the pathway occurring upstream?

Honeybees (*Apis mellifera*) are an excellent comparative model system for studying the evolution of developmental pathways, as both *Apis* and *Drosophila* are long germ band insects (Fleig and Sander, 1986; Sander, 1976), although it is not clear if they evolved this mode of development independently. The lineage leading to honeybees separated from that leading to *Drosophila* about 300 million years ago and from *Nasonia* by 150 million years ago (The Honey Bee Genome Sequencing Consortium, 2006). In honeybees, orthologues of a number of key maternal coordinate genes are missing from the genome (Dearden et al., 2006), while all pair-rule gene orthologues are present, and those that have been studied are expressed with dual-segment periodicity (Binner and Sander, 1997; Osborne and Dearden, 2005a). Likewise, segment polarity genes are conserved and are expressed and function in similar ways to their *Drosophila* orthologues (Beye et al., 2002; Dearden et al., 2006; Osborne and Dearden, 2005a, 2005b). Between the maternal coordinate genes and the pair-rule genes lie the gap genes, which must act to buffer the rapid rate of evolution of the maternal coordinate genes to provide a reasonably stable output for the more sedately evolving pair-rule genes. It is also possible that the *Drosophila* maternal coordinate genes represent an additional, non-conserved component to the pathway and that gap genes in other species may act as maternal coordinate genes. In this paper, we investigate the expression and function of three honeybee gap gene orthologues (*giant*, *Krüppel*, and *caudal*), particularly examining how their expression and function differs from that of *Drosophila* and how that may reflect changes in maternal coordinate gene function. We also examine how these genes regulate *even-skipped*, a key downstream target of all three of these genes in *Drosophila*.

Results

Am-giant plays an essential role in anterior patterning in honeybees

Blast searches identified one gene *GB16015* with similarity to *Drosophila gt*. Phylogenetic analyses using the predicted amino acid sequences (Fig. 1A) demonstrate that GB16015 clusters with other *gt* sequences from insects with high posterior probability, against *Drosophila* Dp1 and GB10413, the most similar genes in the *Drosophila* and honeybee genomes, respectively. We therefore designate the GB16015 as *Am-giant* (*Am-gt*).

In situ hybridization was carried out to determine the expression pattern of *Am-gt* in the honeybee queen ovary and during embryogenesis (Fig. 2A). *Am-gt* mRNA was detected in a subset of nurse cells, those closest to the oocyte and at low levels in the vitellarium. In stage 1 embryos (0–4 hours after egg laying), maternal *Am-gt* RNA is distributed throughout the embryo with higher concentrations in the anterior third (Fig. 2B).

In stage 2 honeybee embryos, as the blastoderm forms (Fig. 2C), zygotic *Am-gt* RNA is expressed in an anterior domain of cells and, more weakly, in a posterior cap of cells that later (late stage 3; Fig. 2D) resolves into a stripe of cells expressing *Am-gt* RNA. By early stage 4, anterior expression of *Am-gt* RNA becomes reduced in cells at the dorsal tip of the anterior end of the embryo (Figs. 2E and F) and is lost from cells in regions where the brain and dorsal head structures develop. By stage 6, *Am-gt* expression is lost from cells at the posterior end of the embryo (data not shown). At stage 7, expression of *Am-gt* RNA remains present in the ventral–anterior region, where the future head structures and gnathal appendages are forming (Fig. 2G). The late embryonic expression of *Am-gt* RNA is in a complex pattern in ventral gnathal regions where the mandibles and maxillae are being patterned and in paired domains in tissue surrounding the labrum (Figs. 2H and I). *Am-gt* expression was not detected anywhere in the embryo after stage 9 (data not shown).

To determine the function of the *Am-gt* in segmental patterning in the honeybee, we injected dsRNA produced from the *Am-gt* gene into just-laid, syncytial, honeybee embryos to trigger gene knockdown by RNA interference (RNAi) (Beye et al., 2002; Wilson and Dearden, 2009). Injected embryos were maintained until hatching to allow examination of larval morphology and compared with those injected with dsRNA from an EGFP gene used as a control. Larvae injected as syncytial embryos with dsRNA targeting the *Am-gt* gene all have missing head and thoracic segments ($n = 56$), while some abdominal segments and the posterior terminal structures are normal (Figs. 2J and K). To determine which segments are affected by reduction in *Am-gt* RNA, we stained *Am-gt* RNAi embryos at stage 9 for RNA from *e30*, a honeybee orthologue of *engrailed*, a segment polarity gene that marks cells in the posterior of every segment (Walldorf et al., 1989). When compared to control-injected embryos (Fig. 2L), *e30*-stained *Am-gt* RNAi embryos are missing *e30* stripes from all head, gnathal and thoracic segments (Fig. 2M). In addition, stripes of *e30* expression normally marking A1 and A2, and A6 and A7 were fused.

Am-Kr is required for patterning the central abdominal region

Blast searches identified one honeybee gene with significant similarity to *Drosophila Krüppel*; *GB16053*. Phylogenetic analyses (Fig. 1B) demonstrate that GB16053 clusters with other Krüppel-like sequences from arthropods and deuterostomes with high posterior

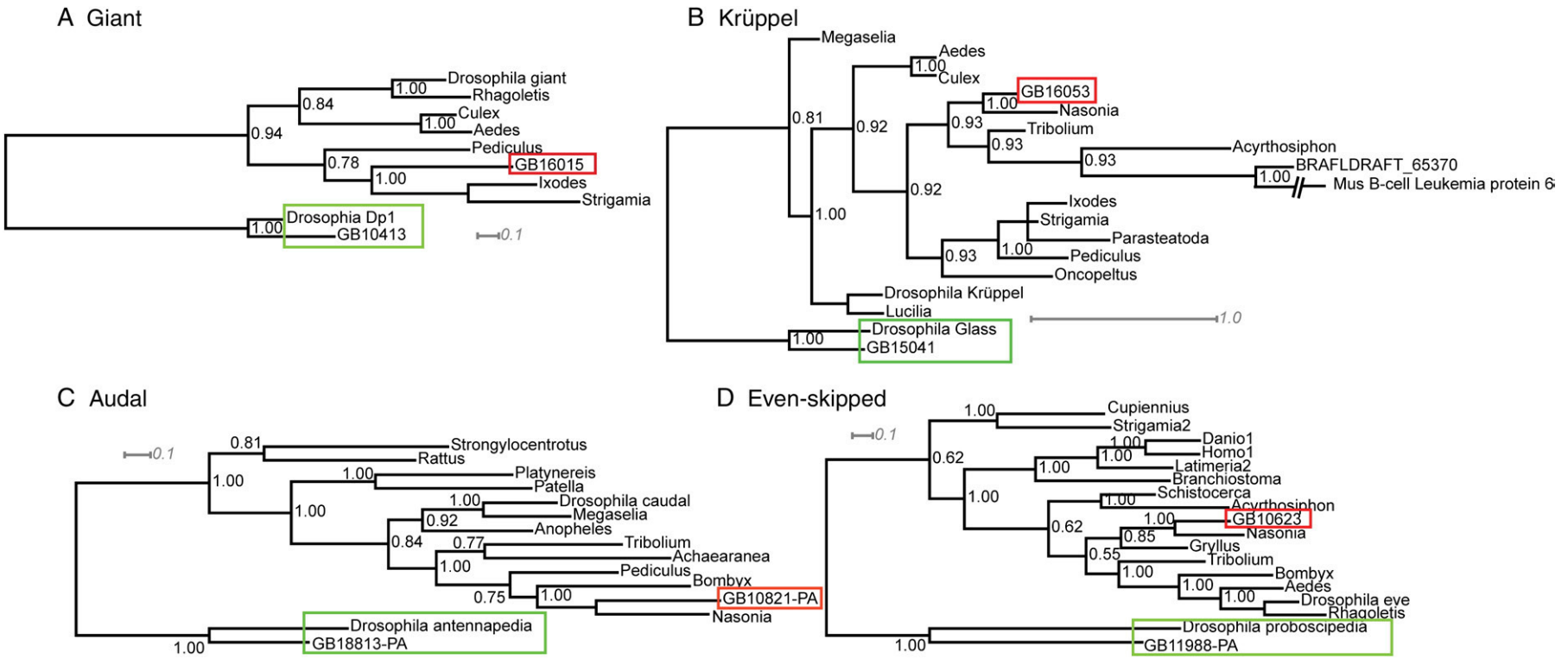

Fig. 1. Phylogenetic analyses of honeybee giant, Krüppel, caudal, and even-skipped orthologues. All phylograms are generated by Bayesian inference of phylogeny; node labels are posterior probabilities. (A) Honeybee giant. Predicted protein sequence from GB16015 (red box), clusters with other giant protein sequences from insects and not with the closely related Dp1 and its honeybee orthologue GB10413 (green box). (B) Honeybee Krüppel. The predicted protein sequence of GB16053 clusters with insect and vertebrate Krüppel genes against *Drosophila* glass and its honeybee orthologue GB15041. (C) Honeybee caudal. GB10821 protein sequence clusters with caudal proteins against *Drosophila* and honeybee antennapedia. (D) Honeybee even-skipped. Predicted protein sequence from GB10623 clusters with even-skipped genes against *Drosophila* and honeybee proboscipedia.

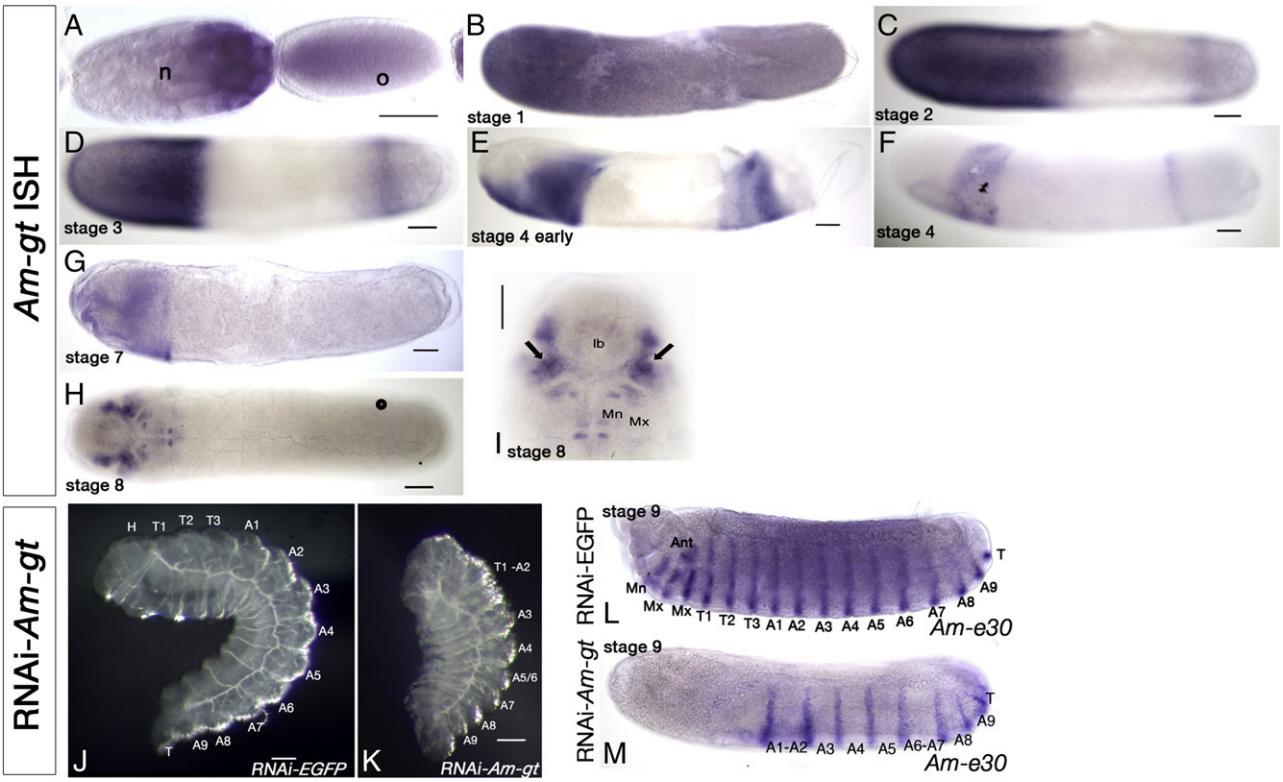

Fig. 2. Expression and knockdown of *Am-gt*. All ovaries/embryos/larvae are oriented with anterior to the left and dorsal up, unless otherwise stated. Scale bars indicate 100 μm. (A) *Am-gt* RNA as detected by *in situ* hybridization is expressed maternally by the nurse cells (n) and loaded into the oocyte (o), where it is detected throughout the oocyte cytoplasm. (B) In just-laid embryos (stage 1), *Am-gt* RNA can be detected throughout the embryo, with a slightly higher concentration in the anterior third. Shortly afterwards (stage 2; C) *Am-gt* RNA becomes up-regulated in cells throughout the anterior half of the embryo and in a cap of posterior cells. By stage 3 (D), expression of *Am-gt* RNA focuses down on cells in the anterior third of the embryo and in a posterior stripe. By stage 4 (E and F), *Am-gt* RNA is lost from the anterior–dorsal–lateral regions of the embryo, where the future brain is patterning. An anterior stripe of expression, and expression along the ventral–anterior of the embryo (where the head structures emerge) remains. Posterior stripe expression ceases just prior to gastrulation (stage 6; G). Late-stage embryonic *Am-gt* RNA (stage 9 (H and I)) expression occurs, in the folds on either side of the future labrum (arrows in I) and where the head appendages are forming. No expression of *Am-gt* detected beyond this stage (data not shown). RNAi knockdown of *Am-gt* expression by injection in just-laid embryos results in loss of anterior segments, and disruption of abdominal ones, in honeybee larvae (J and K). Embryos injected with dsRNA against either EGPF (J) or *Am-gt* (K) were incubated for 72 hours until hatching. *Am-gt*-RNAi larvae lack head and thoracic and anterior abdominal segments. Putative segment assignments in K are based on e30 staining of stage 9 embryos. (L and M) Expression of e30 mRNA in EGFP RNAi-treated (L) and *Am-gt* RNAi-treated (M) embryos at stage 9. Engrailed stripes from head and thorax are missing. Stripes in A1–A2 and A6–A7 segments are missing. Missing segments and stripes determined by morphology and through comparisons between measurements of e30 staining RNAi and wild-type embryos.

probabilities, against a clade containing *Drosophila* glass and GB15041, the most closely related *Drosophila* and honeybee genes. We thus designate *GB16053* as *Am-Krüppel* (*Am-Kr*).

The ovarian and embryonic expression patterns of *Am-Kr* RNA were examined by *in situ* hybridization. In the vitellarium of queen honeybee ovaries, only faint *Am-Kr* RNA expression can be detected indicating a low level of maternal RNA expression (data not shown). Faint staining can also be seen in just-laid embryos (stage 1, Fig. 3A). The first significant expression of the *Am-Kr* transcript is detected in the syncytial blastoderm (stages 2–3) as a broad central domain of expression (Fig. 3B). This domain is maintained through stage 4, when *Am-Kr* RNA also begins to be expressed in cells in a ventral–anterior domain and weakly in cells in the posterior (Fig. 3C). As gastrulation begins (stage 5), the central domain splits into 3 stripes of cells expressing *Am-Kr* RNA (asterisks in Fig. 3D). The central stripes of *Am-Kr* RNA vanish at stage 6 and *Am-Kr* RNA becomes widely expressed in ectoderm on either side of the gastrulation furrow (Figs. 3E and F). The anterior expression domain of *Am-Kr* RNA remains and is present in cells in the labrum and stomodeal invagination, regions that likely correspond to the forming stomatogastric nervous system (SNS) which derives from the labrum and controls the motion of the foregut. In *Drosophila, Krüppel* is also expressed by precursor cells of the SNS (Gaul et al., 1987). The domain of *Am-Kr* RNA expression in the posterior is in regions corresponding to the hindgut, malpighian tubule, and posterior midgut rudiment. Late embryonic *Am-Kr* RNA expression (stage 9; Fig. 3G) was found in the neuroblasts of the brain and CNS.

RNAi knockdown of *Am-Kr* expression was used to determine the function of *Am-Kr* in honeybee segmentation. Larvae derived from *Am-Kr* dsRNA-injected embryos have defective, probably fused, thoracic and central abdominal segments, affecting T1 to at least the A6 segment (Figs. 3H and I) ($n = 45$). Head and gnathal structures in these larvae appear normal including the labrum, mandibles, and maxillae, although it is unclear if internal structures are defective (Figs. 3H and I). Staining of *Am-kr*-RNAi embryos for *e30* revealed that *e30* stripes are present for head appendage segments and for the four most posterior segments (A7–posterior terminus) but that expression is lost in all central regions of the embryo (Fig. 3J). The thoracic and central abdominal regions lost in *Am-Kr* RNAi larvae correspond to the broad central blastoderm domain expression of this gene.

Am-caudal is required for both abdominal and posterior patterning

Phylogenetic analyses (Fig. 1C) indicate that the honeybee gene GB10821 clusters with other caudal sequences with high posterior probability to the exclusion of a clade containing a related homeobox protein, Antennapedia, from *Drosophila* and honeybee (GB18813). We designate the *GB10821* gene *Am-caudal* (*Am-cad*).

We examined the expression of *Am-cad* mRNA in the honeybee queen ovary. *Am-cad* mRNA is highly expressed maternally in the

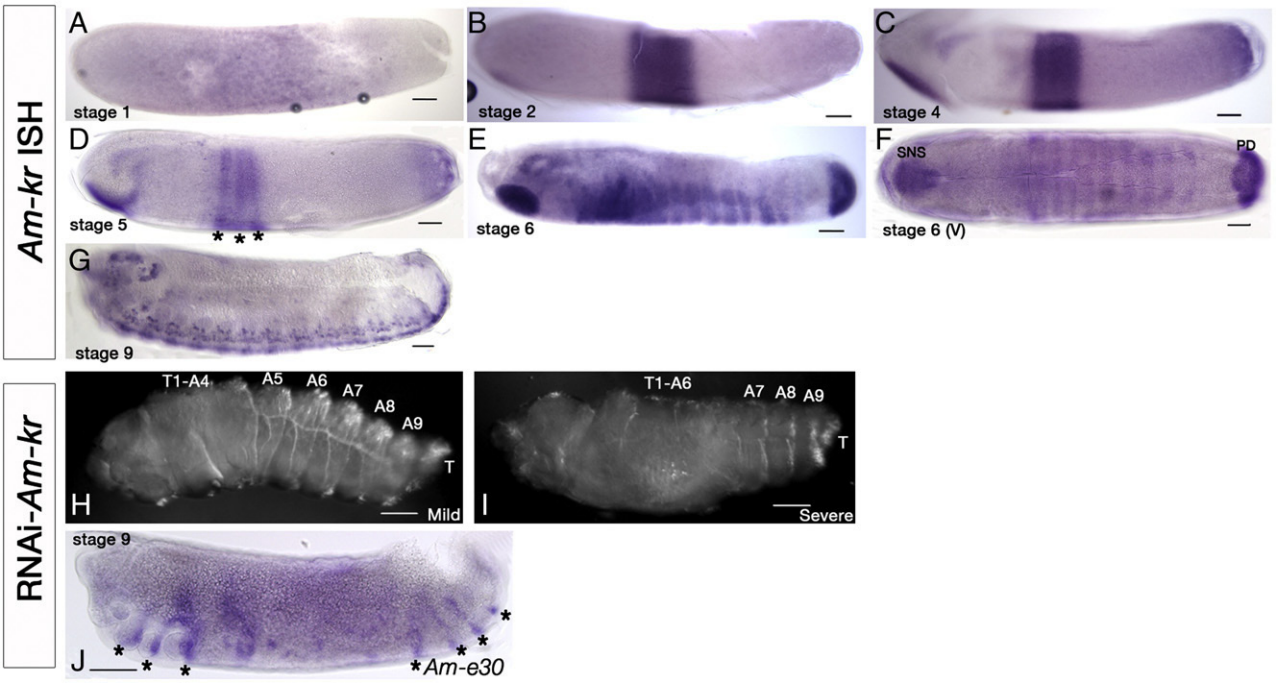

Fig. 3. Embryonic expression and knockdown of *Am-Kr*. All embryos/larvae are oriented with anterior to the left and dorsal up, unless otherwise stated. Scale bars indicate 100 μm. (A) Expression of *Am-Kr* RNA is first faintly detected at stage 1 in cells in a diffuse central domain as energids populate the embryo surface. By stage 2 (B), *Am-Kr RNA* can be detected in blastoderm cells in a broad domain in the center of the embryo. (C) In stage 4 embryos, *Am-Kr* RNA is present in this central domain, in an anterior/ventral group of cells, and in cells in a posterior cap. At stage 5 (D), the central domain expression splits into three stripes (asterisks in D). (E and F) In stage 6 embryos (E—lateral view, F—ventral view), *Am-Kr* RNA is present at low levels throughout the central regions of the ventral ectoderm, in a domain in regions from which the stomatogastric nervous system develops (SNS), and in a posterior domain (PD). By stage 9 (G), *Am-Kr* RNA is restricted to ventral neuroblasts and the SNS. RNAi knockdown of *Am-Kr* RNA in just-laid embryos results in larvae that have normal head and posterior regions but central segments are disorganized and fused (H and I). Putative segment assignments in H and I are based on e30 staining of stage 9 embryos, (H) shows a mild phenotype ($n=6/45$ with a phenotype after injection), (I) shows a more severe phenotype ($n=39/45$). *e30* staining of *Am-Kr* RNAi-treated embryos (J) reveals that while head and posterior stripes of e30 are present (asterisks in J), both thoracic and anterior abdominal segments (A1–A5) are fused together. Missing segments and stripes determined by morphology and through comparisons between measurements of e30 staining RNAi and wild-type embryos.

vitellarium from all the nurse cells and is transported into the oocyte where the transcript is localized, unusually, to the anterior half of each oocyte (Fig. 4A). Fluorescent *in situ* hybridization and confocal microscopy of oocytes indicate that *Am-cad* RNA is localized to the anterior cortex of the oocyte (Fig. 4B). This atypical localization is maintained in freshly laid stage 1 embryos (0–4 hours after egg laying; Figs. 4C and D). *Am-cad* mRNA then migrates in association with dividing nuclei (Fig. 4E and Supplementary Fig. 1) through the syncytial blastoderm to the posterior two-thirds of the embryo by stage 3 (Figs. 4F and G). At this stage, zygotic transcription is initiated, as determined by the appearance of nuclear dots of nascent transcripts, in this broad-expression domain (Fig. 4H). By stage 4, expression is lost in stripes through this region in an anterior-to-posterior succession (Fig. 4I). *Am-cad* RNA then becomes limited to a posterior stripe of expressing cells by stage 6 (as gastrulation occurs), around the developing hindgut and anal plate (Figs. 4J and K). This stripe of expressing cells is maintained until the end of embryogenesis (Fig. 4L) where RNA is present in the ectoderm surrounding the proctodeum (Fig. 4M).

Injection of dsRNA targeting the *Am-cad* gene RNAi results in a range of phenotypes from mild to severe, all with some degree of loss of abdominal patterning. In mildly affected larvae ($n=6$), there is fusion of segments and loss of the very posterior (A7–A9) segments (Figs. 4N and O). In severely affected larvae ($n=30$), there are no visible segments and the posterior terminus of the embryo fails to form at all (Fig. 4P). In severely affected embryos, *e30* RNA expression is not organized into stripes indicating extensive loss of segmental pattern throughout the embryos (Figs. 4Q and R), though in all cases, the head region and brain develop correctly. This indicates that *Am-cad* is required for patterning of both abdominal and posterior terminal structures.

Honeybee gap genes contribute to correct positioning of Am-eve stripe expression

The *Drosophila* orthologues of all the genes examined in this work act to establish the expression of the pair-rule gene, *even-skipped*, in the *Drosophila* embryo (Small et al., 1992). *Even-skipped* is a key primary pair-rule gene that acts, with others, to establish segmentation polarity gene expression in each segment (Fujioka et al., 1995). In *Drosophila*, *eve* expression is regulated through a series of independent and modular stripe enhancers (Small et al., 1993, 1992, 1996). These enhancers have been well characterized in drosophilids (Ludwig et al., 2000; Ludwig and Kreitman, 1995; Ludwig et al., 1998) but have not been investigated in any detail in other organisms. We aimed to determine what role gap genes had on the regulation of the honeybee orthologue of *eve* by knocking down the expression of these gap genes with RNAi and assaying for *eve* RNA expression.

One predicted coding sequence in the honeybee genome, *GB10623*, was identified as being similar to *even-skipped*. Phylogenetic analyses (Fig. 1D) indicate that GB10623 clusters with other *even-skipped* genes, with reasonable posterior probabilities, against a clade containing the related homeobox containing proteins; *Drosophila* proboscipedia, and its orthologue from honeybee GB11988. This phylogenetic analysis indicates that *GB10623* is the most likely orthologue of *Drosophila even-skipped*, and so we name it *Am-even-skipped* (*Am-eve*).

Am-eve expression is first detected at stage 4 (24 hours after laying) in a broad domain of cells throughout the abdominal and posterior regions of the embryo (Fig. 5A). The anterior end of this domain has a broad ring of higher expression running in a ring around the embryo. By early stage 5, all but this anterior band of cells loses *Am-eve* expression and this anterior domain becomes curved. At the

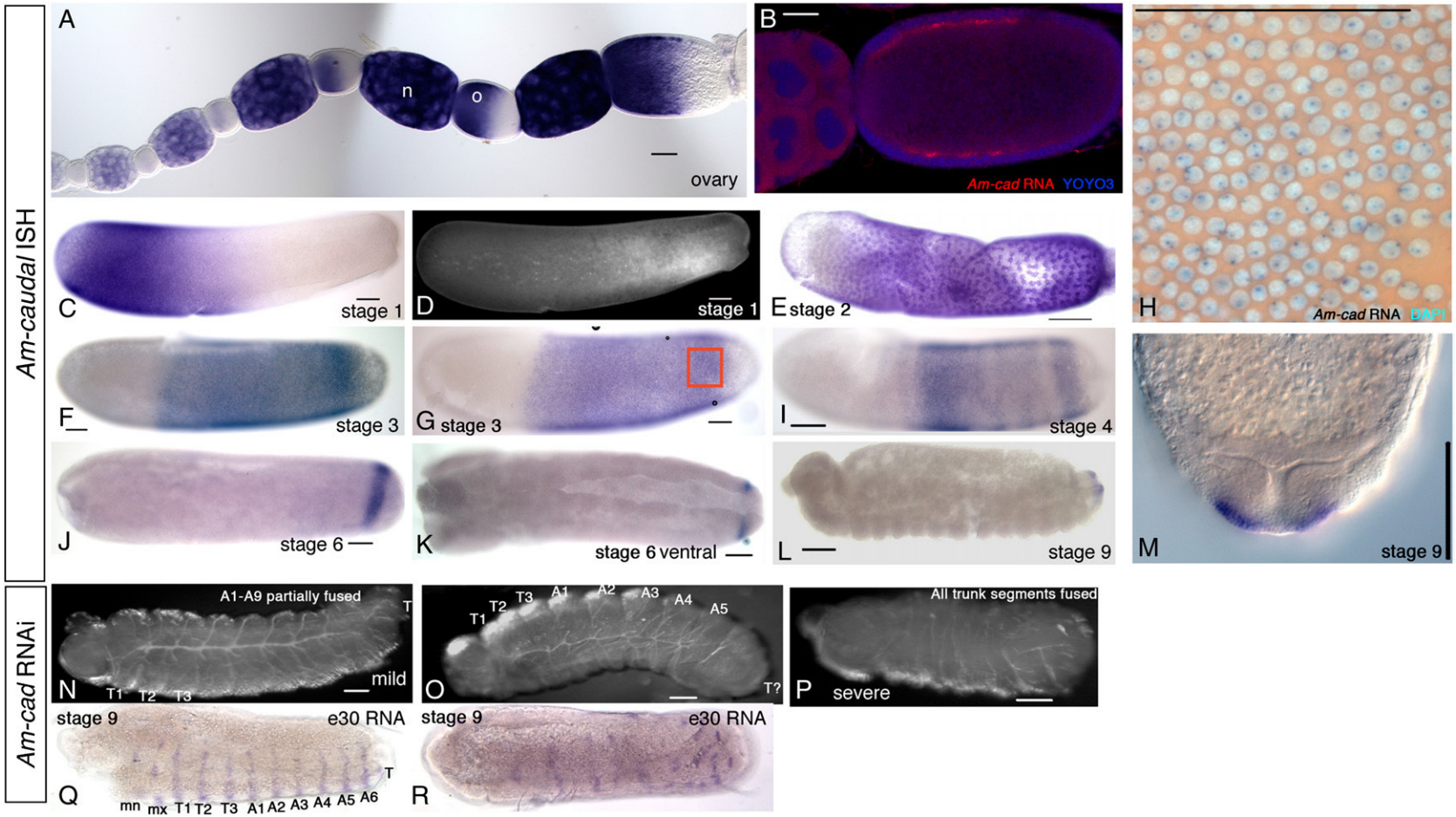

Fig. 4. *Am-cad* expression and RNAi phenotypes. All ovaries/embryos/larvae are oriented with anterior to the left and dorsal up, unless otherwise stated. Scale bars indicate 100 μm. (A) *Am-cad* RNA is expressed by all nurse cells (n) and is transported into the oocyte (o) where it is localized to the anterior. (B) Anterior localized RNA, here detected with fluorescent *in situ* hybridization (red), lies in a thin layer in the anterior cortex of the oocyte. Nuclei stained with YOYO3 (blue). (C) Early stage 1 embryos (DAPI stain of the embryo in C detects approx. 32 nuclei (D)) have maternal *Am-cad* RNA localized to the anterior of the embryo, with the appearance of a gradient anterior high to posterior low. Within a few hours, as energids rise out of the yolk, the transcript relocalizes, in association with energids (E and Supplemental Fig. 1) to the posterior two-thirds of the embryo (F). (G) In stage 3, expression of *Am-cad* RNA is present in a broad central–posterior domain covering two-thirds of the embryo with no expression in the anterior and weaker expression in the posterior (this weaker expression is not just due to lack of cells in this region at this stage). Red box indicates the region magnified in H. (H) Magnification of the boxed region of the stage 3 embryo in G stained for nuclei with DAPI (light blue/white circles) and *Am-cad* RNA (dark dots). At this stage, *Am-cad* RNA can be detected as dots within the circle of the nucleus, as well as more diffusely in the cytoplasm. Nuclear dots indicate that zygotic transcription is active at this stage. (I) Stage 4 embryo showing loss of *Am-cad* RNA from the central abdominal region. Expression fades in stripes. (J) Stage 6 embryo stained for A*m-cad* RNA showing expression only at the posterior end of the embryo as a stripe; this stripe is in ectoderm and is not present in gastrulating cells (K, ventral view). (L) Late stage 9 expression of *Am-cad* RNA is in cells surrounding the proctodeum at the very posterior tip of the embryo (ventral view shown in M, anterior up). RNAi knockdown phenotypes for *Am-cad*. Injection of *Am-cad* dsRNA in just-laid embryos produces a range of phenotypes in larvae. (N–P) These range from mild fusion of posterior segments ($n = 6/36$) (N and O) to disorganization and fusion of all segments and failure of the terminal segments to form correctly ($n = 30$). (P) Segment assignment based on morphology. *e30* RNA staining of mild *Am-cad* RNAi embryos (Q) reveal loss of A7–A9 segments. Severe embryos (R) have disorganized expression of *e30* with no evident stripes.

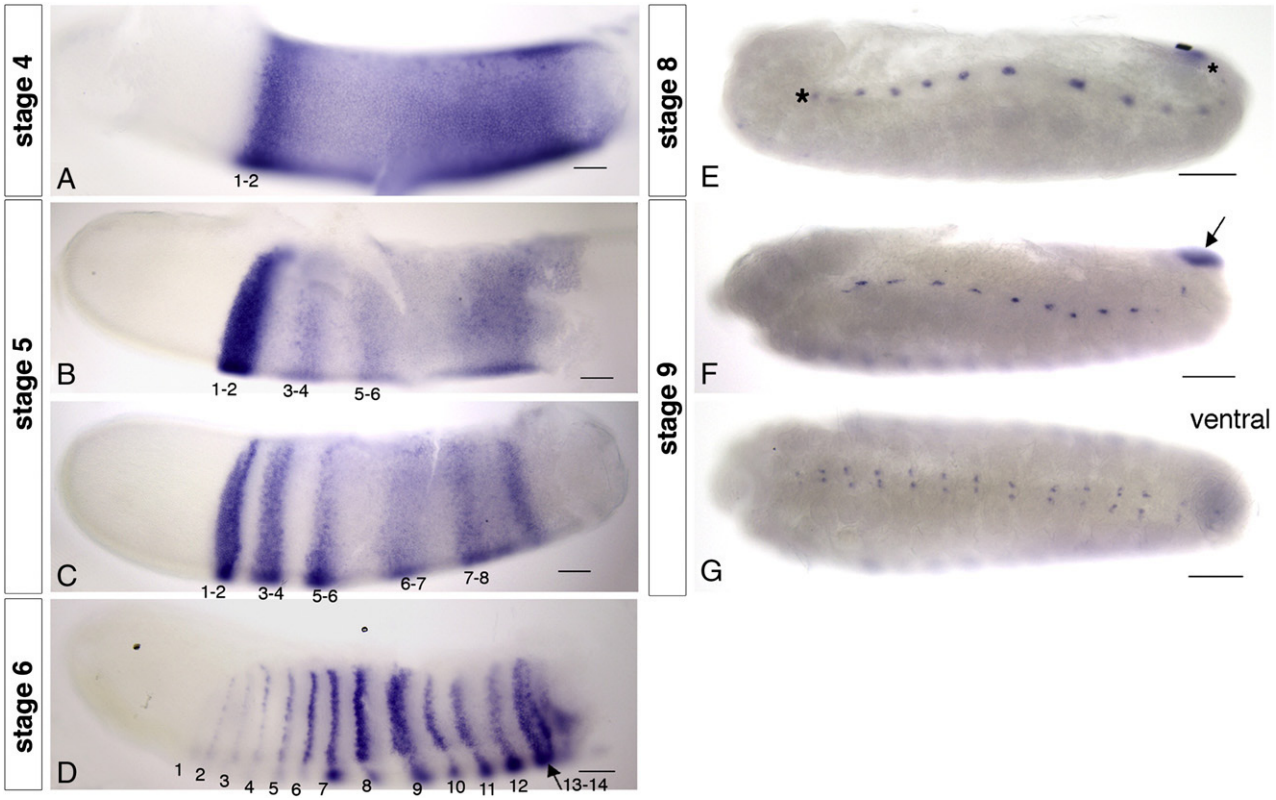

Fig. 5. *Am-eve* RNA expression in honeybee embryos. All embryos are oriented with anterior to the left and dorsal up, unless otherwise stated. Scale bars indicate 100 μm unless otherwise stated. Numbers represent the stripes of *Am-eve* expression. (A) *Am-eve* RNA is first detected at stage 4 in cells in a broad domain covering the posterior two-thirds of the embryo (this embryo damaged at the anterior end). (B and C) Stage 5 embryos stained for *Am-eve* RNA. During stage 5, the expression of *Am-eve* is lost from all but the anterior cells of this broad domain, and wide stripes begin to appear in anterior-to-posterior sequence in the embryo, posterior to the first remaining stripe. These stripes of cells become separated into two stripes through loss of *Am-eve* RNA expression in cells at the center of each stripe. This splitting process follows quickly after the formation of a broad stripe and occurs in an anterior-to-posterior sequence. As the posterior stripes of cells begin to express *Am-eve*, the anterior stripes begin to lose *Am-eve* expression. By stage 6 (D), as gastrulation occurs, the stripes have reached the most posterior forming 14 stripes in total have formed (arrow indicates the final stripe of cells splitting into two). (E) Stage 8 embryos have lost all expression of *Am-eve* in stripes of cells. *Am-eve* RNA is now present in the pericardial cells in each segment (between asterisks) and in a posterior dorsal structure. By stage 9 (F and G), these expression patterns (posterior domain arrowed in F) are joined by expression in segmentally reiterated groups of cells in the central nervous system (G, ventral view).

same time, broad stripes of cells posterior to this initial band begin to form, appearing first as broad stripes that then split, through loss of expression from the cells at the center of the stripe, into thinner, more defined, stripes of cells (Fig. 5B). These broad stripes of cells form in an anterior-to-posterior sequence and split in the same sequence, each broad stripe forming as the stripe anterior to it splits (Fig. 5C). As more posterior stripes of cells form, the expression in anterior stripes of cells is reduced. By gastrulation at stage 6, broad stripes have formed and split all the way along the embryo, with the final stripe splitting as the gastrulation furrow begins to close at the anterior end (Fig. 5D). At this stage, the stripes of *Am-eve* expressing cells run across the ectoderm and the invaginating cells in the gastrulation furrow. Expression in anterior stripes has been lost or reduced to faint RNA expression in a single line of cells, that being the most anterior cells of each stripe. In total, 14 stripes of cells expressing *Am-eve* RNA form though all stripes are only visible very briefly during stage 6. Expression is lost in the anterior-to-posterior sequence as gastrulation is completed.

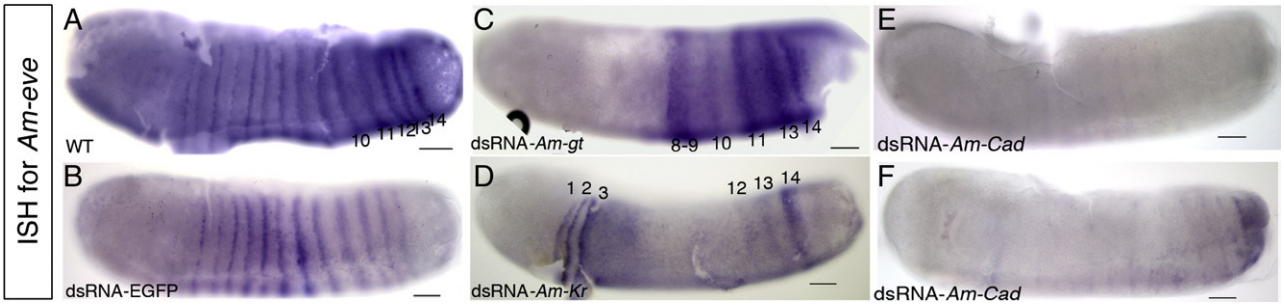

Fig. 6. *Am-gt*, *Am-Kr*, and *Am-cad* are required for *Am-eve* stripe expression. All embryos are oriented with anterior to the left and dorsal up. Scale bars indicate 100 μm. (A) Wild type (WT) *Am-eve* RNA expression detected with *in situ* hybridization at stage 6 when, transiently, 14 stripes of cells are visible along the embryo. (B) The same expression in an EGFP dsRNA-injected honeybee embryo. (C) RNAi knockdown of *Am-gt* in just-laid embryos results in loss of anterior *Am-eve* stripes at stage 6, stripes 8 and 9 fail to split, and stripe 12 is missing. (D) RNAi knockdown of *Am-Kr* in just-laid embryos leads to stage 6 embryos that retain anterior (1, 2, and 3) and posterior (12, 13, and 14) stripes of *Am-eve* RNA-expressing cells but loss or reduction of *Am-eve* expression throughout the central regions of the embryo. (E and F) dsRNA injection against *Am-cad* in just-laid embryos leads to loss of all *Am-eve* stripes (E) or only weak expression of *Am-eve* RNA (F) at stage 6. The increase in *Am-eve* staining in the posterior of the embryo in F occurs in a small number of *Am-cad-RNAi* embryos and is not typical.

After the down-regulation of the early phase of *Am-eve* expression, a second phase of expression occurs. This starts at stage 8 with *Am-eve* RNA detected in discrete clusters of cells located along the dorsal boundary between the embryo and the extraembryonic membranes (Fig. 5E). This is likely to be pericardial cell expression of *Am-eve*. At stage 9, additional expression is seen in the central nervous system (CNS) and at the anal plate. The cells of the CNS expressing *Am-eve* are located in discrete clusters along both sides of the ventral midline (Figs. 5F and G).

To determine the role of *Am-gt*, *Am-Kr*, and *Am-cad* in regulating *Am-eve*, we injected just-laid honeybee embryos with dsRNA targeting each gene and then assayed for *Am-eve* expression at stage 6, when all 14 stripes of *Am-eve* are visible in the embryo. Because of the strong anterior posterior progression of *Am-eve* stripes, it is difficult to interpret missing or fused stripes, especially in the posterior, until all the stripes have formed and split. For this reason, we have examined embryos at the time at which all 14 stripes of *Am-eve* are present. Embryos injected with dsRNA from the *EGFP* gene show no defects in *Am-eve* expression (Figs. 6A and B). Embryos injected with dsRNA targeting *Am-gt* lack all anterior stripes of *eve* expression (which represents the head and thoracic parasegments, stripes 1–6), the central broad stripe and second-to-last two posterior stripes are also wider than normal, indicating a failure to split correctly resulting in fused segments (Fig. 6C). This implies that *Am-gt* is required for correct expression of anterior *Am-eve* stripes and to repress *Am-eve* expression in the central region.

In *Am-Kr* RNAi embryos, the most anterior *Am-eve* stripes are present (corresponding to the mandibular and maxillae segments), but stripes of cells expressing *Am-eve* fail to form in central regions of the embryo and weak expression is detected throughout (Fig. 6D). Three posterior-most stripes form, two faintly and a final stripe, most likely number 14, has normal levels of *Am-eve* RNA expression. This data implies that *Am-Kr* is required for correct *Am-eve* expression in the central regions of the embryo.

Am-cad RNAi-treated embryos have greatly reduced levels of *Am-eve* throughout the embryo. In severe cases no, or extremely weak, expression of *Am-eve* in stripes of cells can be found (Fig. 6E). In more mild cases, faint stripes are visible but are disrupted (Fig. 6F). Thus, *Am-cad* is required for *Am-eve* expression in all segments.

Discussion

Segmentation is a key process in the development of insect embryos and, in arthropods, has a single evolutionary origin. The output of the process is a stereotyped repetitive pattern of segments. That this process is conserved and produces a conserved output would suggest that little evolutionary change in the pathway has occurred. It is clear, however, that over long evolutionary periods, changes in this pathway have occurred (reviewed in Peel, 2004; Peel et al., 2005). Perhaps the most obvious is the shift from short and intermediate germ band types, where only the head or the head and thoracic segments are defined in the blastoderm and the posterior segments form via growth. In contrast, in long germ development, the entire pattern of segments is represented in the blastoderm (reviewed in Peel et al., 2005). Honeybees are Hymenoptera, now thought to be the most deeply branching clade in the holometabolous insects (Krauss et al., 2005; Savard et al., 2006; Zdobnov and Bork, 2007), and thus very distant from *Drosophila*. Comparing with *Nasonia*, another hymenopteran, provides information on changes over shorter evolutionary distances. Honeybees are also long germ band insects (Fleig and Sander, 1986; Sander, 1976), like *Drosophila*, but the distribution of these germ types in the holometabolous insects implies that short or intermediate germ development is likely to be ancestral (Davis and Patel, 2002; Liu and Kaufman, 2005), and thus long germ development in Hymenoptera may have evolved independently to that in *Drosophila*. It has also been discovered that honeybees are missing orthologues of some of the key maternal coordinate genes that act in *Drosophila* to provide the initial patterning information for segmentation (Dearden et al., 2006), raising questions about how early embryogenesis in honeybees is controlled.

Honeybee Krüppel, giant, and caudal act as gap genes during honeybee segmentation

The functional data presented here show that gap genes play a key role in the segmentation of honeybees and knockdown results in gaps in the patterns of segments of larvae, consistent with studies in other insects. In honeybees, despite the absence of many maternal coordinate genes, the *Am-Kr*, *Am-gt*, and *Am-cad* RNAi phenotypes imply that they play important gap gene-like roles in the early stages of segmentation. Zygotic expression of *Am-gt*, *Am-cad*, and *Am-Kr* are broadly similar to these genes in other insects. Indeed, the expression pattern of *Am-Kr* is typical for insect *Krüppel* genes and is similar to the expression of *Drosophila Kr* (Gaul et al., 1987). The RNAi phenotype is also very similar to that of loss of *Kr* expression in *Nasonia* via RNAi (Brent et al., 2007) and the phenotype of *Drosophila* mutants (Wieschaus et al., 1984). Added to this, all three genes have roles in regulating *Am-eve*, the honeybee orthologue of *Drosophila even-skipped*, a primary pair-rule gene and target of these genes in *Drosophila*. Despite these similarities, variations in the expression and function of these genes between *Drosophila*, *Nasonia*, and honeybee exist (Table 1), and these differences are reflected in the way these genes regulate *even-skipped* in the honeybee.

Honeybee even-skipped is regulated by Am-cad, Am-gt, and Am-Kr

Honeybee *even-skipped* RNA is expressed in a pattern similar to *paired* in honeybees (Osborne and Dearden, 2005a) and is similar to that previously published using a cross-reacting antibody raised against *Drosophila* eve (Binner and Sander, 1997). Both *Am-eve* and *Am-prd* are expressed with both dual-segment periodicity and strong anterior–posterior modulation. The most anterior, and first forming, stripe is in the mandibular segment. Stripes then form, first as a broad stripe, defining two segments, which then splits in two, each stripe splitting just after it forms and as the next most posterior broad stripe forms. The only major difference in the mode of *Am-prd* and *Am-eve* expression is that *Am-eve* is initially expressed in a broad domain probably stretching from the presumptive mandibular segment to the posterior of the embryo. This anterior–posterior progression of stripe formation and then stripe splitting is different to *Drosophila eve* where anterior–posterior progress is less pronounced and stripes do not split, is reminiscent of pair-rule gene expression in short germ insects (Choe et al., 2006), and is perhaps suggestive of a clock-and-wave mechanism for the production of segments as seen in vertebrates (Peel et al., 2005).

The striped pattern of *Drosophila eve* expression is controlled via stripe-specific enhancers. Six separate enhancers control *Dm-eve*

Table 1
Summary of phenotypes of mutant or RNAi-treated gap genes in long germ band insects.

	Drosophila	*Nasonia*	Honeybee
Kr	T2–T3 fused A1–A5 fused	T3–A4 fused	T1–A6 fused
gt	Minor head structure defects and A5–A7 fused	Loss of head and T segments A6–A7 fused	Loss of head and T segments A1–A2 and A6–A7 fused.
cad	Loss of all body segments – partial thorax and abdominal segments[a]	Head only	Loss of all body segments – loss of patterning of all segments and terminal region

[a] Loss of both maternal and zygotic *Dm-cad*.

expression and all are dependent upon the co-operative interaction between gap genes and maternal coordinate genes (Small et al., 1993, 1992, 1996). Using bioinformatic methods, we have not been able to identify clusters of transcription factor binding sites around *Am-eve* perhaps supporting the notion that this gene may be regulated via a clock-and-wave mechanism.

Our RNAi experiments with honeybee gap genes, however, argue against this interpretation. In our *Am-Kr* RNAi embryos, both anterior and posterior stripes of *Am-eve* are present while those at the center of the embryo are removed. If *Am-eve* stripes form by a clock-and-wave mechanism then we might expect that blocking *eve* stripe formation in the central region of the embryo would cause loss of pattern in the posterior, as each forming *eve* stripe would be dependent on the one before. This finding thus implies that *eve* stripes in honeybees may be also regulated by stripe-specific enhancers as they are in *Drosophila*.

Our data do, however, imply that there are functional differences between the domains in which honeybee gap genes act to regulate *Am-eve*. *Am-gt* has a role in activating *Am-eve* expression, being required for the formation of anterior stripes and to define the border of the central stripe. In *Drosophila*, *gt* acts as a repressor of *Dm-eve*. *Drosophila gt* mutants exhibit partial loss of stripe 2 and expansion of stripe 5 causing fusion with stripe 6 (Wu et al., 1998). In a *Drosophila Kr* mutant, *eve* stripes 2 and 3 are fused as are 4 and 6 (Frasch et al., 1987). In honeybee, fusion of all central stripes of *eve* expression occurs in the RNAi knockdown. Perhaps the most significant difference is in *caudal*'s influence on *eve*. In honeybee, *Am-cad* RNAi results in a dramatic loss of *Am-eve* expression (and consequently a loss of segmentation) throughout the embryo. In *Drosophila*, *caudal* mutants result in loss of stripes 4, 6, and 7, reduction in stripe 3, and expansion of stripe 5 (Olesnicky et al., 2006). While *Am-Kr*, *Am-gt*, and *Am-cad* all regulate *Am-eve*, they do so in broader domains than their *Drosophila* orthologues regulate *eve*.

Maternal honeybee caudal RNA is localized to the anterior but functions to define abdominal and posterior regions of the embryo

Caudal has an essential and highly conserved role in posterior development (Copf et al., 2004; de Rosa et al., 2005; Dearden and Akam, 2001; Katsuyama et al., 1999; Le Gouar et al., 2003; Marom et al., 1997; Moreno and Morata, 1999; Olesnicky et al., 2006; Schulz et al., 1998; Wu and Lengyel, 1998). Surprisingly, however, honeybee *Am-cad* RNA is initially localized, in the ovary and early embryo, to the anterior. This anterior localization is very unusual and it is not clear what role it plays in *Am-cad* function. As the blastoderm forms, however, this anteriorly placed RNA becomes relocalized to the posterior two-thirds of the embryo and then clears from the most posterior region of the embryo. This relocalization appears to be associated with nuclei arriving at the surface of the egg and resembles, in some ways, the relocalization of *Am-tll* from dorsal regions of the oocyte and just-laid embryo, to the posterior (Wilson and Dearden, 2009).

Despite the anterior localization in the oocyte and freshly laid egg, RNAi knockdown of *Am-cad* from very early stages (stages with anterior RNA) gives uniformly posterior defects. Such injections would be expected to cause degradation of the anterior *caudal* RNA, yet no phenotype in the anterior can be detected. It is possible that this anterior domain of expression has a role in the oocyte, a stage not accessible to RNAi in honeybees, or that this early embryonic domain is refractory to RNAi, but we cannot detect any anterior-specific defects in our treatments. Indeed, in *Drosophila*, *Nasonia*, and honeybee, reduction in caudal activity gives similar phenotypes with defects present only in the posterior. Both *Nasonia* and honeybee use maternal localization, and subsequent migration, of *cad* mRNA to produce a posterior domain of expression. *Nasonia cad* RNA is associated with the oosome (Olesnicky et al., 2006), a subcellular organelle that specifies germ cells but is absent from honeybees (Dearden, 2006). The anterior localization, but posterior function, of *Am-cad* RNA implies that this RNA has no function in the anterior of the embryo but only becomes functional once it has been transported to the posterior. This possibility could be better explored with maternal RNAi, a technique not yet possible in the honeybee.

In addition, *Am-cad* RNA does not appear to be expressed in a gradient. A posterior (high)-to-anterior (low) gradient expression of *caudal* RNA has been reported in all other insects in which it has been studied, including *Drosophila* (Mlodzik and Gehring, 1987), *Tribolium* (Schulz et al., 1998), *Nasonia* (Olesnicky et al., 2006), *Gryllus* (Shinmyo et al., 2005), and *Schistocerca* (Dearden and Akam, 2001). Indeed, *Am-cad* expression, in blastoderm stage embryos, is not present in the posterior-most terminus of the embryo. Initial expression, if not function, of *Am-cad* seems unusual when compared with other insects.

The gaps in segmentation produced by RNAi against *caudal* genes are broader in *Nasonia* than in *Drosophila* (Olesnicky et al., 2006) and broader still in honeybee where all segments from the gnathal segments to the posterior require *Am-cad*. This broad domain of activity is reflected in a broad domain of expression in the blastoderm-stage *Nasonia* and honeybee embryos, much broader that that seen in *Drosophila* and *Tribolium*. The broad domain of activity of *Am-cad* affects the expression of *Am-eve*, where *Am-cad* is required for the formation of all *Am-eve* stripes. This broad effect on *eve* stripes is also seen in the intermediate-germ hemimetabolous insect *Gryllus* (Shinmyo et al., 2005) where caudal is required for all *even-skipped* stripes and expression in the growth zone. *Caudal* thus acts as an activator of pair-rule genes in *Drosophila* (Olesnicky et al., 2006), *Gryllus*, and honeybee. The extensive segmental defects seen in *Am-cad* RNAi embryos are most likely due to the loss of *Am-eve* throughout the embryo at stage 6 and thus the loss and disruption of *e30* stripes at stage 9.

Evolutionary shifts in the timing and domain of gap gene expression and function

Viewed across the extensively studied long germ band holometabolous embryos, the functions of these three gap genes remain relatively conserved (Fig. 7 and Table 1). Perhaps the most obvious trend is that in Hymenoptera, and especially honeybee, *gt*, *Kr*, and *cad* have more extensive activity than their orthologues in *Drosophila*. In the honeybee, the expression domain of *Am-Kr* RNA is broader than in *Drosophila* or *Nasonia* and loss of *Am-Kr* results in the disruption of more segments.

Giant too has a much more significant role in anterior patterning in *Nasonia* and the honeybee compared to *Drosophila*, where only minor defects in head structures are found in *Dm-gt* mutant larvae (Mohler et al., 1989; Petschek et al., 1987). *Gt* is required for the patterning of the head and thoracic segments in both hymenopterans and this is reflected in a larger anterior expression domain (Brent et al., 2007). As discussed above, *Am-cad* too affects more segments and has more extensive function in honeybee than in *Nasonia* (Olesnicky et al., 2006), where it has a more extensive activity than *Drosophila*. Given that recent phylogenetic data imply that the Hymenoptera are the most basal group in the holometabolous insects (Krauss et al., 2005; Savard et al., 2006; Zdobnov and Bork, 2007), it seems likely that these more extensive domains are an ancestral character of the holometabolous insects and that the evolution of segmentation in this group is characterized by a narrowing of the domains in which gap genes act.

These more extensive domains of activity are also present in *Tribolium*, but in this short germ insect, some of these gap genes appear to have different functions. Both *gt* and *Kr* (Bucher and Klingler, 2004; Cerny et al., 2005) have functions that produce homeotic transformations when knocked down or mutant, and indeed the anterior expression of *Tribolium giant* seems to be required for head identity,

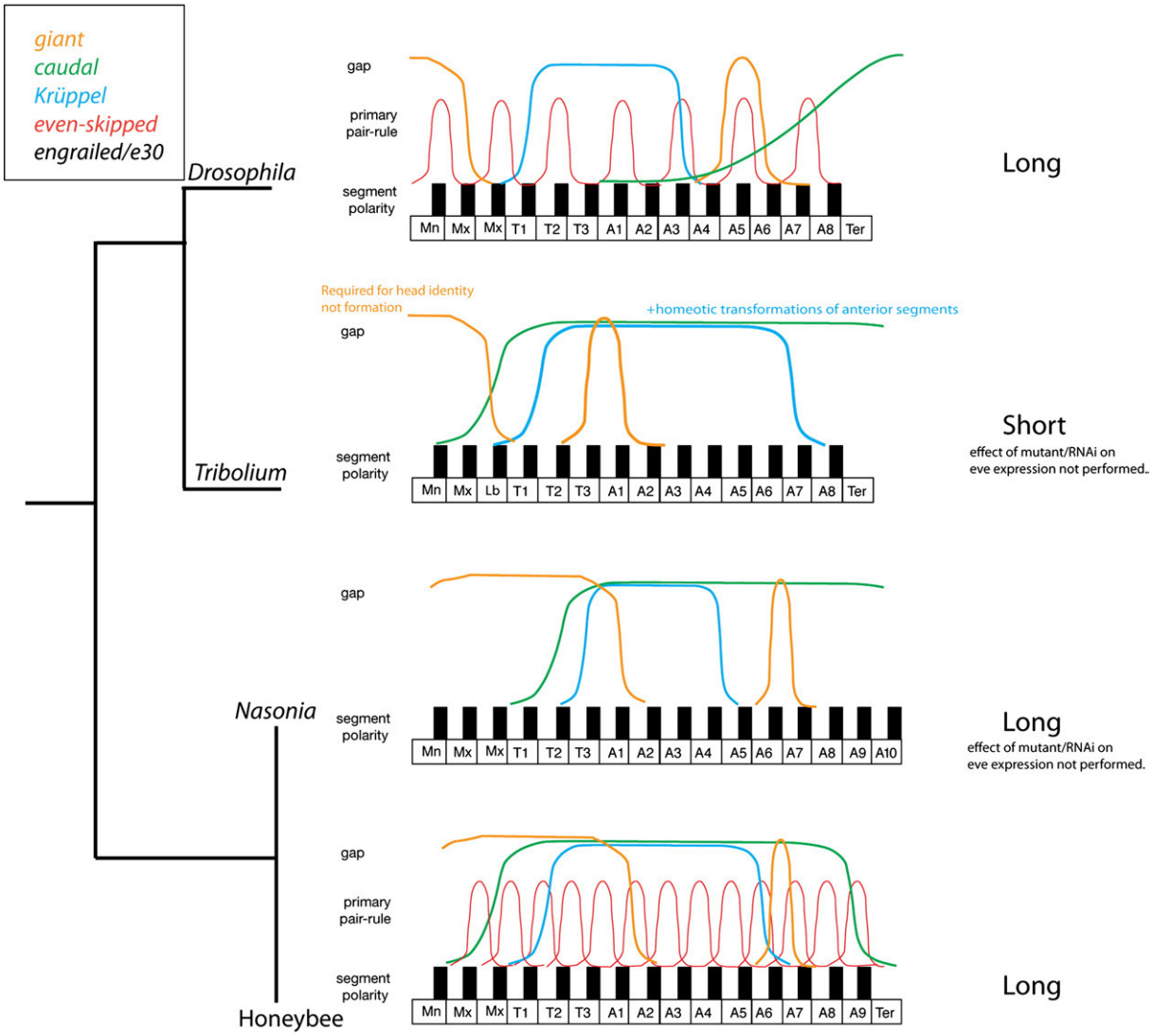

Fig. 7. Gap gene functional domains in holometabolous insects. Cartoon summarizing information on the domains and stripes of *even-skipped* (red, where known) or *engrailed* (black bars) affected by *giant* (orange), *Krüppel* (blue), and *caudal* (green) mutants or RNAi knockdown in *Drosophila*, *Tribolium*, *Nasonia* and honeybee. All of these insects are holometabolous, although *Tribolium* is a short germ insect, the rest use a long germ mode of development. While phylogenetic relationships are shown in the figure, it is important to be aware that long germ band development is unlikely to be the ancestral mode of development in this group, and that long germ development may have evolved independently in Hymenoptera and Diptera.

not formation (Bucher and Klingler, 2004). *Tribolium cad* RNAi embryos (Copf et al., 2004) have a very similar phenotype to honeybee *Am-cad* RNAi embryos in that only head regions form with segments from first maxilla to the posterior being disrupted, a much more extensive phenotype than that seen in *Drosophila cad* mutants. It seems that through the evolution of holometabolous insects, gap gene expression domains are shifting in the embryo.

Why might these shifts in expression domain be occurring? One possibility is that this is due to reorganization of the gap gene regulatory network. In honeybees, for example, no *knirps* orthologue is present in the genome (Dearden et al., 2006). *Knirps* is a repressor of *Kr* (Jaeger et al., 2004) and its absence in honeybee may explain why *Kr* activity is more extensive. It is also possible that changes in the way these genes regulate each other may cause these shifts in gene expression and function.

It is likely that many of these changes reflect differences in the earliest stages of embryogenesis, those controlled by maternal coordinate genes because it appears that these genes evolve more rapidly than later parts of the cascade (Dearden et al., 2006). The more significant roles for *gt* and *cad* might be explained by the absence of *bcd*, which in *Drosophila* regulates many anterior genes, thus relegating *gt* to a less extensive role. Bcd also represses *cad* translation, pushing its activity back from the entire trunk region to just the most posterior abdomen. In *Tribolium*, *Nasonia*, and honeybee, *caudal* has a more extensive and anterior role than in *Drosophila*, perhaps because bicoid is not present to restrict its activity towards the posterior. Perhaps the evolution of *bicoid* has triggered major changes to the function of gap genes in Diptera.

The best evidence for changes in maternal genes leading to gap gene changes are the differences between *Nasonia* and honeybee. In later stages of segmentation, these two Hymenoptera are relatively similar, but the localization of maternal RNAs is very different. In *Nasonia*, *Nv-gt* is localized to the anterior of the oocyte (Brent et al., 2007), whereas there is no localization of maternal *Am-gt* mRNA. In honeybee, *Am-cad* RNA is localized to the anterior, but *Nasonia cad* localizes posteriorly with the oosome (Olesnicky et al., 2006). Finally, *Am-tll* RNA is localized dorsally in the oocyte (Wilson and Dearden, 2009); in *Nasonia*, it is not maternally expressed (Lynch et al., 2006). The expression of many of these genes maternally implies that they may be acting somewhat as maternal coordinate genes. Despite these changes, RNAi against all these genes in *Nasonia* and honeybee gives similar phenotypes implying their function remains the same.

These differences in ways of producing patterning information in the early steps of segmentation suggest that gap genes are acting, in some respects, as buffers between fast-evolving maternal coordinate/axis formation pathways and the need to produce a stable developmental output: segmentation.

Experimental procedures

Cloning of A. mellifera cDNAs

Phylogenetic analyses were carried out on multiple alignments, produced by ClustalX (Thompson et al., 1994), of predicted gap gene proteins using MrBayes (Ronquist and Huelsenbeck, 2003). These analyses were initially carried out under mixed models with default priors to identify the most appropriate model for amino acid substitutions, and then rerun under that model. Final models were Jones et al. (1992) for eve, cad, and gt. The WAG model (Whelan and Goldman, 2001) was used for Kr. Phylograms were displayed using Dendroscope (Huson et al., 2007). Fragments of *A. mellifera* segmentation genes were cloned by RT-PCR from total RNA extracted from honeybee embryos. Oligonucleotide primers were designed based on the predicted coding sequence: *Am-gt* 5′CGAGCCAACCAAATTCATTGGATTC3′ and 5′CGAGATCCCTGCGAGCTTCTCGT3′; *Am-kr* 5′CGTTCGCCGGTATGGAC3′ and 5′CCCCGCCGTGGCAAGAC3′; *Am-cad* 5′ATGGCGGACATCAGCGGGCTCGGTA3′ and 5′TCAGTATACGTGAAACGTTGTACA3′; and *Am-eve* 5′ATTCACCATCGCAGACCCAAT3′ and 5′GCCCTCTCGGATATATCGTTC3′. PCR products were cloned into pGEM T-easy (Promega) and sequenced to confirm identity and sequence orientation. DNA fragments of each gene of interest were also then subcloned into pLitmus38i (NEB) for the production of double-stranded RNA for use in RNAi.

Orthologues of *Drosophila* gap genes were identified using reciprocal tBlastX and Blastp (Altschul et al., 1990) against the honeybee genome and official gene list (The Honey Bee Genome Sequencing Consortium, 2006).

Whole-mount in situ hybridization on honeybee ovaries and embryos

In situ hybridization on honeybee embryos were carried out as described in Osborne and Dearden (2005b). Fluorescent *in situ* hybridization was carried out using the same methods for hybridization, but the DIG-labeled probe was detected with an anti-DIG antibody conjugated to horseradish peroxidase (HRP) (Roche Applied Science). HRP was detected using a Tyramide signal amplification protocol (TSA kit #15, Invitrogen) leading to deposition of Alexa Fluor 594 following the manufacturer's instructions. Specimens were then counterstained with DAPI, mounted in 70% glycerol, and imaged using an Olympus BX61 compound microscope and a DP71 camera. Embryos were staged as per DuPraw (1967).

Honeybee RNA interference

dsRNA was synthesized from cDNAs of gap genes cloned into plitmus38i (NEB) using the MEGAscript RNA kit (Ambion). RNAi was carried out as described in Wilson and Dearden, (2009). dsRNA was injected at 2.5 mg/mL in reverse osmosis H_2O into freshly laid honeybee eggs. Injected embryos were incubated at 35 °C and 80% humidity for 30 hours (and collected for *in situ* hybridization) or until hatching (approximately 70 hours later). For each dsRNA target, between 120 and 300 embryos were injected, with 20% surviving injection.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2009.12.015.

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