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Engrailed expression and body segmentation in the honeybee *Apis mellifera*

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Summary. Honeybee embryos were stained with a monoclonal antibody raised against the *Drosophila engrailed* protein. The antibody was found to label rows of nuclei in the transverse grooves that form the earliest external sign of metameric germ band organization. These grooves demarcate metameric units about seven cell rows wide, of which about three rows with reduced apical cell surfaces account for the grooves. The *en* stripes appear in the grooves as soon as these form and grow from one to about four cells in width and thus completely overlap the groove. During the rudimentary germ band retraction, the grooves shift slightly backwards relative to both the *en* stripes and the tracheal pits. The spatio-temporal pattern by which the series of grooves and stripes arises is quite striking. Both become visible first in the gnathal and thoracic regions, then in the pregnathal parts of the head and in the abdomen. The stripes arise essentially in an antero-posterior sequence. In addition, the earliest stripes to form display a pattern of alternating intensities whereas the later stripes, those in the abdomen, arise with approximately equal strength. The latter trait was earlier observed in the grasshopper, while the former is known from *Drosophila* where, however, the strong stripes correspond to the weak stripes in the honeybee.

Key words: Honeybee – Gastrulation – Segmentation – *Engrailed*

Introduction

Segmentation is one of the basic events in insect embryogenesis (Lawrence 1981; Sander 1988). In *Drosophila* various genes involved in the subdivision of the embryo into visible metamers have been characterized (Nüsslein-Volhard and Wieschaus 1980; Nüsslein-Volhard et al. 1982; Akam 1987; Ingham 1988). Several of these already show a metameric expression pattern in the blastoderm stage (Akam 1987; Ingham 1988; Lawrence and Johnston 1989) while external metameric grooves are not seen before the extended germ band stage (Turner and Mahowald 1977). The correlation of these early patterns with the metameric subdivision of the larval body is well known (Martinez-Arias and Lawrence 1985; Akam 1987; Ingham 1988; Lawrence 1981, 1988; Lawrence et al. 1987; DiNardo and O'Farrell 1989). In some other insects, genetic analysis of segment formation and specification is also under study, for instance in the silk moth (see Tazima 1964), flour beetle (Beeman et al. 1989), grasshopper (Patel et al. 1989), and honeybee (Fleig et al. 1988; Walldorf et al. 1989). Among these, the honeybee may be of interest because formally it represents the extreme of the long-germ type of development, like the fruitfly (Krause 1939) but the metameric pattern of transverse grooves becomes visible at an earlier stage than in *Drosophila* and there is no head involution. The latter trait gives the honeybee an intermediate position between more primitive insects (intermediate and short-germ types) (Krause 1939) and the more specialized higher dipterans. Moreover, the near absence of germ band extension and retraction in the honeybee makes it easier to follow the localization of individual metamers through development. Here I describe the development of *engrailed* protein expression in relation to the metameric pattern of grooves and tracheal pits from gastrulation to the hatching stage.

Materials and methods

Egg collection, incubation, staging, and scanning electron microscopy preparation were done as described previously (Fleig and Sander 1986). Treatment for antibody staining was as follows: dechorionation with NaOCl for 1 min and *n*-heptane phase fixation with 3% formaldehyde in phosphate-buffered saline pH 7.2 for 30 min, followed by removal of the vitelline envelope with tungsten needles on doublestick tape in phosphate-buffered saline pH 7.2. Antibody treatment and labeling were done according to Lawrence et al. (1987).

Results

Gastrulation starts about 33 h after egg deposition (Fleig and Sander 1986), when two longitudinal furrows become visible in the prospective gnathal region. They separate the prospective mesoderm (ventral plate) from the prospective ectoderm (lateral plates) (Fig. 1a). As the furrows extend towards the egg poles, the future germ layers detach from each other and the edges of the ectoderm plates start to move over the mesoderm anlage until they meet each other at the ventral midline; these events, too, occur first in the prospective gnathal and thoracic regions.

During very early gastrulation stages, when separation of the future germ layers has not yet spread to the posterior half of the embryo, metameric units become visible in the gnathal and thoracic regions of the ectoderm plates, demarcated by slight transverse grooves (Fig. 1b, c). Each metameric unit is about seven cell rows wide in the anterior-posterior dimension; two to three rows of cells with smaller apical faces form the groove and the space between two grooves is about four to five cells wide (Fig. 1b, c). The most anterior groove shows an oblique orientation. It separates the anlagen of the antennal and intercalary segments. Together with the well-defined posterior border of the head lobe, this permits the identification of the early metameric units with individual future segments (Fig. 1c).

In this stage *engrailed* stripes can be demonstrated by antibody staining in the grooves (Fig. 1d). The staining shows a double segmental pattern of alternating intensities, the mandibular, labial, and mesothoracic stripes being stronger while the maxillary and prothoracic ones are weaker and perhaps develop a little later; the segmental assignment is based on the assumption that the labeled cells, as in *Drosophila* (Kornberg et al. 1985; Martinez-Arias and Lawrence 1985; Carroll et al. 1988), represent the posterior compartment of the segment. The stripes, especially the weaker ones, seem to consist at first of a single irregular row of cells. The stripes in the forehead region (prospective intercalary, antennal, and perhaps labral segments) are weaker or not visible at all, and the abdominal part of the embryo does not yet show any *engrailed* staining. In the thoracic region, weak *en* stripes are also evident in the mesoderm anlage (Fig. 1d).

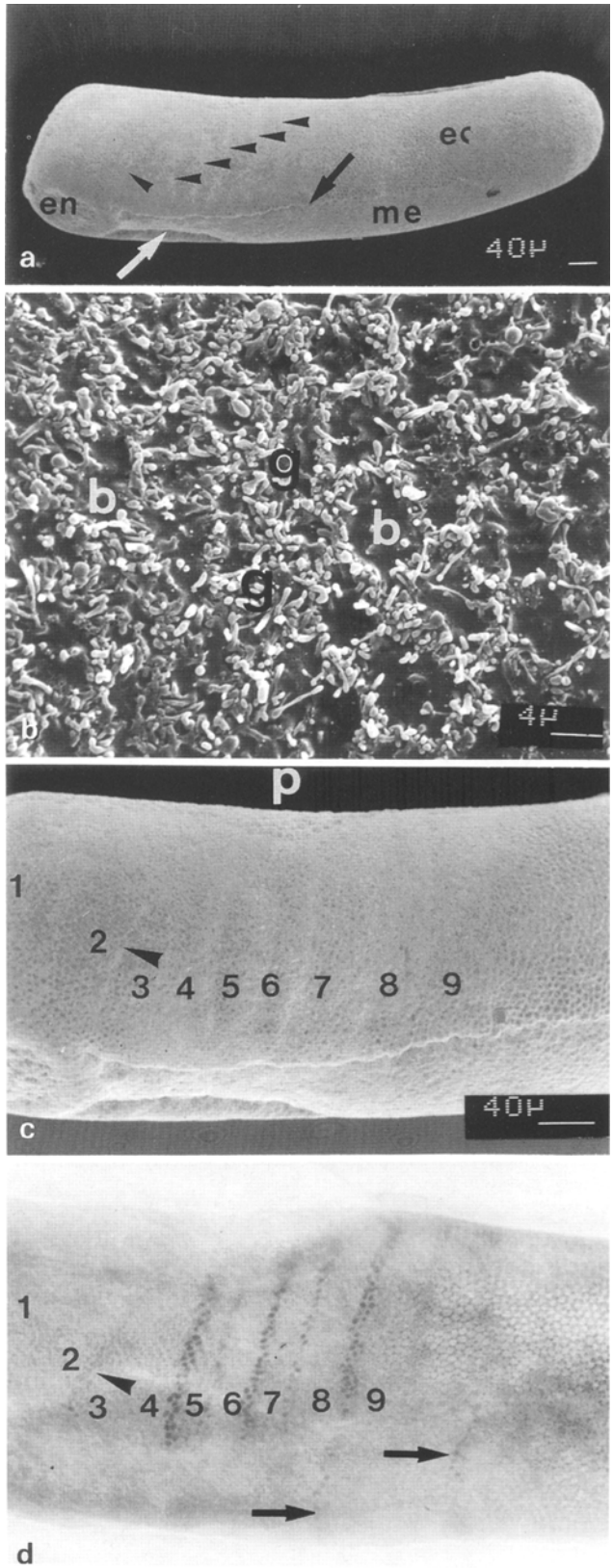

Fig. 1. a Scanning electron microscopy (SEM) preparation of whole embryo, early gastrulation stage, ventrolateral view. Anterior is to the *left* and ventral to the *bottom* (as in all following photographs). Two gastrulation furrows (*arrows*) separate the prospective ectoderm (*ec*) from mesoderm anlage (*me*); *en* is the future anterior entoderm. Transverse grooves are already visible in this early stage in the gnathal and thoracic regions of the future ectoderm (*arrowheads*). Age 33 h (at 35° C, as in all other figures). **b** Detail of **a**; apical surface of cells is smaller in the grooves (*g*) than between the grooves (*b*). **c** Detail of **a**; assignment of grooves to future segments: labral (*1*), antennal (*2*), intercalary (*3*), mandibular (*4*), maxillary (*5*), labial (*6*), prothoracic (*7*), mesothoracic (*8*), metathoracic (*9*). Landmarks for identifying segment anlagen are the oblique orientation of the groove between the antennal and intercalary segment anlage (*arrowhead*) and the wedge of large preserosal cells (*p*) at the posterior margin of the head lobe. **d** *Engrailed* stripes in gnathal and thoracic regions during early gastrulation. A double-segmental pattern of alternating strong (two cells wide) and weak (one cell wide) stripes is visible; note also the labeled stripes in the mesoderm anlage (*arrows*)

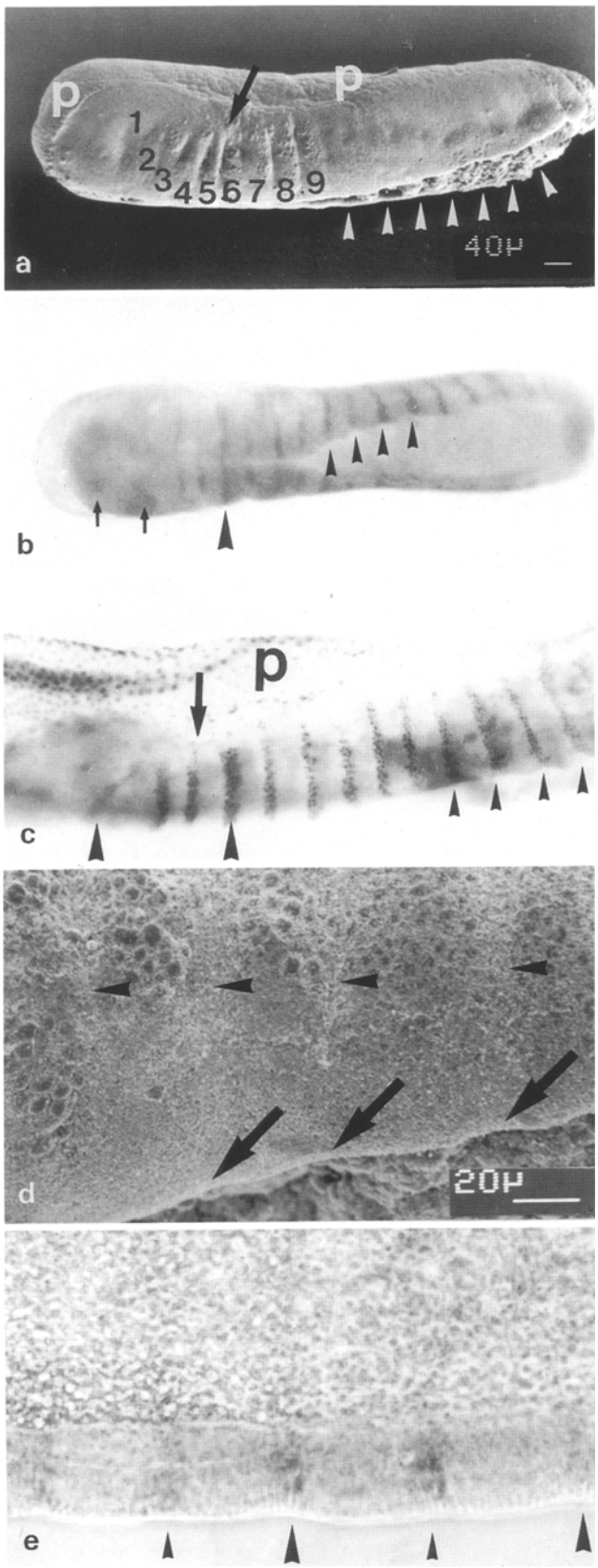

Fig. 2. **a** SEM preparation of whole embryo during gastrulation, lateral view. Metameric grooves are seen in the prospective ectoderm and also (*arrowheads*) in the mesoderm anlage not yet covered by ectoderm. Globular protrusion of cells in the future ectoderm indicates mitoses. The wedge of preserosal cells (*p*) at the posterior margin of the head lobe, between maxillary and labial segment anlage, is marked by an *arrow*. Age 35 h. **b** Same stage, ventral view. Note *engrailed* stripes in all body regions. In the pregnathal head, the presumed labral and antennal stripes are very weak (*arrows*) while the intercalary stripe is not yet visible. Alternating of strong and weak stripes is evident in the gnathal and thoracic regions. Identification of stripes is based on the labial stripe, marked by a large *arrowhead*. The mesodermal layer no longer shows any label. Indentations along the medial margin of the ectoderm plates (*arrowheads*) reveal the position of the grooves. **c** Slightly older stage, lateral view. Antennal stripe (*arrowhead at the left*) and posterior margin of head lobe (*arrow*) can be used as landmarks. Gnathal, thoracic, and abdominal stripes are of almost equal strength, they are two or three cells wide. Indentations (*arrowheads*) of the ventral margin of the ectoderm anlage show the position of the grooves. Note the strong label in the nuclei of the preserosa (*p*). **d** Ventrolateral detail of gastrulating embryo. The indentations are marked by *arrows* and the grooves in the future ectoderm by *arrowheads*. **e** Ventral detail of optical section, late gastrulation stage. Those cells which form the grooves stain for *engrailed*. Deep and shallow grooves show an alternating pattern (*large and small arrowheads*)

As gastrulation continues, grooves and *engrailed* stripes are seen along the whole body (Fig. 2a, b). The stripes in the abdomen all originate with equal intensity and arise one after the other in antero-posterior sequence. Soon afterwards the double segmental pattern of the stripes vanishes and all stripes except those in the procephalon and labial segment are about three cells wide. During gastrulation each segment anlage is about ten cells wide (Fleig et al. 1988) after mitoses start again (Fig. 2a). In the procephalon, where the grooves are smaller or not visible at all in the scanning electron microscope, staining intensity is weak and only a few cells are marked. The labial stripe, on the other hand, remains broader than all the others (Figs. 2b, c, 3b). In later stages of gastrulation the *engrailed* label is no longer seen in the incipient mesoderm (Fig. 2b) although the visible part of this layer is clearly subdivided by grooves and bulges in a metameric manner (Fleig and Sander 1986) (Fig. 2a). During gastrulation the ectodermal grooves can be seen in the light microscope when the medial rim of the advancing ectoderm plate is in focus (Fig. 2b, d), and this establishes that the *engrailed* stripes at this stage coincide with the grooves (Figs. 2b, e, 3d, e). From mid-gastrulation onward, all nuclei in the preserosa and later in the serosa express *en* protein (Figs. 2c, 3b, c).

During late gastrulation, and especially in the young germ band, adjacent grooves transiently alternate in depth (Fleig and Sander 1986) (Fig. 3a). In contrast to this, the *engrailed* stripes in the young germ band stage are of equal width and strength in both thorax and abdomen (Fig. 3b). They are about four cells wide and the nuclei along the posterior border show weaker staining (Fig. 3c). Comparison of the early 'double segment' pattern of *engrailed* intensity in the gnathal and thoracic regions with the subsequent pattern of alternating groove depths reveals that the weak grooves correspond to the formerly strong stripes, and vice versa.

In the fully extended germ band only the last three of a total of ten abdominal segments form a cap around

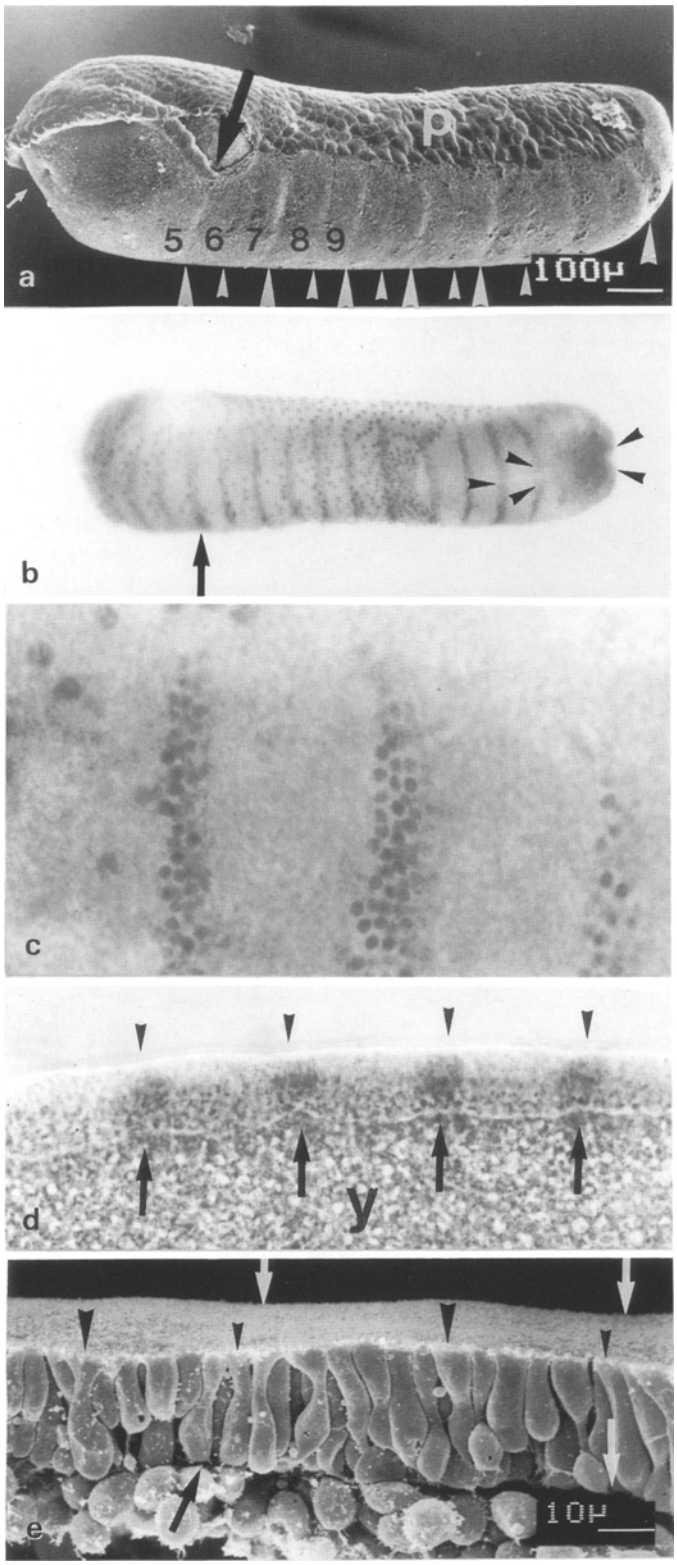

Fig. 3. **a** SEM preparation of whole embryo during late gastrulation, lateral view. The head lobe extends around the anterior pole, the oral pit (*small arrow*) and the posterior margin of the head lobe (*large arrow*) are visible. A double-segmental pattern of alternating deep and shallow grooves is shown (large and small arrowheads). The first double metamere contains the anlagen of the labial and prothoracic segments. The preserosa starts to expand ventrally around the anterior pole (*left*) and over the head lobe. Globular protrusions in the posterior region of the prospective ectoderm result from mitoses. Age 41 h. **b** Embryo slightly older than in **a**, ventral view. Gastrulation is not yet finished in the posterior part (*arrowheads* mark edges of advancing ectoderm). The serosa with its labeled nuclei has moved over the anterior two-thirds of the embryo ventrally. Labial stripe marked by an *arrow*. **c** Detail of **b** in the region not yet covered by the serosa, except in the *upper left* corner where labeled serosa nuclei are visible. The stripes are now three to four cells wide, the label in the nuclei is strong along the anterior border (*left*) and weak along the posterior border (*right*). **d** Optical section, detail of a young germ band showing ectoderm and yolk (*y*). Grooves are hardly visible along the outer ectoderm face (*arrowheads*), but are clearly visible along the inner face (*arrows*). **e** Detail of SEM preparation, stage and view as in **d**, fracture through lateral ectoderm (columnar cells at the top) and mesoderm (globular cells below). The grooves are visible along the outer and inner face of the ectoderm (*arrows*). The apical surfaces of the columnar ectoderm cells are larger in the area between the grooves (*large arrowheads*) than in the grooves (*small arrowheads*)

the posterior pole of the yolk system and, in contrast to *Drosophila*, no segments but only the anlagen of Malpighian tubules and hindgut reach the dorsal egg side. All grooves are then of equal depth (Fig. 4). Soon thereafter the ten pairs of tracheal invaginations form one after the other, beginning with the most anterior in the mesothorax and progressing to the last one located in the eighth abdominal segment. The invaginations appear laterally in a row and each of them is located half-way between two grooves (Fig. 4). Some hours later eight additional pairs of lateral invaginations appear transiently in line with the tracheal pits. They occupy the anterior slope of each groove between segments a1/a2 and a8/a9 (Fig. 5a); the cells invaginating there will probably give rise to oenocytes (Schnetter 1935). These invaginations provide a good marker to show that during germ band retraction, which is rather inconspicuous in the honeybee (Figs. 4, 5a), the *en* stripes are still congruent with the grooves (Fig. 5b, c). Some time later these secondary invaginations are no longer seen on the surface of the embryo (Fig. 6a). At dorsal closure the relative position of the grooves seems to have changes with reference to both the tracheal pits and the *en* stripes. The deepest stretch of the grooves now coincides with the posterior stripe border and the pits are located somewhat closer to the anterior groove than before (Fig. 6a, b, c).

Discussion

With antibodies against *Drosophila engrailed* protein (DiNardo et al. 1985), the onset of *engrailed* expression has also been studied in embryos of two other arthropods, namely the crayfish *Procambarus* and the grasshopper *Schistocerca* (Patel et al. 1989). In all three species, the *engrailed* antibody stains at first only a single line of nuclei per segment anlage (Karr et al. 1989; Foe 1989; Patel et al. 1989). This is also true for the honeybee, where the stripe then expands in two steps to its final width of about four cells. At least the first of these steps, from one to three cell rows, must involve recruit-

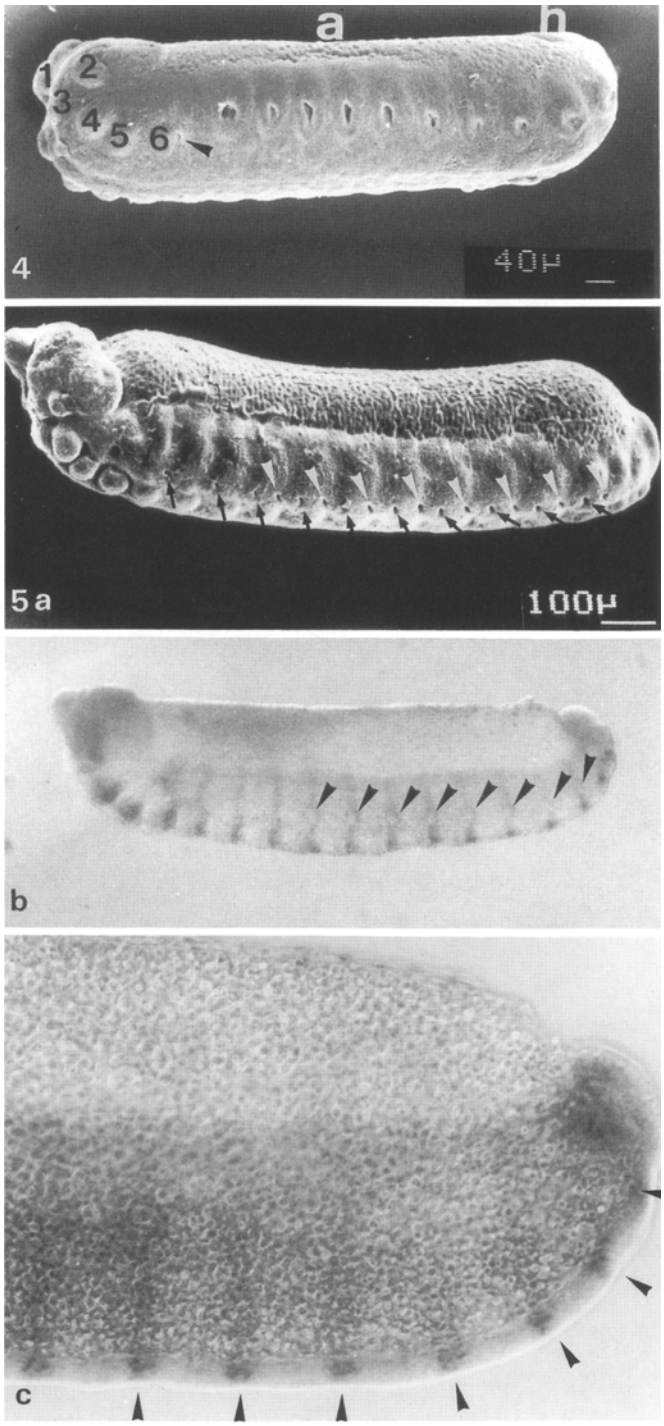

Fig. 4. SEM preparation of young germ band stage, lateral view. In this and the following (older) stages the serosa is removed. The invagination of the future silk gland (*arrowhead*) is visible behind the anlage of the labium. The ten tracheal invaginations are each located half-way between two grooves; note the gradient in their development from mesothorax to the eighth abdominal segment. The anlagen of hindgut and Malpighian tubules have moved around the posterior pole on the dorsal side (*h*); this is the most extended stage of the honeybee germ band. The amnion (*a*) covers the dorsal part of the yolk as a transient dorsal closure (Fleig and Sander 1988). Age 45 h

Fig. 5. a SEM preparation of late germ band stage, lateral view. Head appendages are more advanced than in Fig. 4, the rudiment of intercalary segment (bulge of tritocerebrum) is no longer visible. Hindgut anlage now occupys the posterior pole; tracheal pits (*arrows*) are slightly closer to the anterior than the posterior groove, especially in the thorax. Between the first and ninth abdominal segments, eight secondary invaginations are visible, each in the anterior flank of a groove (*arrowheads*). Age 54 h. **b** Embryo slightly younger than in **a**, note slightly dorsal position of the hindgut anlage; lateral view. The *engrailed* stripes are located within the grooves, forming a weak anterior and a strong posterior branch around each secondary invagination (*arrowheads*). Ten abdominal stripes (and segments) are visible. **c** Same stage as in **b**, posterior part, lateral view. The position of the *engrailed* stripes in the grooves (*arrowheads*) is evident in this optical section

ment of new cells, since it takes place before the onset of post-blastodermal mitoses. Mitoses in the germ band stage (Fleig and Sander 1986) may account for the second and final increase in the number of labeled cells per stripe. As in *Schistocerca* (Patel et al. 1989), staining tends to be stronger in the anterior than in the posterior nuclei of a given stripe during early germ band stages.

In the honeybee the *engrailed* stripes arise sequentially, as they do in the fruitfly (DiNardo et al. 1985; Karr et al. 1989) and the grasshopper (Patel et al. 1989), albeit in a different pattern. Onset and strength of *en* expression in these species seem to correlate roughly with the largest prospective appendage buds. In the honeybee the mandibular buds are most prominent and it is perhaps in this region that the first stripe occurs. In the fruitfly the maxillary buds are the largest, and the first and broadest stripe is found in the corresponding region (DiNardo et al. 1985; Karr et al. 1989). In the grasshopper, where the leg buds are the largest, the earliest stripe appears in the first thoracic segment (Patel et al. 1989).

During formation of the stripe pattern, the honeybee seems to combine traits of both the fruitfly and the grasshopper. In the gnathocephalon and thorax, adjacent stripes alternate in intensity, as in the fruitfly (Weir and Kornberg 1985; DiNardo et al. 1985), whereas the abdominal stripes appear in antero-posterior succession without evident alterations in strength, a mode observed in grasshopper (Patel et al. 1989) and leafhopper (my unpublished results). The appearance of a pair-rule trait in the anterior but not in the posterior regions of the honeybee germ band poses the interesting question whether pair-rule genes in this species might be involved solely, or predominantly in subdividing the anterior body regions. A difference between fruitfly and honeybee patterning mechanisms is indicated by the fact that the strong stripes in the honeybee share segmental identity with the weak stripes in the fruitfly, and vice versa.

The spatial relationship between *engrailed* stripes and transverse grooves could reveal the position of the grooves within the future segment pattern if *engrailed* expression in *Apis* were to coincide with the posterior compartment, as in *Drosophila* embryos (Kornberg et al. 1985; Martinez-Arias and Lawrence 1985; Carroll et al. 1988). In crayfish, grasshopper, and leafhopper the grooves arise just behind the *engrailed* stripes, i.e. in intersegmental positions (Patel et al. 1989; my unpublished results) while in the honeybee they appear first *within* the *engrailed* stripes, and shift rather late to the

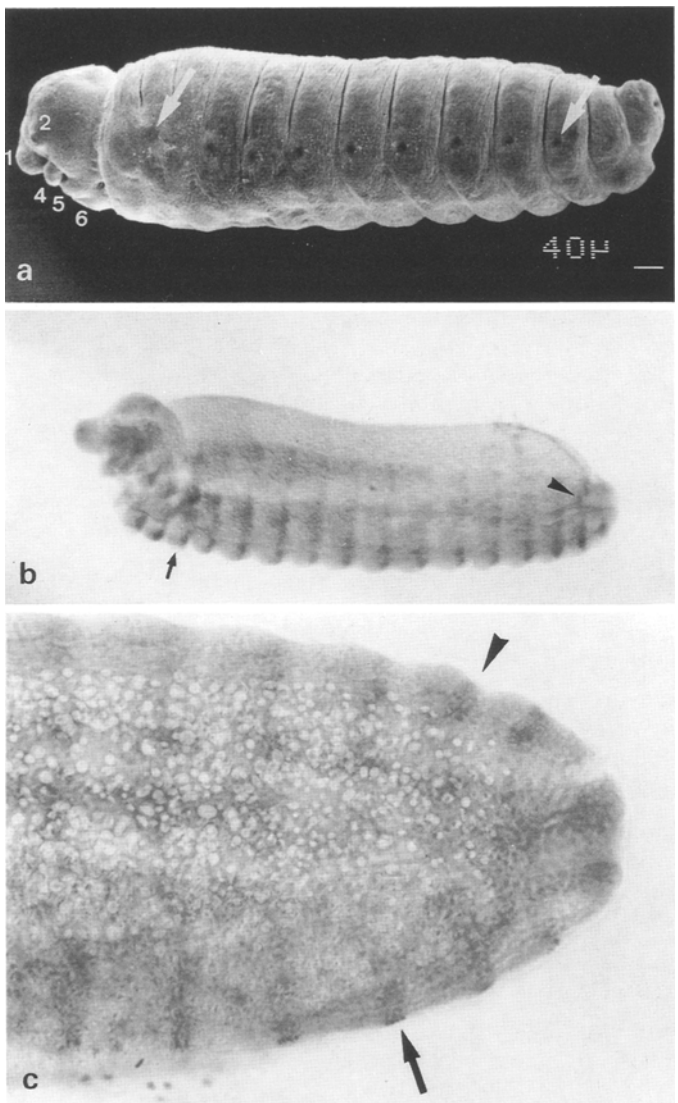

Fig. 6. a SEM preparation of a larva about to hatch, lateral view. Tracheal pits located in the anterior half of each segment, the first and ninth are marked by *arrows*. Age 63 h. **b** Same stage as in **a**, lateral view. The *engrailed* stripes terminate just in front of the bottom of each groove. Ten abdominal stripes are visible. The labium at the rear end of the (somewhat damaged) head is marked by an *arrow*. At the posterior the hindgut and part of the Malpighian tubules (*arrowhead*) are visible in optical section. **c** Same stage as in **b**, lateral view of posterior part of embryo. The *engrailed* stripes are located anterior to the deepest point of the grooves (see *arrowhead*). The stripes are about four cells wide (*arrow on bottom*)

intersegmental position. In *Drosophila*, however, the spatial rearrangement of grooves is even larger (Turner and Mahowald 1977; Petschek et al. 1987), because parasegmental (primary) grooves in front of the *engrailed* stripes are later replaced by intersegmental (secondary) grooves behind the stripes (Martinez-Arias and Lawrence 1985; Lawrence et al. 1987; Lawrence 1988; Lawrence and Johnston 1989). A similar difference exists with respect to the tracheal pits, which in the honeybee remain on the body surface. They shift only somewhat anteriorly with reference to the grooves, whereas in the fruitfly they are taken up by the secondary grooves during germ band retraction (Turner and Mahowald 1979; Martinez-Arias and Lawrence 1985; Petschek et al. 1987).

The discussed differences between *Apis* and *Drosophila* could mainly be due to the unusual depth of the secondary grooves in *Drosophila* noted already by Martinez-Arias and Lawrence (1985). This in turn might be related to head involution. During this event the grooves become shallow again (Turner and Mahowald 1979; Petschek et al. 1987). Perhaps the epidermis of the thorax and abdomen stretches flat in order to compensate for the epidermis internalized with the head. A correlation of excessive groove depth with the process of germ band retraction is less likely. In the Colorado beetle *Leptinotarsa decemlineatea*, where germ band extension and retraction are as extensive as in *Drosophila*, the grooves fail to swallow up the tracheal pits and remain shallow throughout (my unpublished results, see Fig. 1 in Sander 1988).

To sum up, the honeybee, in its mode of forming the individual *engrailed* stripe, follows rules apparently shared by all arthropods studied so far. The development of its stripe pattern, however, and the topographical relations between incipient stripes and transverse grooves place it somewhere between the fruitfly and lower insect forms like the grasshopper.

Expression of *engrailed* in embryonic envelopes has to my knowledge not been described before. In the honeybee the large nuclei of the serosa stain as soon as this layer becomes morphologically distinct (preserosa, Fleig and Sander 1986). The nuclei of the amnion fail to stain. This may be connected to the fact that the amnion in the honeybee forms dorsally right from the beginning (Fleig and Sander 1986). In the Colorado beetle the amnion in this position also does not express *en* activity while at an earlier stage, when it is still in the ventral position, its nuclei stain with the *en* antibody (my unpublished observation).

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