Diversity in insect axis formation: two orthodenticle genes and hunchback act in anterior patterning and influence dorsoventral organization in the honeybee (Apis mellifera)

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SUMMARY
Axis formation is a key step in development, but studies indicate that genes involved in insect axis formation are relatively fast evolving. Orthodenticle genes have conserved roles, often with hunchback, in maternal anterior patterning in several insect species. We show that two orthodenticle genes, otd1 and otd2, and hunchback act as maternal anterior patterning genes in the honeybee (Apis mellifera) but, unlike other insects, act to pattern the majority of the anteroposterior axis. These genes regulate the expression domains of anterior, central and posterior gap genes and may directly regulate the anterior gap gene giant. We show otd1 and hunchback also influence dorsoventral patterning by regulating zerknäult (zen) as they do in Tribolium, but that zen does not regulate the expression of honeybee gap genes. This suggests that interactions between anteroposterior and dorsal-ventral patterning are ancestral in holometabolous insects. Honeybee axis formation, and the function of the conserved anterior patterning gene orthodenticle, displays unique characters that indicate that, even when conserved genes pattern the axis, their regulatory interactions differ within orders of insects, consistent with relatively fast evolution in axis formation pathways.

KEY WORDS: Axis formation, Developmental hourglass, Anterior patterning, Dorsoventral patterning, Extra-embryonic membranes, Segmentation

INTRODUCTION
The genes that control axis formation in the fruit fly Drosophila melanogaster are often missing from the genomes of other insects (Dearden et al., 2006; The Honey Bee Genome Sequencing Consortium, 2006; Richards et al., 2008; The International Aphid Genomics Consortium, 2010) implying that axis formation is a relatively fast evolving pathway. Although variation exists in axis formation, some molecules, such as those encoded by orthodenticle (Otd) genes, seem to have conserved roles (Schröder, 2003; Lynch et al., 2006; Schinko et al., 2008; Lemke and Schmidt-Ott, 2009; Kotkamp et al., 2010; Nakamura et al., 2010).

Otd genes have a long evolutionary history; patterning the anterior in vertebrates (Mercier et al., 1995; Pannese et al., 1995; Ang et al., 1996) and invertebrates (Chuang et al., 1996; Stormaiuolo et al., 1998; Wada and Saiga, 1999; Nederbragt et al., 2002). Insect genomes often have two Otd genes, otd1 and otd2. The function of otd1 has been examined in a small group of insects including some Diptera (Finkelstein and Perrimon, 1990; Finkelstein et al., 1990; Royet and Finkelstein, 1995; Lemke and Schmidt-Ott, 2009), the beetle Tribolium castaneum (Schröder, 2003; Kotkamp et al., 2010), the jewel wasp Nasonia vitripennis (Lynch et al., 2006) and the cricket Gryllus bimaculatus (Nakamura et al., 2010). In these insects knockdown of otd1 leads to anterior defects consistent with a role in anterior patterning. In Drosophila, a single Otd gene is present in the genome, ocelliless (oc), an otd1 ortholog, that acts in head patterning although not early in development (Finkelstein and Perrimon, 1990; Finkelstein et al., 1990; Wieschaus et al., 1992). In Drosophila, this early anterior patterning role has been taken by bicoid, a hox3-derived transcription factor whose DNA-binding domain has evolved to be much like Otd (Finkelstein et al., 1990; Mercier et al., 1995; Klein and Li, 1999). It is suggested that changes in Bicoid protein sequence have led to it ‘taking over’ the anterior patterning role by regulating genes ancestrally downstream of oc (Dearden and Akam, 1999; Stauber et al., 1999; Brown et al., 2001; Stauber et al., 2002).

In comparison, otd2 has been less characterized. In Tribolium and Nasonia, otd2 is expressed late in embryogenesis, with no probable role in anterior patterning (Lynch et al., 2006; Schinko et al., 2008). otd2 is missing from the Drosophila genome and has not been examined in Gryllus. The pea aphid (Acyrthosiphon pisum) has only one Otd gene, otd2 (Huang et al., 2010; Shigenobu et al., 2010; The International Aphid Genomics Consortium, 2010), which is not expressed early in anterior regions (Huang et al., 2010).

Alongside otd1, hunchback (hb) has been implicated in anterior patterning in a number of insects (Wolff et al., 1995; Patel et al., 2001; Mito et al., 2005). In Nasonia, Nv-hb acts with Nv-otd1 in anterior patterning (Lynch et al., 2006). In Drosophila, hb is a direct target of Bicoid, and patterns the anterior (Simpson-Brose et al., 1994). In Tribolium, hunchback (Tc-hb) has been reported to act with otd1 to regulate anterior patterning (Schröder, 2003), but recent evidence indicates that Tc-hb actually regulates trunk specification, with anterior segments being transformed to abdominal fates in an RNAi knockdown (Marques-Souza et al., 2008). Tc-hb knockdown also causes expansion of trunk Hox gene expression towards the anterior, and reduction in the expression of the thoracic gap gene Krüppel (Marques-Souza et al., 2008). This is inconsistent with Tc-hb acting to pattern the anterior; indeed Tc-hb knockdown does not cause loss of the anterior expression of a zygotic anterior gap gene, giant (Tc-gt). Trunk patterning may be a...
more ancestral function of \( hb \) than anterior patterning as it is also seen in widely diverged insects such as \( Oncopeltus \) and \( Gryllus \) (Liu and Kaufman, 2004; Mito et al., 2005).

Although \( otd1 \) genes are a common element in insect anterior patterning, their actions differ, implying the interactions between \( otd1 \) proteins and their targets differ between species. In \( Tribolium \), RNA interference (RNAi) targeting maternal and zygotic \( Tc-otd1 \) partially mimics the \( bcd \) mutant phenotype in \( Drosophila \) (Schröder, 2003). Double knockdown of \( Tc-otd1 \) and \( Tc-hb \) leads to loss of anterior structures (Schröder, 2003), but whereas the defects produced suggest an anteroposterior patterning role, knockout of \( Tc-otd1 \) causes only minor anteriorward shifts in gap gene expression. Indeed, the anteroposterior phenotype of \( Tc-otd1 \) knockdown in \( Tribolium \) can be recapitulated by double knockdown of two dorsoventral patterning genes, \( zen \) (\( Tc-\text{zen} \)) and \( Short \text{on gastrulation} \) (\( Tc-Sog \)) (Kotkamp et al., 2010). The defects in dorsoventral patterning appear to cause loss of pattern from the anterior.

In \( Nasonia \), \( orthodenticle-1 \) (\( Nv-otd1 \)) and \( hunchback \) (\( Nv-hb \)) together pattern the anterior of the embryo (Lynch et al., 2006). \( Nv-otd1 \) RNA is localized to the anterior and posterior poles of the oocyte (Lynch et al., 2006) and patterns both ends of the embryo. RNAi against \( Nv-otd1 \) gives both anterior and posterior patterning defects, but when in combination with RNAi against \( Nv-hb \), the anterior defects are more severe. Consistent with this, \( Nv-otd1 \) is required for \( giant \) (\( Nv-gr \)) expression (Lynch et al., 2006) the key anterior gap gene in this species (Brent et al., 2007).

The diversity in the regulation of axis formation in holometabolous insects is consistent with previous findings that, in insects at least, genes in pathways that act early in development are less conserved than later ones (Dearden et al., 2006; Wilson et al., 2010), and with recent molecular evidence for a ‘developmental hourglass’ model of development (Hatzkan-Covo et al., 2005; Cruickshank and Wade, 2008; Kalinka et al., 2010). Diversity in genes involved in axis formation indicates that these pathways are fast evolving. Such relatively fast evolution might also be detected in changes in the regulatory interactions of conserved genes involved in axis formation.

**MATERIALS AND METHODS**

**Isolation of honeybee genes**

\( Am-otd2 \) was amplified from honeybee cDNA using the following oligonucleotides primers: CCCTACGGCGCCCTCAAGAC, amotx25; TCCCCGGGTTGGCGGACTA, amotx23. \( Am-hb \) was amplified from honeybee cDNA using: CACGGCAGGATGGGAGTA, amhbRNA5; GATCTGGCAAATGGGAGATA, amhbRNA3. Cloning of other honeybee genes has been described previously (Osborne and Dearden, 2005b; Osborne and Dearden, 2005a; Dearden et al., 2006; Wilson and Dearden, 2009; Wilson et al., 2010).

**Phylogenetic analysis**

ClustalX (Thompson et al., 1994) alignments of full-length protein sequences were analyzed using MrBayes (Ronquist and Huelsenbeck, 2003) under the WAG model (Whelan and Goldman, 2001).

**In situ hybridization**

In situ hybridization was carried out as described previously (Osborne and Dearden, 2005b; Dearden et al., 2010).

**RNAi knockdown in honeybee embryos**

RNAi was performed as described previously (Wilson and Dearden, 2009; Dearden et al., 2010). Injected embryos were incubated at 35°C until the desired stage of development (24 hours for stage 4; 30-35 hours for stages 5-6). Stage 9 (65 hours) and later embryos were mounted in oil, or fixed and DAPI stained, and then photographed using an Olympus BX61 microscope. Hatched larvae were mounted in oil and photographed using a Leica dissecting microscope and digital camera. In the case of stained embryos at least 50 examples of each phenotype were examined and representative examples photographed.

**Drosophila transgenesis, immunohistochemistry and in situ hybridization**

Genomic regions around \( Am-otd1 \) were cloned into pCaSpeR-hs43-lacZ or pHpelican and used to transform \( w^{1118} \) Drosophila (Rubin and Spradling, 1982). Transgenic lines were crossed to mutants for \( causal \) (\( cad \)), \( hb \), \( zerknüllt \) (\( zen \)) and \( Otd \) (\( cad^{19} \), \( hbb^{3} \), \( zen^{2} \)). \( Drosophila \) in situ hybridization was carried out using established protocols (Patel, 1994).

**Identification of cis-regulatory motifs**

ClusterDraw (Papatsenko, 2007) was used to identify binding-site clusters for transcription factors in the giant locus of \( A. mellifera \) and \( D. melanaster \). Binding site motifs used are available at http://line.bioinfolab.net/webgate/submit.cgi.

**RESULTS**

**Identification of honeybee Otd and \( hb \) orthologs**

Blast searches (Altschul et al., 1990) identified two predicted honeybee genes with similarity to \( Drosophila \) \( Otd \): GB16866 and GB11566. Phylogenetic analysis (Ronquist and Huelsenbeck, 2003) of aligned orthodenticle and vertebrate Otx protein sequences indicate GB16866 clusters with a clade of \( orthodenticle-1 \) sequences and GB11566 clusters with \( orthodenticle-2 \) sequences (see Fig. S1A in the supplementary material). We designate GB16866 as \( Am-orthodenticle-1 \) (\( Am-otd1 \)) and GB11566 as \( Am-orthodenticle-2 \) (\( Am-otd2 \)).

Blast searches of the honeybee genome identified one predicted gene, GB19977, with similarity to \( Drosophila \) \( hb \). Phylogenetic analysis of aligned hunchback protein sequences indicates GB19977 clusters with other insect hunchback proteins (see Fig. S1B in the supplementary material). We designate GB19977, \( Am-hb \).

**Expression of \( Am-otd1 \), \( Am-otd2 \) and \( Am-hb \)**

To determine whether \( orthodenticle \) and \( hunchback \) orthologs in the honeybee are expressed in patterns consistent with anterior specification we examined RNA expression using in situ hybridization. \( Am-otd1 \) is expressed by nurse cells of the honeybee queen ovary and accumulates throughout the cytoplasm of the oocyte at all stages (Fig. 1A). Maternal RNA is enriched in the anterior of the syncytial blastoderm embryo within a few hours of the egg being laid (Fig. 1B; stage 1) where it is associated with energid cytoplasm. \( Am-otd1 \) RNA becomes enriched in the anterior half of the embryo with highest concentration at the anterior pole (Fig. 1C; stage 2). Expression at the pole is reduced by stage 4 (Fig. 1D), after which it is upregulated in a triangular anterior domain and weakly at the posterior pole (stage 5; Fig. 1E). Posterior expression vanishes by stage 6, when faint expression is seen on the boundaries of the gastrulation furrow (Fig. 1F). At stage 9 \( Am-otd1 \) RNA is detected in the CNS (Fig. 1G).

\( Am-otd2 \) RNA is expressed by posterior nurse cells (Fig. 1H) and is present in the oocyte (Fig. 1J) and freshly laid egg (Fig. 1K). \( Am-otd2 \) expression fades in an anterior to posterior sequence after egg laying (Fig. 1K,L). At stage 4, \( Am-otd2 \) RNA expression appears in cells at the anterior and posterior poles (Fig. 1M). Anterior expression resolves into a triangular domain, similar to \( Am-otd1 \). A cap of cells at the posterior expresses \( Am-otd2 \) at stages 5 and 6 and faint expression is seen at the anterior end of the edges of the gastrulation furrow (Fig. 1N,O). At stages 8 and 9, \( Am-otd2 \) RNA is limited to the developing CNS (Fig. 1P,Q).
Am-hb RNA is present throughout maturing oocytes and nurse cells (Fig. 1R). It is ubiquitous in early embryos and comes to be associated with nuclei, with stronger staining in the anterior two-thirds of the embryo (Fig. 1S, T). By stage 2, Am-hb RNA is enriched in the anterior of the embryo (Fig. 1T). By stage 4 it becomes enriched in a broad anterior stripe and a weaker posterior one (Fig. 1U). This posterior expression later (stage 6) expands to a cap of cells, but weak Am-hb expression is detected throughout the embryo (Fig. 1V). In later stages Am-hb is expressed in the CNS (Fig. 1W).

RNAi knockdown of Am-otd1, Am-otd2 and Am-hb

If Am-otd1, Am-otd2 and Am-hb act to pattern the anterior, then removing their function using RNAi early in development should lead to defects in anterior patterning.

Am-otd1 knockdown produces two phenotypic classes. Compared with controls (Fig. 2A), mildly affected larvae (Fig. 2B) show anterior defects, although not loss of segments, whereas the majority of larvae (Table 1) are more severely affected, lacking head, thoracic and abdominal segments (Fig. 2C). In anterior regions, compared with controls (Fig. 2D), mildly affected embryos at stage 9 retain all segments but have dorsally duplicated gnathal segments (Fig. 2E), whereas severely affected individuals have no anterior segments (Fig. 2F). In situ hybridization for e30 RNA, a honeybee ortholog of engrailed (Walldorf et al., 1989; Dearden et al., 2006) allows better definition of segments (Fig. 2F). In mildly affected larvae, e30 stripes have an unusual orientation being focused around a dorsal thoracic region (Fig. 2H), which is a phenotype associated with gnathal duplications (Fig. 2E) and ectopic anterior tracheal pits (circled in Fig. 2I). Severely affected embryos stained for e30 RNA have staining in only the most posterior abdominal segments, indicating extensive loss of pattern from the anterior (Fig. 2J).

Knockdown of Am-otd2 by RNAi also produces mild and severe phenotypes. In mildly affected larvae, the labrum is lost and head structures, resembling mandibles or maxillae, are shifted...
Fig. 2. RNAi-mediated knockdown of Am-otd1. Embryos and larvae are oriented with anterior left, dorsal up. (A) EGFP RNAi control larva. (B) Mildly affected larva injected as an embryo with Am-otd1 RNAi, showing loss of anterior structures. (C) Severely affected larva with loss of anterior segments, thoracic and A1-A6 abdominal segments. (D-F) Head regions of control injected (D) and Am-otd1 RNAi (E,F) stage 9 embryos. In mild Am-otd1 RNAi knockdown a dorsoventral mirror-image duplication of anterior structures occurs (E). Normal gnathal limbs are labeled (mn, mandible; mx1, maxilla; lb, labium; T1, 2, 3; thoracic limb buds) duplicated gnathal appendages, identified by examination of their morphology, are labeled mm*, mx1*, lb*. The line of symmetry for the duplication is marked with a dotted line. The region from which the anterior-most structures are missing is marked with an asterisk. In severe cases (F) anterior structures and gnathal and thoracic limb buds are missing. (G) e30 RNA expression in a EGFP RNAi stage 9 embryo. (H) e30 RNA staining of a mildly-affected Am-otd1 RNAi stage 9 embryo showing disruption of the e30 pattern in anterior regions. (I) DAPI-stained embryo similar to H; ectopic tracheal pits are circled. (J) e30 RNA staining of a severely affected Am-otd1 RNAi stage 9 embryo. Scale bars: 100 μm. Ant, antennal segment; T, Telson.

toward the anterior (Fig. 3A). In mildly affected embryos anterior stripes of cells expressing e30 RNA are shifted to more anterior positions (Fig. 3B). In severe phenotypes, all anterior segments are missing and posterior segments appear twice-normal size (Fig. 3C), implying fused or expanded segments. The presence of the telson suggest that these are fused segments rather than expanded ones with segments missing at the posterior. e30 RNA staining reveals the loss of every second stripe of e30 throughout the abdomen, and loss or reduction of anterior stripes (Fig. 3D). We examined this unusual phenotype in Am-otd2 knockdown embryos for expression of Am-paired (Am-prd) and Am-even-skipped (Am-eve), orthologs of Drosophila pair rule genes (Osborne and Dearden, 2005a; Wilson et al., 2010). Am-prd RNA is expressed after stage 6 in 15 stripes of cells (Fig. 3E) (Osborne and Dearden, 2005a). In Am-otd2 RNAi-injected embryos, 10 faint stripes of Am-prd RNA are detected (Fig. 3F). Am-eve RNA expression normally occurs in six broad stripes of cells across the embryo that split into two in an anterior to posterior progression (Fig. 3G) (Wilson et al., 2010). In Am-otd2 RNAi embryos, the most anterior and posterior stripes of Am-eve expression appear normal and split, but central abdominal stripes have weaker expression and do not split (Fig. 3H).

Both Am-otd1 and Am-otd2 are expressed in posterior domains from stage 4 (Fig. 1E,N), similar to Nv-otd1 expression in Nasonia, but no posterior defects are seen with RNAi knockdown (Figs 2 and 3). To determine whether these genes are acting redundantly we carried out double RNAi knockdown. Even severely affected double-knockdown embryos, with extensive loss of anterior structures and fusion of abdominal segments, have a segmented posterior region as shown by e30 RNA (Fig. 3I) and DAPI staining (Fig. 3J). Despite expression in posterior regions neither orthodenticle gene appears to contribute to posterior segmentation.

RNAi knockdown of Am-hb causes the majority of embryos to die before stage 9, before they develop the required morphology to distinguish affected segments. More mildly affected embryos show a range of phenotypes, from a lack of head and gnathal segments (Fig. 4A), to loss of anterior segments all the way to the posterior abdomen (Fig. 4B). Staining these embryos at stage 9 for e30 RNA, and comparing them with controls (Fig. 4C) indicates mildly affected embryos have defective anterior segments, fusions of T3 to A1, and loss of A8 and A9 (Fig. 4D), whereas severely affected embryos have only two or three of the most posterior segments (Fig. 4E).

### Table 1. Larval, or stage 9 embryo, RNAi phenotype frequency

<table>
<thead>
<tr>
<th>RNAi target</th>
<th>Mild-moderate phenotype</th>
<th>Severe phenotype</th>
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<tbody>
<tr>
<td>Am-otd1</td>
<td>19 (19%) with loss of brain only 16 (16%) with loss of brain and duplication of head structures</td>
<td>64 (65%) with no anterior development, loss of fusion of abdominal segments and terminal segments normal</td>
</tr>
<tr>
<td>Am-otd2</td>
<td>48 (70%) with loss of brain structure and normal segments</td>
<td>21 (30%) with loss of anterior structures and fusion of segments, but normal terminal structures</td>
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<tr>
<td>Am-hb</td>
<td>15 (25%) with anterior structures missing, ten segments remaining and normal terminal structure</td>
<td>45 (75%) with only three or four segments remaining and terminal segment</td>
</tr>
<tr>
<td>Am-otd1+ Am-otd2</td>
<td>15 (35%) with loss of brain and duplication of head structures</td>
<td>27 (64%) with loss anterior structures and segments, loss of abdominal segments or fusion of some segments</td>
</tr>
</tbody>
</table>

Table refers to injected embryos allowed to hatch or that die at late stage 9.
RNAi knockdown of *Am-otd1*, *Am-otd2* and *Am-hb* all indicate roles for these genes in maternal patterning of anterior regions of the embryo, but the defects in severe cases are extensive and stretch into the abdominal segments.

**Regulation of gap gene expression by anterior patterning genes**

If *Am-otd1*, *Am-otd2* and *Am-hb* act as maternal anterior patterning genes they should regulate the expression of gap genes. Knockdown of these genes should lead to changes in the expression levels and boundaries of gap genes. We examined the effect of knockdown on *Am-Kruppel (Am-Kr)*, *Am-caudal (Am-cad)* and *Am-giant (Am-gt)* expression; genes previously shown to act as gap genes in the honeybee (Wilson et al., 2010).

In control-injected embryos, *Am-Kr* is expressed in a broad central domain at stage 5 where it acts to pattern thoracic and anterior abdominal segments (Fig. 5A). At stage 6 this domain splits into three stripes (asterisks in Fig. 5B). At this time a posterior cap and an anterior domain of expression are detected (Fig. 5B) (Wilson et al., 2010). *Am-cad* is required for abdominal patterning in the honeybee and is expressed from the central to the posterior regions (Fig. 5C) (Wilson et al., 2010). *Am-gt* RNA is expressed in a broad anterior domain and posterior stripe by stage 3. Later, *Am-gt* RNA expression is lost from the anterior dorsal region of the embryo (Fig. 5D) (Wilson et al., 2010).

Knockdown of *Am-otd1* results in loss of anterior *Am-Kr* expression (arrowhead in Fig. 5E,F) and loss or reduction in expression in the central domain (asterisks in Fig. 5E,F). The anterior border of *Am-cad* expression is shifted posteriorly (arrowheads in Fig. 5C,G) with expression being lost from central regions (Fig. 5G). *Am-gt* expression in *Am-otd1* knockdown embryos is missing from the anterior, and ectopic expression appears in central regions of the embryo (Fig. 5H arrow).

In the majority of *Am-otd2* knockdown embryos, expression of *Am-Kr* and *Am-cad* is normal. In a small proportion (13/45) of injected embryos, however, the central domain of *Am-Kr* expression domain shifts slightly toward the posterior (Fig. 5I) and, in stage 6, the central domain fails to split into stripes and anterior expression is lost (Fig. 5J). In a small proportion of embryos (9/25) weak *Am-cad* expression extends toward the anterior from the normal central domain and expression is reduced in the posterior half of the embryo (Fig. 5K). In many *Am-otd2* RNAi embryos, *Am-gt* expression is present at the anterior but fails to be downregulated in the anterior-dorsal region (corresponding to the position of late *Am-otd2* expression; arrow in Fig. 5L), and ectopic expression continues to the trunk regions.

*Am-hb* RNAi knockdown embryos have a reduction in expression in the central domain of *Am-Kr* expression (Fig. 5M) at stage 4 and a loss of anterior (arrowhead) and central domain expression at stage 6 (asterisk in Fig. 5N). *Am-cad* RNA expression
is reduced through central regions of the embryo, remaining at the posterior, where it expands to the very posterior terminus (Fig. 5O). The anterior domain of Am-gt is lost in Am-hb RNAi embryos (arrowhead in Fig. 5P).

Zygotic gap-gene domains that regulate patterning along the honeybee embryo are strongly affected by Am-otd1 and Am-hb knockdown, and to a lesser extent by Am-otd2.

Am-otd1 and Am-hb are required for correct extra-embryonic membrane patterning

In Tribolium, Tc-otd1 regulates the expression of Tc-zen and Tc-sog (Kotkamp et al., 2010). To determine whether a similar linkage exists in the honeybee we examined the expression of Am-otd1 RNAi embryos. Honeybee sog is not expressed in early embryogenesis (M.J.W. and P.K.D., unpublished data). Am-zen RNA is expressed in a dorsal domain that extends from the anterior, along the dorsal surface, to the posterior (stage 4, Fig. 6A,B) (Osborne and Dearden, 2005b; Dearden et al., 2006). In Am-otd1 RNAi embryos, Am-zen RNA expression is lost from only the anterior and posterior poles (Fig. 6C). We also examined Am-zen expression in Am-hb RNAi embryos. Knockdown of Am-hb results in expansion and disorganization of Am-zen expression (Fig. 6D,E) and extra-embryonic membranes (evidenced by the more widely spaced serosa cells; Fig. 6F). Am-zen mRNA is lost from the anterior in Am-hb RNAi embryos but becomes expressed across central regions and expanded dorsally. Both Am-otd1 and Am-hb thus contribute to the regulation of Am-zen and extra-embryonic membrane patterning.

In Tribolium, RNAi knockdown of Tc-otd1 leads to ectopic apoptosis contributing to the anterior patterning defect. To determine whether this also occurs with Am-otd1 knockdown, we examined RNAi-treated embryos with DAPI and found that cell death is increased in Am-otd1 embryos in anterior regions (see Fig. S2 in the supplementary material).

To determine whether, as in Tribolium, loss of Am-zen is responsible for the anteroposterior patterning defects found in Am-otd1 and Am-hb RNAi embryos, we examined the phenotype of Am-zen knockdown. If Am-zen is responsible for the defects in anterior posterior patterning, then knockdown should lead to anterior segmentation defects and changes in the expression of gap and pair-rule genes.

Am-zen RNAi embryos survive to hatchling but have a deformed head in dorsal regions and extended embryo flanks (Fig. 6G), causing them to curve differently to control larvae (Fig. 6H). DAPI staining at stage 9 indicates that the dorsal side of the embryo collapses in mild cases (Fig. 6J), and, in severe cases, the germ rudiment extends all the way to the dorsal side of the embryo (Fig. 6K,L) unlike the control (Fig. 6I). All segments are present in Am-zen RNAi embryos (Fig. 6J-L), pair-rule gene expression is normal (Fig. 6M) and e30 stripes extend further toward the dorsal side of the embryo, indicating that dorsal embryonic tissue extends more dorsally (Fig. 6N).

As Tribolium zen knockdown produces defects in anteroposterior patterning we examined the regulation of key anteroposterior patterning genes in Am-zen knockdown embryos (Fig. 7) expecting that they would be affected by loss of Am-zen expression as in Tribolium. In Am-zen knockdown embryos, Am-Kr and Am-cad expression domains are indistinguishable from those in wild-type embryos (Fig. 7A,B). Anterior expression of Am-tailless (Am-tll) is reduced and shifted anterodorsally (Fig. 7C) compared with wild-type embryos (Fig. 7D). Am-gt expression is normally absent from dorsal regions of the embryo (Fig. 7E) but in Am-zen knockdown, expression is expanded into cells in anterodorsal regions (Fig. 7F-I). Thus although Am-zen is required for patterning of the extra-embryonic membranes, loss of Am-zen does not affect segmentation, does not have a significant effect on gap gene expression, and is not responsible for the loss of anterior patterning seen in Am-otd1 RNAi embryos.

Regulation of Am-gt expression

Our experiments suggest that Am-otd1, Am-otd2 and Am-hb regulate Am-gt (Fig. 5). To determine whether this regulation is direct and indicative of a role in regulating key anterior gap genes, as expected for their maternal anterior patterning function, we searched for cis-regulatory motifs (CRMs) that regulate the expression of Am-gt. We searched for clustered transcription-factor binding sites for known regulators of giant upstream of Am-gt on the assumption that the target binding sites of potential regulators remained the same as their Drosophila orthologs, using ClusterDraw (Papatsenko, 2007) (see Fig. S3 in the supplementary material). A 4 kb region (Am-gt CRM) indicated in this analysis was cloned upstream of a lacZ reporter gene and used to produce transgenic Drosophila lines.

lacZ expression driven by the Am-gt CRM was examined by in situ hybridization (Fig. 8). Weak expression of lacZ transcripts was detected in stage 1 embryos (Fig. 8A), with significant expression appearing by stage 4 in an anterior domain (Fig. 8B,C). At stage 5, lacZ is expressed along the dorsal midline in the developing aminoserosa (Fig. 8D). We crossed the Am-gt CRM reporter into mutants homozygous for transcription factors that potentially regulate binding to this CRM. In Otd mutants, lacZ is absent in the anterior
but remains in the dorsal midline (Fig. 8E). In *hb* mutants *lacZ* expression is reduced through the embryo (Fig. 8F). In *zen* mutants, *lacZ* expression is lost from the aminoserosa (Fig. 8G). In *Drosophila*, *cad* has been implicated in activating the posterior domain of *gt* (Schulz and Tautz, 1995). *Am-gt* CRM expression in *cad* mutants is upregulated throughout the embryo, consistent with a role for *Am-cad* as a repressor of *Am-gt* (Fig. 8H). To test this interaction in the honeybee, RNAi knockdown of *Am-cad* resulted in a posterior shift of the posterior border of the anterior. (*I*) *Am-Kr* RNA expression in *Am-cad* RNAi stage 5 embryo showing a posterior shift in the anterior boundary of *Am-cad* expression. (*H*) *Am-gt* RNA expression in an *Am-cad1* RNAi stage 5 embryo. *Am-gt* expression is lost, with ectopic expression in trunk regions of the embryo (arrow) and the posterior stripe shifting to the anterior. (*J*) *Am-Kr* RNA expression in *Am-cad2* RNAi stage 5 and 6 embryos. *Am-Kr* expression is reduced and the central band of expression is slightly shifted towards the posterior (I); no splitting of the central domain into stripes occurs and anterior expression is lost (arrowhead, J).

**DISCUSSION**

**Diversity in the roles of Otd genes in insect anterior patterning**

We have shown that *Am-otd1, Am-otd2* and *Am-hb* have anterior patterning roles in honeybee embryos. We have also shown that *Am-otd1, Am-otd2* and *Am-hb* probably directly regulate the expression of the key anterior gap gene *giant*. RNAi knockdown of these genes, especially *Am-otd1* and *Am-hb*, affect the expression domain of every gap gene we have examined, including anterior and more posterior-acting genes. Given this evidence we propose that *Am-otd1* and *Am-otd2* together with *Am-hb* act as maternal anterior patterning genes in the honeybee embryo.

Although this is consistent with the composite anterior patterning system identified in *Nasonia* (Lynch et al., 2006), it is clear that the regulatory interactions, and functions, of these genes in honeybees differ from all other described insects.

The effects of knockdown of either *Am-otd1* or *Am-hb* are more extensive than in any other insect reported. Severely affected larvae from embryonic *Am-otd1* RNAi have anterior defects stretching all the way to eighth abdominal segment, and similarly *Am-hb* RNAi embryos retain only the three to four most-posterior segments. *Nv-otd1* knockdown in *Nasonia* leads to loss of the anterior and gnathal regions, as well as defects in the most posterior segments. *Nasonia* *hb* knockdown produces, at most, anterior, gnathal and...
Am-otd2 has acquired unique functions in honeybee embryogenesis. It is maternally expressed and required for both anterior patterning and segmental patterning in abdominal regions. In Tribolium, Nasonia and Acrythosiphon, Otd2 is reported as being expressed late in embryogenesis with no possible role in axis formation (Yuebing et al., 1996; Lynch et al., 2006; Schinko et al., 2010). In honeybee Am-otd2 regulates known gap genes and affects the expression of pair-rule genes leading to defects in segmentation. In contrast to Am-otd1, Am-otd2 appears to act as a repressor, restricting expression of Am-gt and Am-cad.

Some of the activity of both honeybee Otd genes and hb appear to be transduced through giant. In honeybees, as in Nasonia, giant is a key regulator of anterior fate required for head and thorax formation (Brent et al., 2007; Olesnicky and Desplan, 2007; Wilson et al., 2010). The Am-gt CRM we have identified has predicted binding sites for both hb and Otd, and removing these proteins in Drosophila embryos changes the expression of a reporter gene driven by the honeybee giant CRM. Both Am-otd1, Am-otd2 and Am-hb knockdown affect Am-gt expression, indicating this CRM construct accurately reflects the regulation of Am-gt, and regulation by Am-otd1, Am-otd2 and Am-hb is likely to be direct.

Thoracic defects. RNAi against both genes gives broader defects, though rarely as extensive as individual knockdown in honeybee (Lynch et al., 2006). Mutants of the presumed replacement for otd1 in Drosophila, bicoi, also only affect anterior patterning of the anterior abdominal segments (Struhl et al., 1989). In crickets RNAi knockdown of Gb-otd1 leads to loss of anterior head regions (Nakamura et al., 2010). In honeybees definition of the anterior seems a crucial event in patterning the entire body, and in these embryos, anterior patterning defines most of the abdominal segments. This unusual extent of the influence of anterior patterning must be related to the unusual function of honeybee segments. This unusual extent of the influence of anterior patterning defines most of the abdominal embryogenesis. It is maternally expressed and required for both anterior patterning and segmental patterning in abdominal regions. In Tribolium, Nasonia and Acrythosiphon, otd2 is reported as being expressed late in embryogenesis with no possible role in axis formation (Yuebing et al., 1996; Lynch et al., 2006; Schinko et al., 2010). In honeybee Am-otd2 regulates known gap genes and affects the expression of pair-rule genes leading to defects in segmentation. In contrast to Am-otd1, Am-otd2 appears to act as a repressor, restricting expression of Am-gt and Am-cad.

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Finally, in Nasonia, Nv-otd1 is required for both anterior and posterior patterning, and the RNA is tethered to a posterior organelle, the oosome, in oocytes (Lynch et al., 2006). In honeybees both Am-otd1 and Am-otd2 are expressed in posterior regions, but no evidence of RNAi disruption of segmentation can be seen in the posterior, though these genes have later roles in this region, as shown by the loss of Am-zen expression in the posterior of Am-otd1 knockdown embryos.

**Regulation of dorsoventral gene expression by orthodenticle is an ancestral character**

In Tribolium, anteroposterior patterning defects caused by Tc-otd1 knockdown appear to be due to interactions with dorsoventral patterning (Kotkamp et al., 2010). Tc-otd1 activates zen and sog, and these genes specify anterior fate. This regulatory linkage has not been investigated in Nasonia, and is not present in Drosophila, suggesting that it might be a quirk of Tribolium biology, perhaps related to the anterior placement of extra-embryonic membranes in Tribolium embryos (Falciani et al., 1996).

However, we show that some of the regulatory linkages identified in Tribolium are also present in the honeybee. Am-otd1 regulates zen, and zen expression regulates Am-gt, as shown by our Am-gt CRM experiments. Zen is the ancestor of bcd (Stauber et al., 1999), but it is not bcd that is regulating our CRM fragment because lacZ is not expressed early enough, nor in a concentration-dependent way. This linkage between Otd genes and zen may be intact in some Diptera because in the cyclorrhaphan fly Episyrphus, otd1 overexpression causes both reduction of the serosa and zen expression (Lenke and Schmidt-Ott, 2009).

The dorsoventral duplication of gnathal segments seen in mild Am-otd1 knockdown is probably related to this dorsoventral patterning effect. The duplication is not, however, due solely to loss of Am-zen, because although the embryo extends more dorsally in Am-zen knockdown, it does not have the gnathal duplications seen in Am-otd1 knockdown. We suggest that mild knockdown of Am-otd1 may disrupt the identity of the anterior regions, perhaps due to loss of the highest concentrations of an Am-otd1 gradient, leading to the uncovering of a anterodorsal patterning centre as suggested by Am-e30 staining in Fig. 2H. Loss of Am-zen expression as a result of Am-otd1 knockdown, leads to ectopic dorsal expansion of the embryo, which now, because of the absence of Am-otd1, takes up a ventral, gnathal fate.

We show that the cross-talk between anteroposterior and dorsoventral patterning, seen in Tribolium, is conserved in honeybee. Honeybees are hymenoptera and considered to be the most basal branch of holometabolous insects (Krauss et al., 2005; Savard et al., 2006; Zdobnov and Bork, 2007), indicating this is an ancestral character in holometabolous insects. It seems that Otd
genes in the ancestor of holometabolous insects regulated both anteroposterior and dorsoventral systems, but in Tribolium, the contribution of odd to anteroposterior patterning has been reduced.

The evolution of anterior patterning and buffering

The regulation of anterior fate in the honeybee is similar to anterior patterning in Tribolium, Nasonia, and the honeybee, but the honeybee system is distinctly different. These differences are related to both the nature and strength of the regulatory interactions between anterior patterning molecules. We have shown that some of these changes are caused by functional changes in the regulatory regions driving expression in these genes.

These findings indicate that, as in Nasonia, Otd genes act, with hh, as maternal anterior patterning genes in the honeybee, but that the details of this regulation differs between these species. This diversity indicates that anteroposterior axis formation is an evolutionarily labile pathway, despite being required as the foundation of later, highly conserved, patterning. An important challenge for the future is to understand how changes in this regulatory network are buffered to produce the conserved gene expression outputs of both segmentation and dorsoventral patterning.

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