

Regeneration in the medial–lateral axis of the insect thoracic segment

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Summary

We have studied pattern regulation in the medial–lateral axis of the insect segment by grafting legs of beetle larvae (*Tenebrio molitor*) in different orientations into different positions medial and lateral to the leg site. The Boundary Model and Polar Coordinate Model of the insect appendage predict various patterns of supernumerary leg regeneration, and these grafts were designed to test the predictions.

When a larval leg is grafted with normal anterior–posterior orientation medial to the normal leg, larvae and subsequent adults bear the graft plus a supernumerary leg. This is located where the lateral edge of the grafted leg confronted medial thorax (from the leg base across to the midline) and is orientated as a mirror image of the graft. The tarsal structure of supernumeraries resulting from grafts of the mesothoracic leg onto the metathorax shows that the supernumeraries may be derived from the graft, the host site or from both sources. Similarly, when a leg is grafted lateral to the leg site, a supernumerary forms at the confrontation between the medial edge of the graft and lateral thorax (from leg base across to the dorsal tergite). These results agree with the predictions of both models and would indicate that the compartments or the positional values extend out from the leg to the midline and the edge of the tergite.

The two models differ in their predictions for the number, position and orientation of supernumeraries following 180° rotation of the grafted leg. When the rotated graft is placed lateral to the leg, larvae and adults form a single supernumerary which, in accordance with the Polar Coordinate Model, is lateral to the graft and orientated as a mirror image of it. However, the results of the corresponding medial graft cannot be readily explained by either model. Larvae form a single supernumerary either posterior or medial to the graft, suggesting a modified model with unequally spaced positional values, but the subsequent adult supernumeraries are almost all located medially.

Experiments involving a graft placed medial to the leg site frequently show duplication of the adult midline suture, an extra branch forming between the thorax and the graft or supernumerary leg. In this case, as in the regeneration of the dorsal midline, the extreme medial structure is formed between two more lateral regions, which need not come from opposite sides of the body, but must have opposite mediolateral polarities.

At present, no model can adequately explain all the results of grafting and extirpation on the insect ventral thorax.

Key words: pattern formation, regeneration, insect segment, *Tenebrio molitor*.

Introduction

Understanding the mechanisms by which cells adopt fates appropriate to their position is a major problem in developmental biology. Insect epidermal cells secrete a richly patterned cuticle and the insect epidermis has been a convenient and rewarding system for the study of the cell interactions involved in the formation, growth and elaboration of spatial patterns.

In the early insect embryo, successive bands of cells become determined to form the segments (e.g. meso- and metathorax) and, in *Drosophila*, the evidence from surgical experiments, clonal analysis and patterns of early gene expression indicate that separate anterior (A) and posterior (P) lineage compartments are established at around that time, probably as an integral part of segment formation (see Martinez-Arias & Lawrence, 1985). Groups of cells in appropriate regions on the thoracic segments form the legs

and the wing primordia or, in extreme holometabolous insects (e.g. *Drosophila*), the corresponding imaginal discs. The leg and wing discs each consist of A and P compartments, so they presumably arise astride the A/P boundary, and Meinhardt (1983, 1984) has proposed the Boundary Model which directly relates compartments to the formation of appendages. He suggests that each segment consists of (at least) *three* transverse compartments (A, P and S) and that (at least) A is also subdivided into medial (M) and lateral (L) compartments. The appendage will form with its distal tip at the junction of the A/P and M/L boundaries. In insects that are able to regenerate, any subsequent juxtaposition of cells from the AM, AL and P compartments will create an extra tip, explaining the formation of branched supernumerary limbs after damage in imaginal discs (e.g. Girton, 1981) or after grafting in larval legs (e.g. Bohn, 1965). Similarly, surgical operations that completely remove an S compartment of the thorax will confront cells from the P compartment of one segment with those of the AM and AL compartments of the next. This will result in the regeneration of a complete A/P reversed supernumerary leg, as is frequently observed after such extirpations (Bohn, 1974; French & Rowlands, 1986).

An alternative view of the initial development and regeneration of insect appendages is provided by the Polar Coordinate Model (French *et al.* 1976). Grafting experiments on the larval leg (and comparable excision and mixing experiments on imaginal discs) indicate that stable cellular 'positional values' are graded down and around the appendage. Interaction between cells with different values results in local growth and intercalary regeneration, forming the intervening proximal–distal or circumferential values or, if those values are already present on adjacent cells, completing the distal tip of the limb (Bryant *et al.* 1981). If the embryonic primordium is initially established as a few cells bearing very different positional values, interactions similar to those producing intercalary regeneration could control normal growth and pattern formation within the appendage (Bryant & Simpson, 1984). This model does not specifically involve compartments but, like the Boundary Model, it can explain most of the patterns of supernumerary leg regeneration found in damaged imaginal discs or grafted larval legs (Bryant *et al.* 1981) and also after excisions in the anterior–posterior axis of the thorax (French & Rowlands, 1986).

In the present experiments, we have performed various grafting operations on beetle larvae, placing legs in different orientations into different positions

across the medial–lateral axis of the thoracic segment, in order to compare the regeneration of supernumerary legs and other cuticular features with the predictions of the two models.

Materials and methods

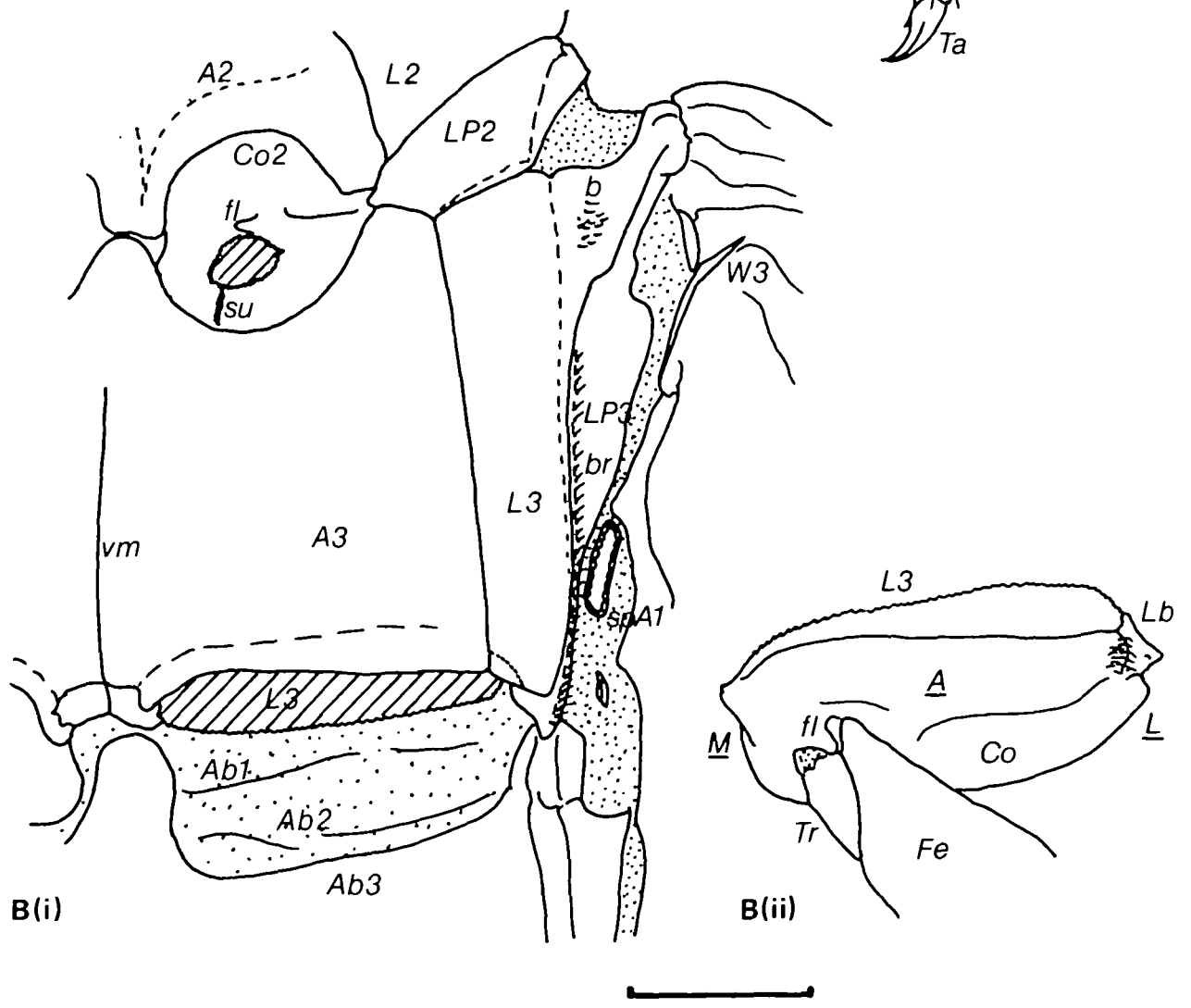
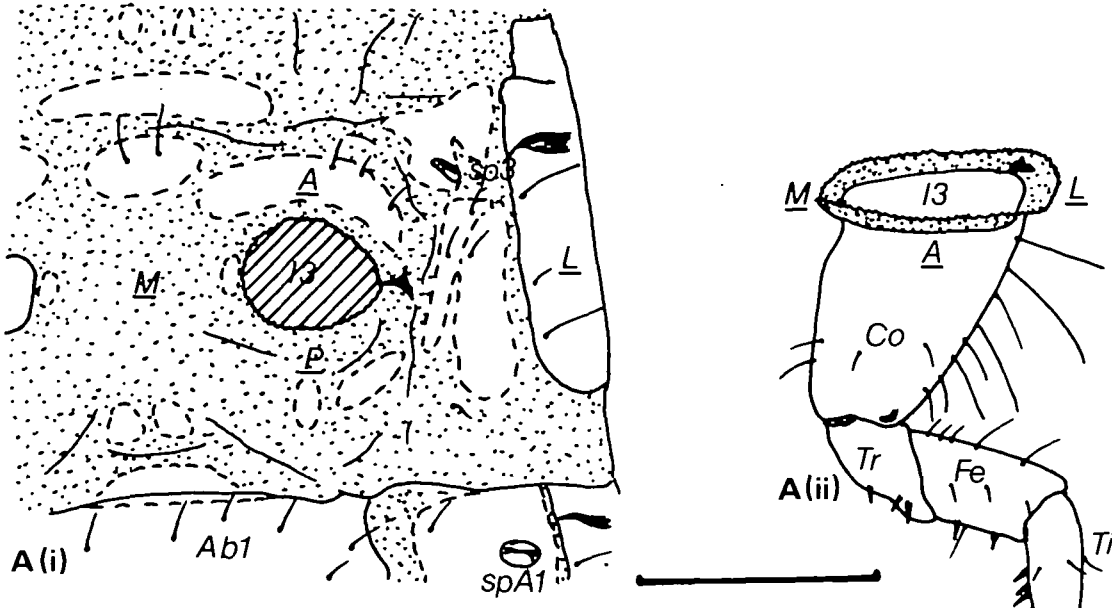
Operations were performed on larvae of the beetle, *Tenebrio molitor* (see French & Rowlands, 1986 for details of rearing, anaesthetization and care of operated animals). Larvae were used 1–2 days after moulting and animals judged to be in their penultimate larval instar were chosen as hosts. However, selection by size was not reliable; some animals metamorphosed at the next moult while others went through one or two further larval instars before pupation. Donors of an earlier instar were used, so that the small graft legs would fit into the chosen sites on the larger host animals.

Grafting operations were performed using fine forceps and knives made from chips of razor blade. The mesothoracic or metathoracic leg plus a tiny collar of surrounding thoracic cuticle was removed from the donor (see Fig. 1Aii) and grafted in the appropriate orientation into a hole prepared in the desired position lateral or medial to the host left metathoracic leg. The collar of the graft was pushed under the rim of host cuticle and was secured as the haemolymph dried. In some experiments, the adjacent host leg was held down with insect wax (Krogh & Weis-Fogh, 1951) to facilitate grafting and to ensure that the grafted leg was not disturbed.

Many of the operated animals that moulted into a further larval instar were examined for the presence of the graft and any regenerated structures. A few were fixed as larvae but most were left to develop into the adult stage.

Animals were partially dissected to remove internal organs and fixed in Carnoy or 100% ethanol for examination of external cuticular features. Some were further

Fig. 1. The structure of the larval (A) and adult (B) ventral thorax of *Tenebrio molitor: camera-lucida* drawings of the thorax and the detached metathoracic larval (L3) and adult (L3) legs. Unsclerotized or weakly sclerotized cuticle is stippled. The metathoracic leg bases are cross hatched, as is the cut distal end of the adult mesothoracic coxa (Co2). The larval metathoracic spiracle (sp3) and the first abdominal spiracles (spA1) are shown. The leg segments are coxa (Co), trochanter (Tr), femur (Fe), tibia (Ti) and tarsus (Ta). The adult meso- and metathoracic coxae bear a flange (fl) on the anterior (A) face, a suture (su) on the posterior (P) face and a patch of bristles (Lb) on the lateral (L) edge. *M* is medial. *Ab1*, *Ab2* and *Ab3* are the first, second and third abdominal segments and *W3* is the metathoracic wing. Anterior and lateral to the adult legs are large sclerites, the meso- and metasternum (*A2*, *A3*), the meso- and meta-episternum (*L2*, *L3*) and the meso- and metasternal epimeron (*LP2*, *LP3*). Between the two metasterna is the deep midline suture (*vm*). Sclerite *L3* bears an anterior tuft of bristles (*b*), and *LP3* bears a line of bristles (*br*) set on a cuticular ridge.



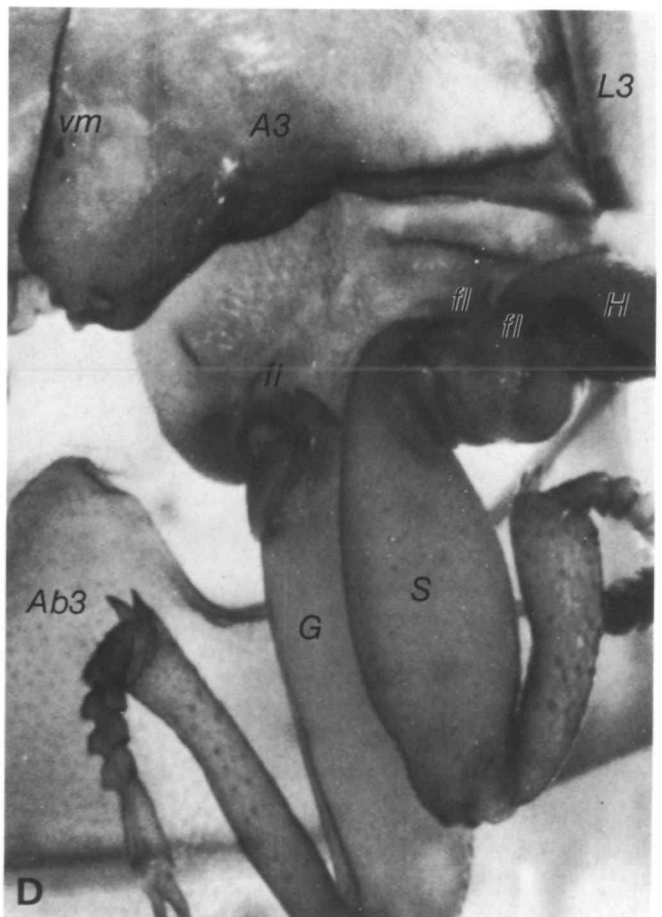
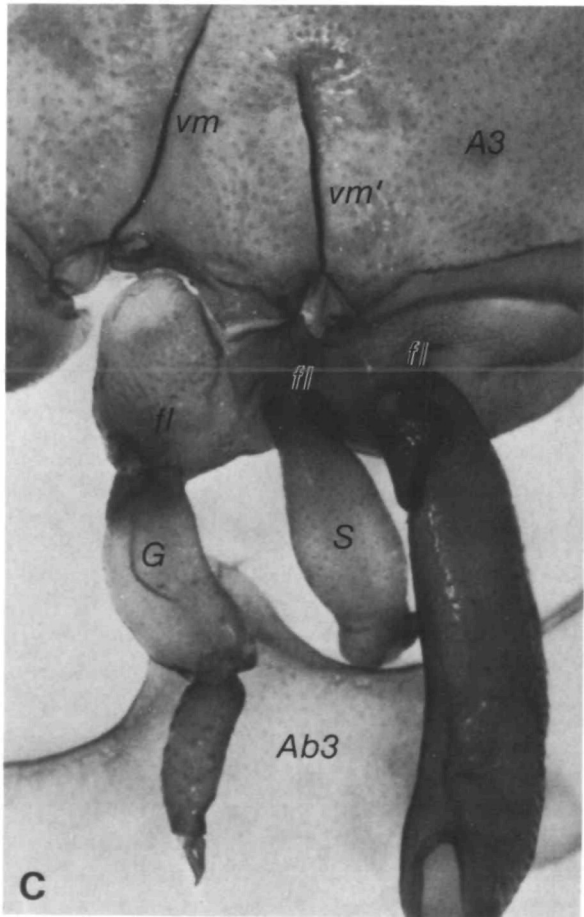
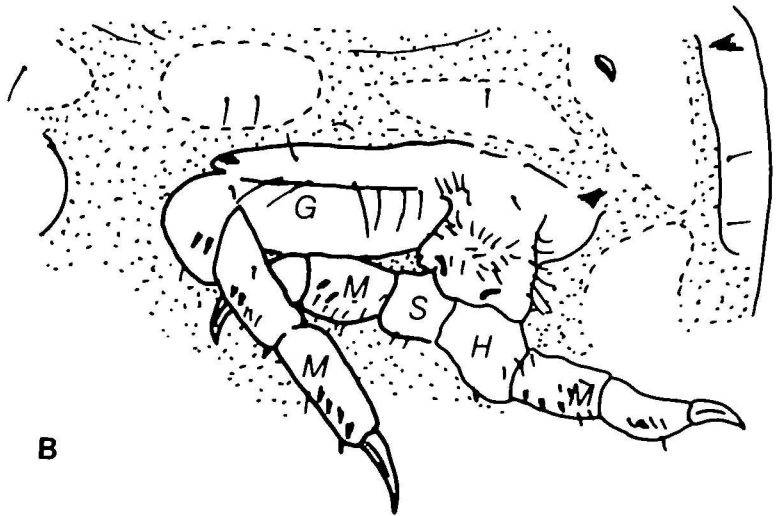
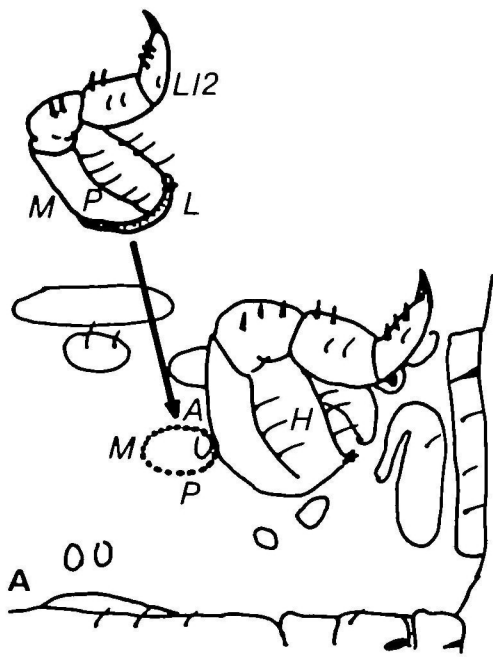


Table 1. Adult structures resulting from a graft leg placed, in normal anterior–posterior orientation, medial (Expts M1, M2, M3, M4) or lateral (Expt L1) to the host leg

Expt	No.	no G	G, no super	G, one super		
				no.	super position	super orient
M1	55	12	22	21	19-L 1-LA 1-A	16-L, 1-LP, 2-? 1-LA* 1-L*
M2	60	8	13	39	34-M 3-MA 2-?	28-M, 3-MP, 3-? 2-MA*, 1-M* 2-?
M3	70	40	2	28	26-M 2-MA	25-M, 1-MP 2-M
M4	52	20	3	29	22-M 1-P 6-?	17-M, 2-MP, 3-? 1-P* 6-?
L1	95	48	15	32	25-L 2-LA 4-A 1-?	23-M, 2-A 1-MP, 1-LA 3-A*, 1-P 1-?

No. refers to the total number of operated animals surviving to adult, and comprises those with no sign of the graft or any extra leg structures (*no G.*), those with a graft but no supernumerary (*G, no super*) and those with a graft plus a supernumerary (*G, one super*)
Super position is the position of the supernumerary relative to the grafted leg (*A* is anterior, *MA* is medial–anterior etc. as in the figures). *Super orient* is the orientation of the medial face of the supernumerary relative to the body axes of the host (in Expt M3 the position and orientation are given, as in the other grafts, relative to the left side of the host body although, because the graft was placed on the midline, the supernumerary is to the right of the midline).
The majority result for each graft is given in bold type. ? refers to cases where the position and/or orientation is not scoreable because of damage. In cases marked * the position and/or orientation of the graft leg has changed since the operation.

dissected, digested in KOH and sclerites were mounted in Gum Chloral for detailed examination of cuticular structures.

Results

(A) The structure of the larval and adult ventral thorax

The structure of the ventral metathorax of the larva and the adult beetle are shown in Fig. 1. The larval

Fig. 2. Experiment M1. (A) Schematic diagram of the operation, grafting the left mesothoracic leg (*LI2*) just medial to the host left leg (*H*), keeping the anterior–posterior (*A/P*) and medial–lateral (*M/L*) axes aligned. (B) *Camera-lucida* drawing of the metathorax of an operated larva after two moults, showing the graft leg (*G*) and the supernumerary (*S*) lateral to the graft and fused with it at proximal coxa level. Supernumerary and host (*H*) legs are fused to distal coxa level. The orientation of the medial face (*M*) of the supernumerary shows it to be a mirror-image of graft and host legs. (C,D) Two resulting adults, showing medially orientated graft (*G*) and host (*H*) legs, plus laterally orientated supernumerary (*S*). All legs are normally orientated in the *A/P* axis, as shown by the anterior position of the flange (*f*). In C, supernumerary and graft coxae are fused together but separated from the host coxa by a supernumerary midline suture (*vm'*) while in D all coxae are fused. Other abbreviations as in Fig. 1.

legs are surrounded by flexible, lightly sclerotized cuticle with small sclerites and bristles in particular positions, enabling reliable location of graft sites. The adult legs are deeply set in sockets and associated with large sclerites with characteristic cuticular features (see French & Rowlands, 1986). The coxa of the metathoracic or mesothoracic leg has an anterior face bearing a flange, while the posterior face is rounded with a suture. These various cuticular features allow the identification of the position and orientation of supernumerary adult legs produced after grafting legs into different positions on the larval thorax. The legs are very similar in structure but their tarsi are subdivided into five (mesothoracic) or four (metathoracic) segments so, in experiments involving a grafted mesothoracic leg, the origin of supernumerary legs can be determined (see French, 1986).

(B) Graft series M; leg placed medial to the leg site

Graft M1

The left mesothoracic leg was grafted in control orientation into a site just medial to the host left metathoracic leg (Fig. 2A).

As shown in Table 1, of the operated animals surviving to adult and retaining the grafted leg, about 50% also bore a regenerated supernumerary leg. In some other animals, there were signs that a graft or a

supernumerary had been broken off, leaving a wound. The grafted and supernumerary legs shared a large fused coxa which was either completely separate from or fused to that of the adjacent host leg. In most cases, the supernumerary was positioned *lateral* or approximately lateral to the graft, between it and the host leg, where the lateral side of the grafted leg base confronted thorax just medial to the host leg. In most cases the supernumerary was orientated as a mirror

image of the graft and host legs, with its medial face and claws pointing towards the *lateral* side of the animal (Fig. 2C). The distal parts of the supernumerary were usually damaged or absent, but in 5/8 scoreable cases the tarsi had four segments (indicating the supernumerary was host derived), in two cases they had five segments and the remaining animal had a composite supernumerary tarsus (indicating dual origin).

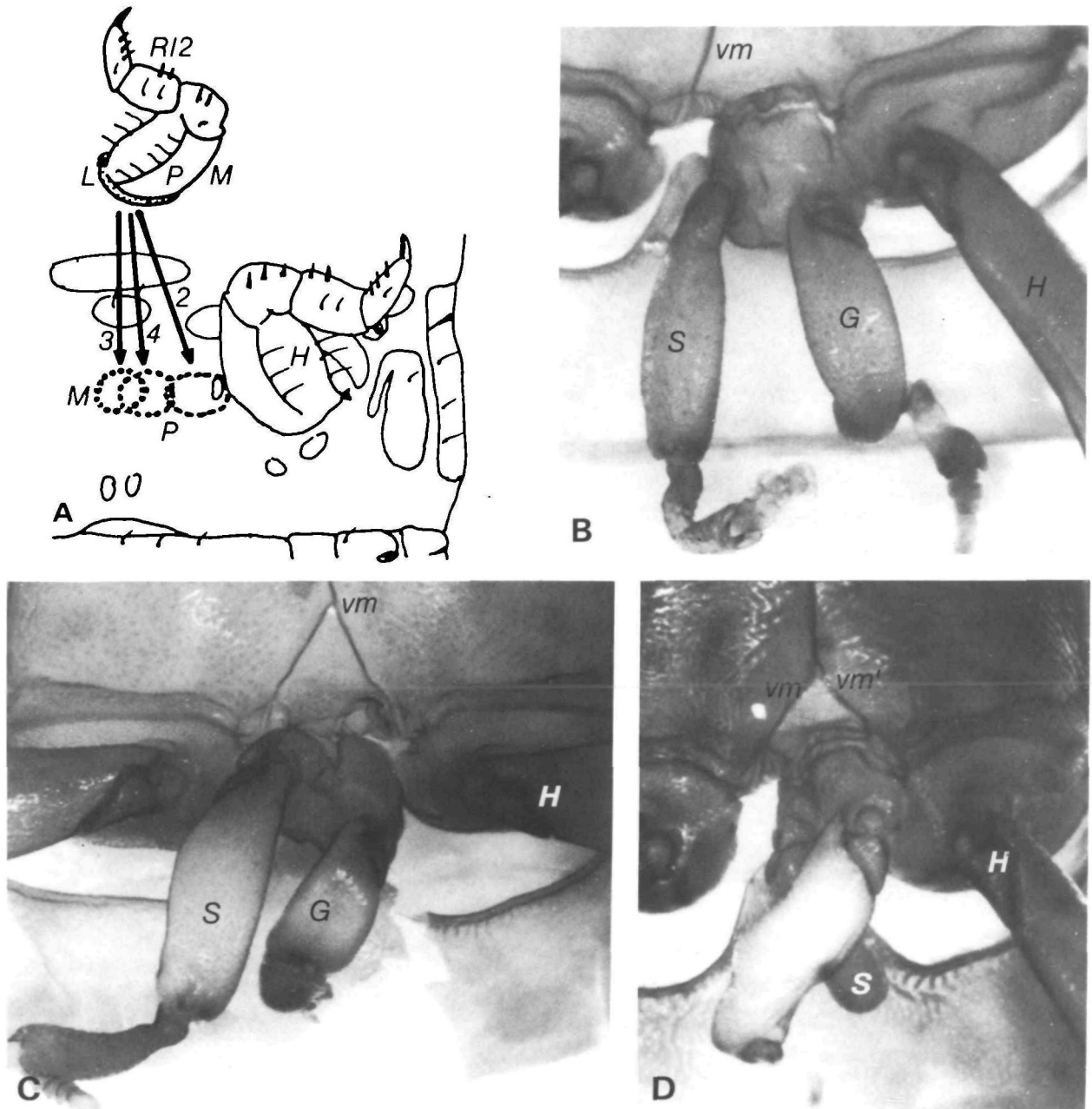


Fig. 3. Experiments M2, M3 and M4. (A) Schematic diagram of the operation, grafting the right mesothoracic leg (*R12*) into three positions medial to the host left leg (2, graft M2; 3, graft M3; 4, graft M4), thereby reversing the medial-lateral axis of the graft relative to the host site. (B,C,D) Adults resulting from graft M2 (B), M3 (C) and M4 (D), showing graft and host legs plus, in each case, a medially orientated supernumerary (*S*) medial to the graft. In B the coxae of graft and host legs are fused, while in C and D they are separate and there is an extra midline suture (*vm'*).

Apart from the presence of a graft, and often a supernumerary leg, the other morphological abnormality often present was a duplication of the deep suture found in the ventral midline between the large A3 sclerites anterior to the metathoracic legs (see Fig. 1). The supernumerary suture was formed in 7/21 animals with supernumerary legs but only in one case bearing only the graft (and this animal had clearly lost a supernumerary structure). The suture was formed only in cases where the host and supernumerary coxae were completely separate (Fig. 2C), it was positioned between them and often the anterior end joined the normal suture. In animals that were dissected and digested with KOH, it was evident that a supernumerary suture was associated with partial duplication of the large internal apophyses which invaginate at its posterior end.

Some of the operated animals that moulted into larvae were examined before being left to undergo metamorphosis and, in 2/8 animals with surviving grafts, a laterally orientated supernumerary larval leg was already evident lateral to the graft (Fig. 2B).

Graft M2

The right mesothoracic leg was grafted, in reversed medial–lateral orientation, just medial to the host left metathoracic leg (Fig. 3A).

Of the operated animals that completed metamorphosis and retained the grafted leg, 75% bore a supernumerary leg which was usually formed *medial* to the graft, at the confrontation between the lateral side of the grafted leg base and thoracic tissue midway between the leg and the ventral midline (Table 1). In most cases, the supernumerary was orientated as a mirror image of the graft, with claws pointing *medial* (Fig. 3B). In 2/9 scoreable cases, the supernumerary tarsus was four-segmented (host-derived), in two cases it was graft-derived and in five cases it was composite with the four-segmented part on its posterior, medial or lateral side.

As in experiment M1, there was sometimes a duplication of the midline suture (in 8/39 animals bearing a supernumerary leg and 3/13 bearing only the graft). The suture was only formed when graft and host coxae were completely separate and it was positioned between them.

Some of the operated animals were scored as larvae and 12/13 of those with a surviving graft already had a supernumerary leg or rudimentary lobe in a medial or medial/anterior position (with the claw pointing medially in the two scoreable cases).

Graft M3

The right mesothoracic leg was grafted, maintaining its orientation, to a site directly on the host ventral midline (Fig. 3A).

Of the operated animals that completed metamorphosis with a surviving graft, 75% had a regenerated supernumerary leg. In most animals the supernumerary was a mirror image of the grafted leg, to its right, and hence had formed where the lateral edge of the grafted leg base confronted thoracic tissue just to one side of the midline (Table 1; Fig. 3C). In seven animals the supernumerary bore a complete, scoreable tarsus and one of these was five-segmented (graft-derived) but the others were composite with a host contribution on the posterior, medial or lateral side.

In 26/30 of the successfully grafted animals the midline suture was duplicated, with one branch running between the supernumerary and host right coxae and the other between the graft and host left coxae (Fig. 3C).

Many of the operated animals were scored first as larvae, and most of those with a surviving graft had a supernumerary leg or lobe to the right of the graft or approximately in that position. In those cases where orientation was scoreable, the supernumerary was usually a mirror image of the graft with the claw pointing to the midline.

Graft M4

The right mesothoracic leg was grafted, in reversed medial–lateral orientation, to a site on the left side of the host ventral midline (Fig. 3A).

Almost all of the operated animals completing metamorphosis with a surviving graft also bore a supernumerary leg, usually *medial* to the grafted leg (Table 1). The supernumerary therefore usually developed at the confrontation between the lateral edge of the grafted leg base and the ventral midline of the thorax (Fig. 3D). In most cases, the supernumerary was orientated with its medial face and claws pointing *medially*, and the tarsus was of dual graft and host origin in the one complete scoreable leg.

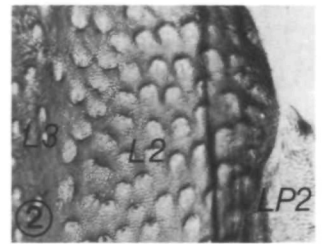
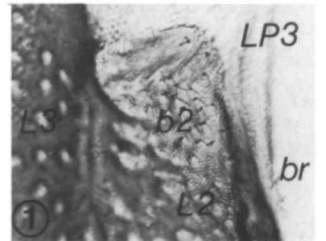
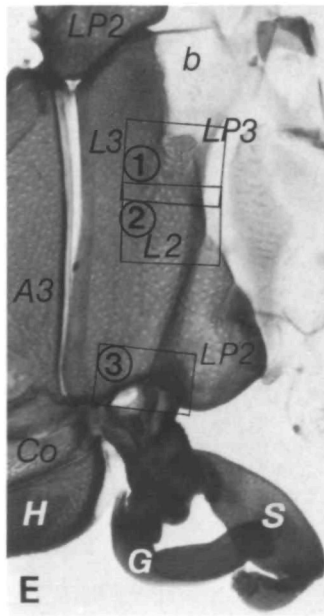
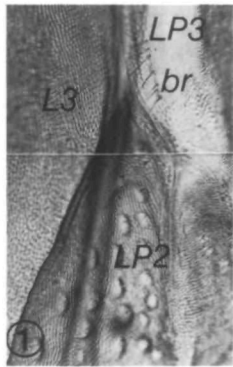
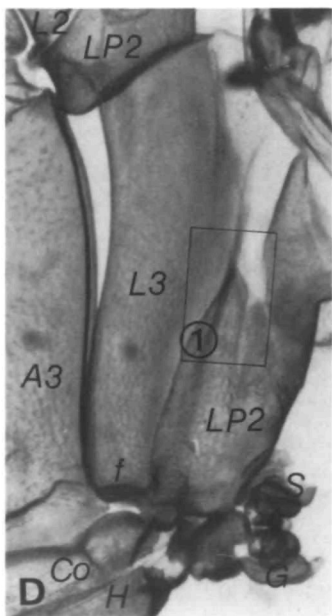
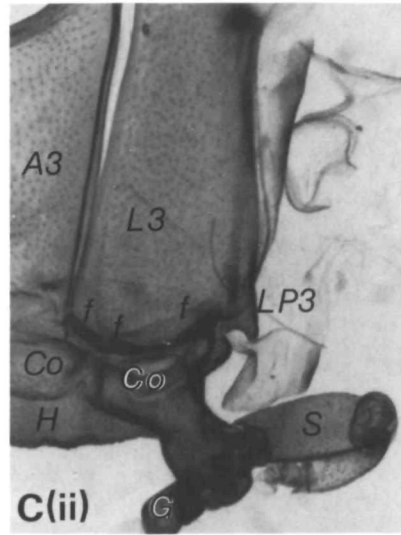
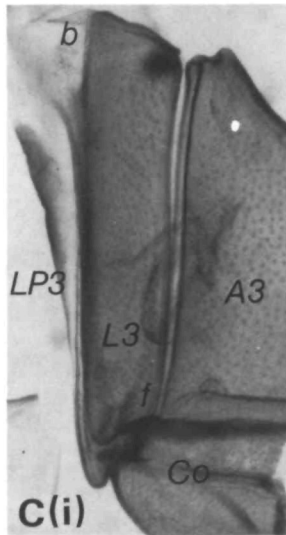
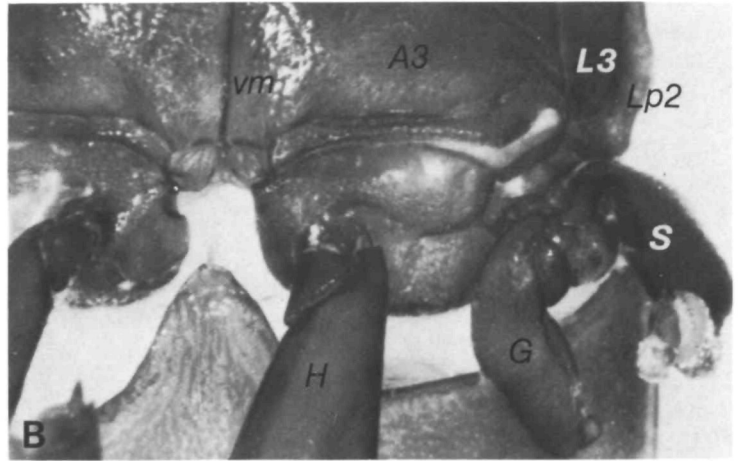
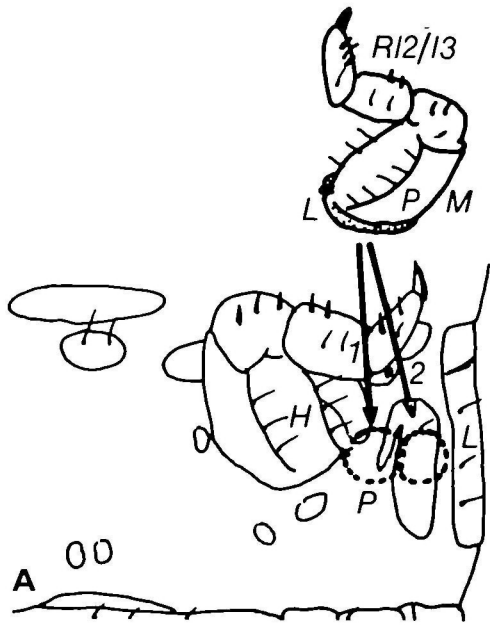
As in the previous grafts, the operation also affected the sclerites anterior to the legs, as a supernumerary midline suture was formed between the graft and host coxae in 20/29 animals.

The major finding from this series of experiments is that an interaction between the lateral edge of the base of a grafted leg and any area of medial thorax (from the leg base right across to the ventral midline) results in the formation of a supernumerary leg which may be derived from the graft, from the host site or from both sources.

(C) *Graft series L; leg placed lateral to the host leg*

Graft L1

The right mesothoracic or metathoracic leg was grafted in reversed medial–lateral orientation to a



site just lateral to the host left metathoracic leg (Fig. 4A).

Only about 50% of the resulting adults retained the grafted leg and most of these also bore a regenerated supernumerary leg, usually situated *lateral* to the graft (Table 1). Hence the supernumerary usually formed from an interaction between the medial edge of the grafted leg base and the thoracic tissue well lateral to the normal leg site. Most supernumeraries were mirror images of the graft, with claws orientated towards *medial* (Fig. 4B). Only one supernumerary tarsus was complete and scoreable in animals resulting from grafts of the mesothoracic leg, and this was five-segmented (graft-derived).

Many of the operated animals showed abnormalities in the structure of the lateral sternites L3 and LP3 (see Fig. 1). These were particularly frequent when there was no extensive fusion between host and graft coxae, so that the graft (plus supernumerary, if any) was positioned very laterally on the animal. In 8/9 animals retaining a grafted *metathoracic* leg (and in several animals having no surviving graft) sclerite LP3 was normal but the posterior end of L3 was very broad, bearing two or three copies of the characteristic fold of cuticle normally found only in its medial/posterior corner (Fig. 4Cii). In one animal, sclerite L3 was normal but the posterior tip of LP3 was triplicated. In 25/28 animals retaining a grafted *mesothoracic* leg (and even in 12/42 having no surviving graft) the characteristic posterior part of sclerite LP3

was completely absent and replaced by a much broader, darkly pigmented sclerite which could be interpreted as part of the mesothoracic sclerite LP2 (Fig. 4D). This was frequently accompanied by a broadening of the posterior end of sclerite L3 and in some cases part of this sclerite was clearly replaced by part of L2 (Fig. 4E). These sclerite abnormalities are considered further in the Discussion.

Most of the operated animals were scored as larvae before their metamorphosis and 54/72 of those with a surviving graft bore a supernumerary leg, usually anterior/lateral or lateral to the graft. Larval supernumerary legs were usually poorly developed but, in scoreable cases, they were usually orientated toward medial or posterior/medial.

Graft L2

The right mesothoracic leg was grafted in reversed medial–lateral orientation to a site well lateral to the host left metathoracic leg (Fig. 4A).

From a total of 55 operated animals, 30 completed metamorphosis but none retained the grafted leg and the only sign of the operation sometimes present was a wound or small lump of tissue situated midway up sclerite L3. Some larvae were fixed and examined before metamorphosis, and 10/13 of those with surviving grafts also had a supernumerary leg or lobe in a *lateral* or anterior/lateral position relative to the graft. Supernumeraries were usually poorly developed and, in the few scoreable cases, their orientation was variable.

These experiments show that interaction between the medial edge of a grafted leg base and the lateral thorax (extending from the leg base almost across to the edge of the tergite) can result in the formation of a supernumerary leg (although very laterally positioned legs are lost during metamorphosis).

The M and L series involve grafting a leg in normal anterior–posterior orientation to various sites across the medial–lateral axis of the ventral thorax. The position and orientation of most of the resulting supernumeraries agree with the predictions of both the Boundary and Polar Coordinate Models (see Fig. 5A). However, if a similar transplantation also involves anterior–posterior reversal of the graft leg, the predictions of the two models differ regarding the number, position and orientation of supernumeraries, as shown in Fig. 5B.

(D) *Graft series R; 180°-rotated leg placed medial or lateral to the host leg*

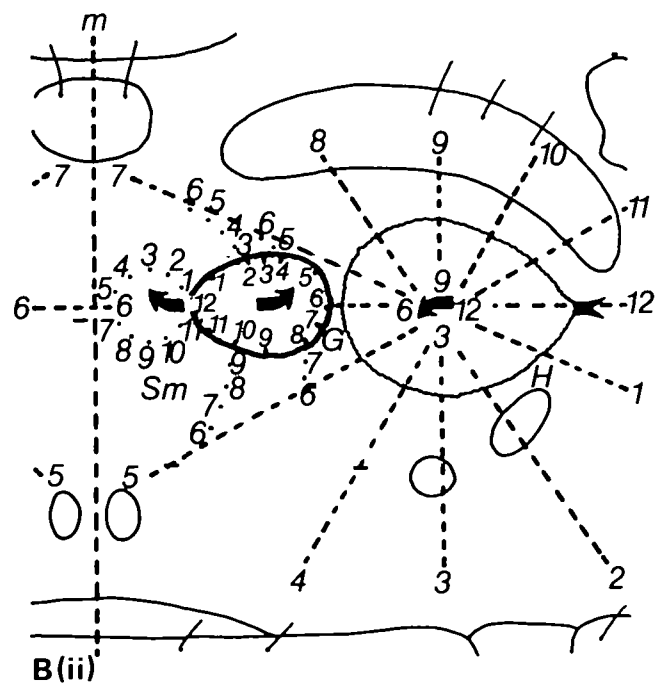
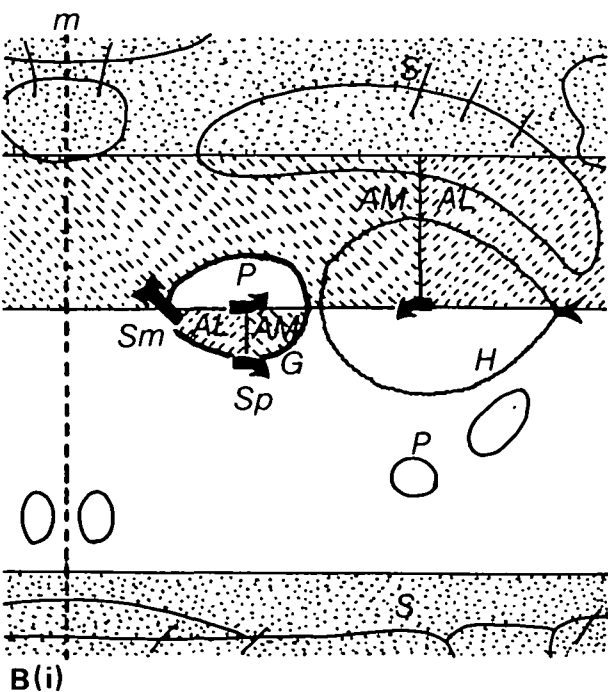
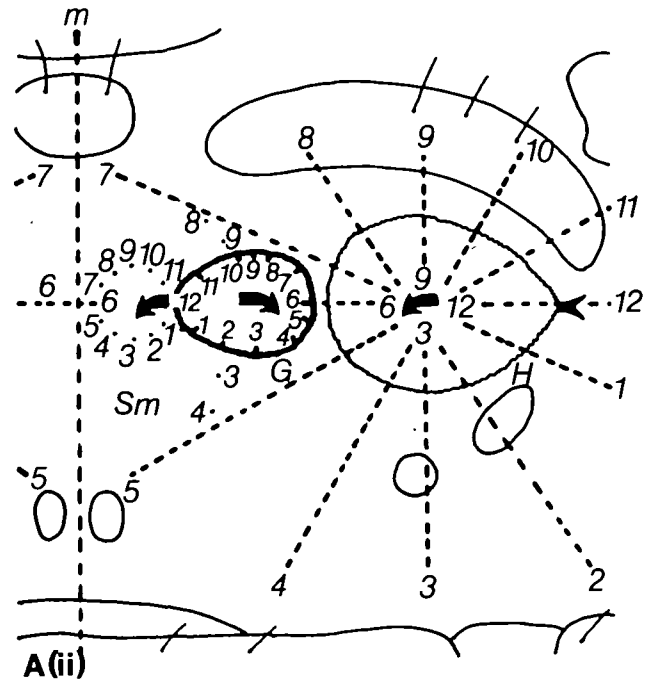
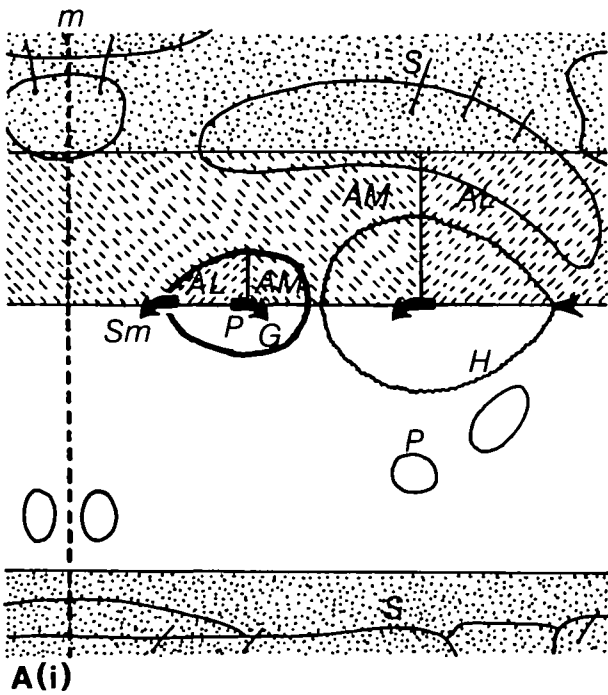
Graft MR

The left mesothoracic or the left metathoracic leg was rotated 180° (thereby reversing its anterior–posterior and medial–lateral axes) and grafted medial to the host left metathoracic leg (Fig. 6A).

Fig. 4. Experiments L1 and L2. (A) Schematic diagram of the operation, grafting the right meso- or metathoracic leg (*RL2/L3*) into two positions lateral to the host left leg (1, graft L1; 2, graft L2), reversing the medial–lateral axis of the graft relative to the host site. (B) Adult resulting from graft L1, showing host leg (*H*), grafted mesothoracic leg (*G*) and a laterally positioned, medially orientated supernumerary (*S*). *A3* and *L3* are metathoracic sclerites and *LP2* is mesothoracic (see Fig. 1). (C–E) Adults resulting from graft L1-mounted cuticle preparations with inset photographs of particular areas. (C) Control right side (*Ci*) and experimental left side (*Cii*) of animal with a grafted metathoracic leg. In *Cii* the posterior part of sclerite *L3* is broadened with a clear triplication of the cuticular fold (*f*). *LP3* is normal. (D,E) Two adults resulting from a mesothoracic leg graft and showing some mesothoracic structures on the lateral sclerites. In D, sclerite *L3* is normal in structure but the posterior part of *LP3* is replaced by cuticle characteristic of *LP2*. Inset ① shows the junction between this partial *LP2* and the normal anterior part of *LP3*, with its line of bristles (*br*). In E sclerite *LP3* is partially replaced by *LP2* but also the lateral part of *L3* is replaced by *L2*. Insets ②, ③ show various parts of the junction between meso- and metathoracic structures (*b2* are bristles resembling those found in the anterior–lateral region of *L2*).

As shown in Table 2, of the operated animals that completed metamorphosis and retained the graft, most also bore a *single* regenerated supernumerary leg in a *medial* (or approximately medial) position. In most cases, the medial face and claws of the supernumerary were orientated towards *medial* (or approximately medial), and the cuticular structures of the coxal apex showed that the anterior-posterior axis of the supernumerary, like that of the graft leg,

was reversed relative to the host. In a few animals, the graft legs were misaligned and there was a single supernumerary variable in position and orientation. The supernumerary tarsus was complete and scoreable in five animals resulting from grafts of the mesothoracic leg, and these were all five-segmented (graft-derived). As in the M series of grafts, the MR transplantation could result in duplication of the midline suture, but only rarely (ten cases). Usually



the host, graft and supernumerary coxae were fused together, with no supernumerary suture (Fig. 6C).

Many of the operated animals were scored as moulted larvae, prior to metamorphosis and, as shown in Table 2, just over 50% of them had regenerated a supernumerary leg or lobe. One exceptional animal had three supernumerary structures and another had two. However, in this experiment, unlike the others, the position and orientation of larval supernumeraries was *not* in accordance with the structures scored on the adults. In most cases, the larval structures were in a *posterior* or approximately posterior position and were orientated towards *lateral*, whereas in only around 30% of cases were they approximately medial in position and in orientation (Table 2). Some of these larvae were followed individually through metamorphosis and scored as adults. *Medial-orientated-medial* adult supernumeraries were formed by 7/7 animals previously bearing *posterior-orientated-lateral* larval structures (Fig. 6B,C), by 3/6

with posterior supernumeraries of unscorable orientation and by 2/3 with no larval supernumeraries (the remaining adults having lost graft and supernumerary legs).

Graft LR

The left metathoracic leg was rotated 180° and grafted lateral to the host left metathoracic leg (Fig. 7A).

Few of the operated animals surviving to adult had retained the grafted leg, but most of these also bore a single regenerated supernumerary leg, and many of the others had a wound indicating that tissue had been lost. In all cases where the graft had not become misaligned, the supernumerary was in a *lateral* position with its medial face pointing *medially* on the animal (Table 2; Fig. 7), and in scoreable cases the anterior–posterior axis, like that of the graft, was reversed relative to the host.

As in the L1 graft, the LR transplantation frequently resulted in an abnormality in the lateral sclerites. Most adults with a supernumerary (and many of those with no surviving graft or supernumerary) had a duplication or triplication of the posterior end of sclerite LP3, although sclerite L3 was usually normal (Fig. 7).

Many animals were scored as larvae before being followed through metamorphosis and, as shown in Table 2, over 50% of them had regenerated a supernumerary leg or lobe, usually in a lateral (or lateral–posterior) position and orientated *medially*. Some of these larvae were followed individually and scored as adults. *Lateral-orientated-medial* adult supernumeraries were formed by 1/10 animals previously bearing similar larval structures, by 1/2 with lateral-orientated-posterior larval structures and by 2/11 with no larval supernumeraries (the remaining adults, plus two derived from larvae with posterior supernumeraries, having lost graft and regenerated legs).

The results show that, after 180° rotation of the graft, the single supernumerary structure present on the *adult* animal is in very good agreement with predictions from the Polar Coordinate Model (Fig. 5B). However, when scored before metamorphosis, the MR graft, but *not* the LR graft, occasionally results in multiple supernumeraries, and the majority of the single supernumeraries correspond in position and orientation to one of the structures predicted by the Boundary Model.

Discussion

Regeneration of supernumerary legs

In these experiments, we have grafted larval beetle legs into various positions across the medial–lateral

Fig. 5. Interpretation of leg-grafting experiments: schematic drawings of ventral thorax, host (*H*) and graft (*G*) legs and predicted position and orientation of supernumeraries. In the Boundary Model (*Ai*, *Bi*), the thorax consists of *S*, *A* and *P* compartments, with *A* subdivided into *AM* and *AL*, and the leg is situated at the intersection of the *A/P* and *M/L* boundaries. In the Polar Coordinate Model (*Aii*, *Bii*) the circumferential positional values (*1–12*) of the leg fan out onto the surrounding thorax. The orientation of a limb is indicated by the curved arrow, and *m* is the ventral midline.

(A) Experiment M2 (right leg grafted, *M/L* reversed, medial to the host left leg). The Boundary Model (*Ai*) predicts confrontation of the *AM*, *AL* and *P* compartments medial to the grafted leg, forming a left supernumerary leg (*Sm*) orientated like that of the host. In the Polar Coordinate Model (*Aii*) intercalation will occur between graft and surrounding thorax – only some intercalated values are shown (e.g. 9,8 will form between graft 10 and thorax 7, while 3,4 will form between 2 and 5). The pattern of shortest-route intercalation will generate a complete circumference, and hence a supernumerary left leg (*Sm*) medial to the graft (see French *et al.* 1976). Similarly, the two models have identical predictions for grafts M1, M3, M4, L1 and L2.

(B) Experiment MR (left leg grafted, 180° rotated, medial to the host left leg). The Boundary Model (*Bi*) predicts a right supernumerary leg (*Sp*) posterior to the graft and orientated with claws pointing laterally, plus a duplicated supernumerary (because of the arrangement of compartments *AM*, *P*, *AL*, *P*) medial to the graft (*Sm*). The pattern of intercalation in *Bii* predicts only one right supernumerary (*Sm*) medial to the graft with claws pointing medially. Similarly, for graft LR the Boundary Model predicts a posterior supernumerary orientated laterally plus a lateral duplicated supernumerary, while the Polar Coordinate Model predicts only a lateral supernumerary orientated medially.

axis of the ventral thoracic segment and analysed the regeneration of supernumerary legs in the larvae and resulting adults. In the beetle, unlike extreme holometabola such as *Drosophila*, the adult thoracic sclerites and leg sockets form from the tissue of the larval thorax (French & Rowlands, 1986) and adult legs are derived from their larval predecessors (Huet & Lenoir-Rousseaux, 1976). Operated animals moulting into further larval instars often have a supernumerary bulge or larval leg and, after metamorphosis, they usually bear a corresponding adult supernumerary. The frequency of supernumerary formation is variable between experiments and probably has little significance, since supernumeraries may first appear either after one or after several moults, and supernumerary (and graft) legs can be

lost, particularly during metamorphosis. The adult metathoracic leg is confined within a deep socket and the extra graft and supernumerary legs are often damaged and are rarely complete to tarsal tip.

Supernumerary distal parts are regularly regenerated following contralateral grafting operations which reverse one transverse axis of the distal part of an insect leg (Bohn, 1965; Bullière, 1970; Bart, 1971; French, 1984). Reversal of the A/P axis leads to supernumeraries of host handedness and orientation forming in A and P positions, while M/L reversal causes them to regenerate in M and L positions. Similarly, A/P or M/L reversal of a complete *Tenebrio* leg grafted to the contralateral leg site resulted in A and P or in M and L supernumerary legs, suggesting that the arrangement of positional values

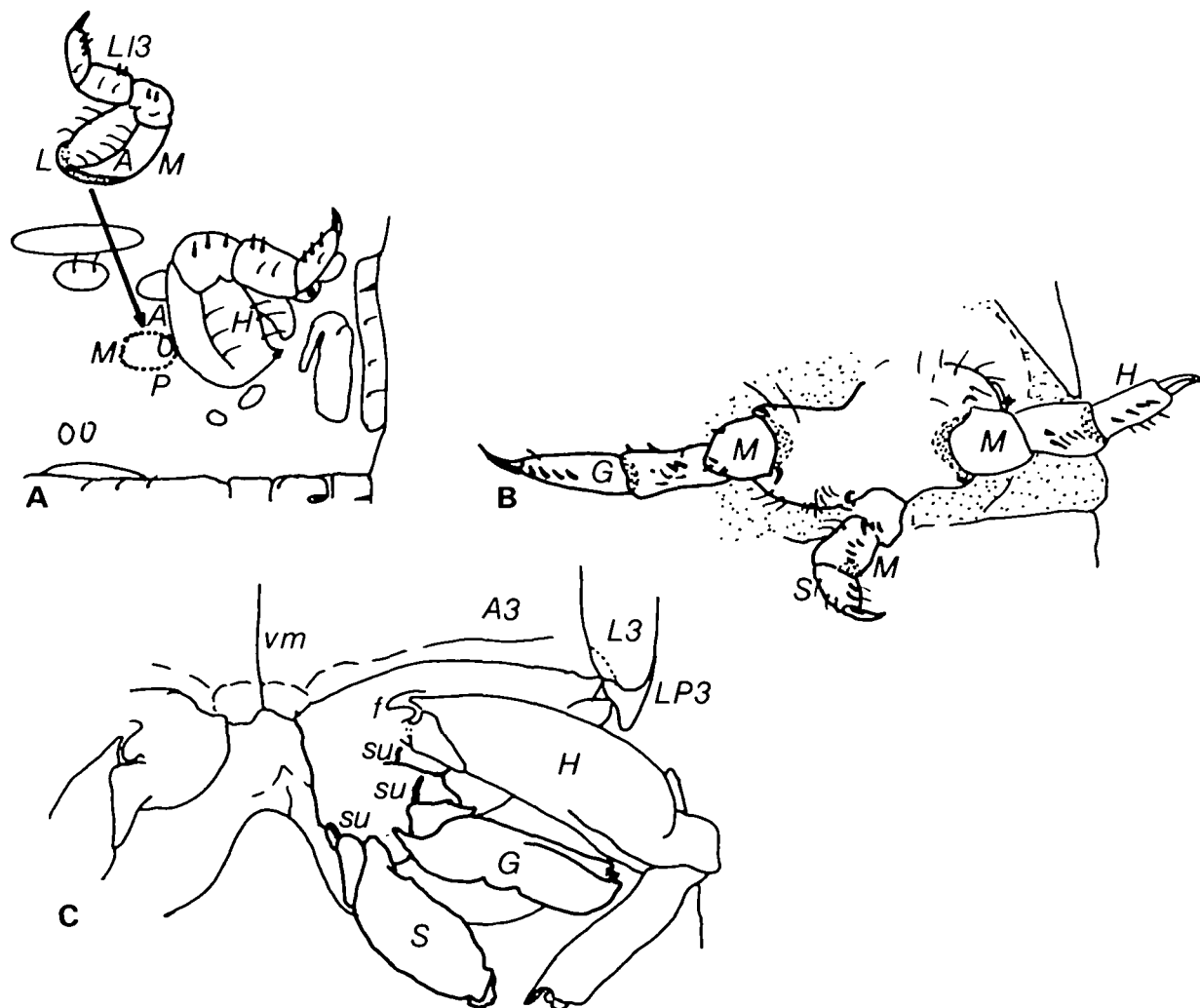


Fig. 6. Experiment MR. (A) Schematic diagram of the operation, grafting the left metathoracic leg (*L3*) medial to the host left leg and rotating it by 180°, thereby reversing both the anterior–posterior (*A/P*) and medial–lateral (*M/L*) axes of the graft. (B,C) *Camera-lucida* drawings of one resulting animal at larval (B) and adult (C) stages. (B) The larval supernumerary (*S*) is positioned approximately posterior to the graft and its medial face (*M*) is orientated laterally on the host. (C) The adult supernumerary (*S*) is approximately medial to the graft and orientated medially. The positions of the anterior coxal flange (*fl*) and posterior suture (*su*) show that the host leg is normally orientated, while the graft and supernumerary are both *A/P* reversed relative to the host.

Table 2. Larval and adult structures resulting from a 180°-rotated left leg placed medial (Expt MR) or lateral (Expt LR) to the host left leg

Expt	No.	no G	G, no super	G, one super		
				no.	super position	super orient
<i>MR</i>						
Larvae	113	9	38	64 (2)	34-P 7-LP 12-M 6-MP 2-MA 3-var	19-L, 3-P, 1-LP, 1-LA, 10-? 4-L, 1-LP, 1-LA, 1-MP 4-M, 1-MA, 1-LA, 1-L, 5-? 1-M, 1-L, 4-? 1-MA, 1-? 3-var
Adults	147	72	11	64	41-M 9-MA 8-MP 6-var	35-M, 4-MP, 2-? 7-MA, 2-M 5-MP, 2-M, 1-LP* 6-var*
<i>LR</i>						
Larvae	51	7	16	28	14-L 6-LP 6-P 2-AL	6-M, 2-P, 6-? 4-MA, 2-M 1-A, 5-? 1-P, 1-?
Adults	58	26	11	21	14-L 5-M 1-A 1-P	11-M, 3-? 4-L*, 1-MP* 1-A* 1-A*

For the larvae *No.* refers to the operated animals that were scored as larvae (most of which were left to metamorphose), and for the adults *No.* refers to the total number surviving to adult stage. Abbreviations as in Table 1. *var* refers to a small heterogeneous group of animals each with one supernumerary, very variable in position and orientation. The two larvae in parentheses had more than one supernumerary: one had two (P, orientated towards L; M, orientated MA), and the other had three (A, orientated A; M, orientated A; MP, orientated MP).

extends from the leg onto the immediately surrounding thorax (French, 1986). In the present experiments, the grafted leg is placed away from the normal leg site, and supernumeraries can form where lateral leg base interacts with medial thorax, across to the ventral midline (expts M1–M4), or where medial leg base confronts lateral thorax, across to the edge of the tergite (expts L1, L2). A similar result has been found in crustaceans, where a supernumerary leg is regenerated at the confrontation of medial thorax and the lateral face of a grafted leg (Mittenthal & Trevarrow, 1984).

The adult meso- and metathoracic legs differ in the structure of the tarsus. The M and L series of experiments involve grafting a larval mesothoracic leg into the metathorax, and examination of the structure of the tarsus of distally complete adult supernumeraries shows that they can be regenerated from the tissue of the grafted leg, of the thoracic site or of both. In previous experiments when a leg was grafted A/P reversed into the leg site, the resulting A and P supernumeraries were reliably half-and-half in origin with the borders just posterior to midlateral and midmedial (French, 1986 – see also French & Rowlands, 1986). However, the M and L legs resulting from an M/L reversal were very variable in

origin, just like the present legs formed at a medial–lateral confrontation. Taken together, these results suggest that the larval leg and thorax is subdivided into A and P lineage compartments which are respected during regeneration, but give no indication of a comparable subdivision into M and L lineage compartments. Similarly, clonal analysis of the developing *Drosophila* imaginal discs clearly demonstrates the A and P compartments but gives little evidence for a comparable M–L subdivision (reviewed by Brower, 1985). It should be noted that this is not strong evidence against the Boundary Model of appendage initiation (Fig. 5A) since, in that model, ‘compartments’ may be labile and need not correspond to lineage restrictions (Meinhardt, 1984).

The number, position and orientation of supernumerary legs found following grafts in normal A/P orientation (Table 1) are in excellent agreement with the predictions of both the Boundary and Polar Coordinate Models (Fig. 5A). In terms of the models, this would indicate that the transverse compartments or circumferential positional values extend right across the larval ventral thorax, from the midline to the edge of the dorsal tergite, which is the primordium of the adult wing (Hundertmark, 1935).

The models differ in their predictions for grafts MR and LR, which involve 180° rotation of the grafted leg (Fig. 5B), and in most respects the results are compatible with the Polar Coordinate Model. For the LR graft the result is clear; larvae and adults form only one supernumerary with the appropriate location and orientation (Table 2). Interpretation of the MR experiment is confused by the fact that the adult result (which is clear and agrees with the Polar Coordinate Model) does *not* correspond with the larval results (which are somewhat variable and agree with the predictions of neither model).

MR larval supernumeraries are predominantly either posterior-orientated-lateral or medial-orientated-medial. The Boundary Model predicts that both these supernumeraries should form, while the Polar Coordinate Model predicts only the medial one (Fig. 5). It is not likely that animals are restricted to one supernumerary by general factors such as haemolymph supply, since legs grafted to the leg site regularly result in the regeneration of two supernumeraries (French, 1986). However, the MR supernumeraries do seem to be mutually exclusive since, if they formed independently with probabilities fitting the frequency of the no-supernumerary result, about 15% of animals should have both.

One problem in formulating and testing the predictions of the models is that they are very similar and very 'adaptable', with the results of some experiments depending critically on assumptions concerning the spacing of compartment borders or positional values. As illustrated in Fig. 8, a modified spacing of positional values in the Polar Coordinate Model can give the observed larval MR supernumeraries, without affecting the predictions for the other experiments. The Boundary Model can be modified in a similar way. If the AM/P boundary is moved so that it passes anterior to the graft site, only the posterior supernumerary will form in experiment MR, while if it is completely posterior to the graft site only the medial supernumerary regenerates. However, it does not seem possible to modify the Boundary Model so that *either* one or the other supernumerary is generated, and it seems unlikely that the variability in results (of only this experiment) could arise from variability in location of the graft.

MR adult supernumeraries are positioned and orientated medially and it is not clear how the position and orientation of the supernumerary can change during metamorphosis (Fig. 6B,C). The graft and supernumerary legs (and usually also the host leg) are fused together at the base in both larval and adult stages, and the observed changes cannot be achieved through rotation of the entire structure. In some way, the supernumerary must move around the base of the graft. It is possible that a posterior larval

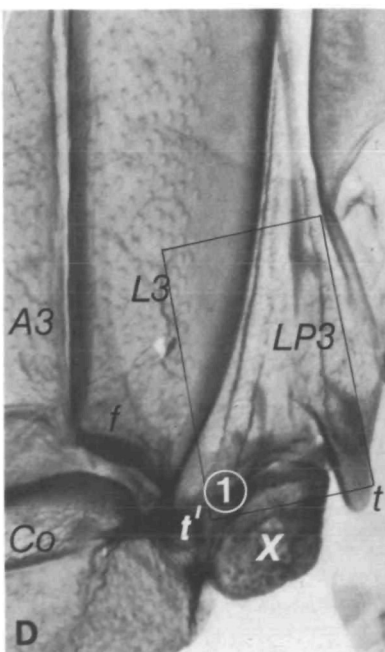
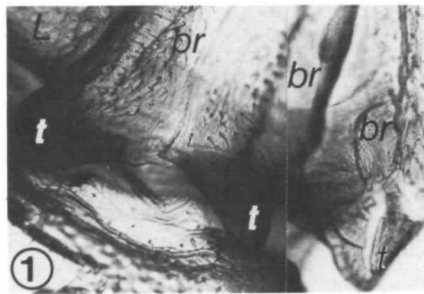
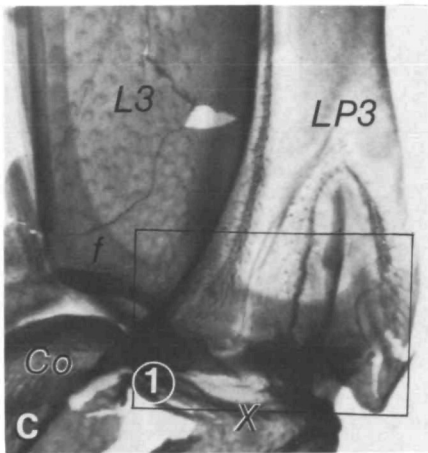
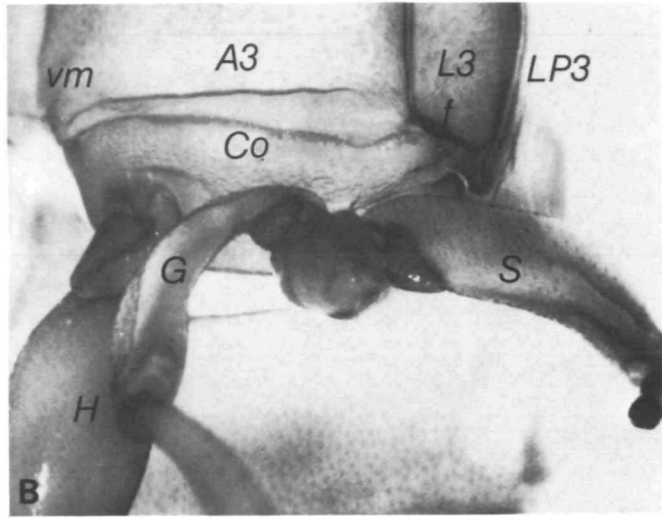
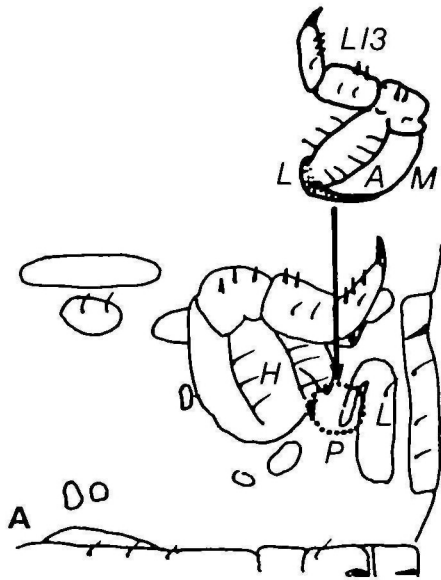
structure does not develop directly into a medial adult leg, but that the former is lost at metamorphosis and the latter regenerates anew. It is not clear why a larval posterior supernumerary should be lost in this way, as larval and adult supernumeraries regularly form in this position after A/P reversal or 180° rotation of a leg grafted to the leg site (French, 1986). Furthermore, if positional values are stable within the epidermis and are arranged as in Fig. 8 (in accordance with the larval structures), why should *all* legs regenerating during metamorphosis do so in the medial position?

The modified Polar Coordinate Model (Fig. 8) can explain most of the present results from grafting a leg medial or lateral to the leg site but, like other current models, it fails with the 180°-rotated leg to leg site graft (French, 1986). The model predicts medial and lateral supernumeraries, orientated medially, while the experiment produces two supernumeraries (occasionally one or three) in a wide variety of positions and orientations (French, 1986).

Regeneration of the ventral midline

A feature of the present results that is not predicted by any current model of pattern regulation is the formation of a supernumerary branch of the ventral midline suture between the medial faces of the host and an M/L reversed (graft or supernumerary) coxa (see Figs 2, 3). Since the suture is in the midline of the bilaterally symmetrical animal it would not be expected to form by intercalation between two regions with more lateral positional values. In experiments M2–M4 it forms between left thorax and right grafted leg (with a frequency that increases as the thoracic site becomes more medial). However, interaction between epidermal cells from opposite sides of the body is *not* required, as the suture forms in graft M1, between host and the supernumerary formed after a left leg has been grafted to the left side of the thorax. These results seem directly comparable with regeneration of the ecdysial line after various grafting and excision experiments on cockroaches (Shelton, 1979). This dorsal midline structure forms where

Fig. 7. Experiment LR. (A) Schematic diagram of the operation, grafting the left metathoracic leg (*LL3*) lateral to the host left leg and rotating it by 180°, thereby reversing the anterior–posterior (*A/P*) and medial–lateral (*M/L*) axes of the graft. (B) Adult resulting from graft LR, showing host leg (*H*), grafted leg (*G*) and laterally positioned, medially orientated supernumerary (*S*). In this specimen lateral sclerites *L3* and *LP3* are normal. (C,D) Mounted cuticle preparations of specimens showing a triplication of the posterior end of sclerite *LP3*. In C there are three clear lines of bristles (*br*) and three posterior tips (*t* – see inset $\text{\textcircled{D}}$), while in D the triplication extends further anteriorly but two of the tips are partially fused (*t'*). In both specimens most of the graft and supernumerary legs are broken off (*X*).



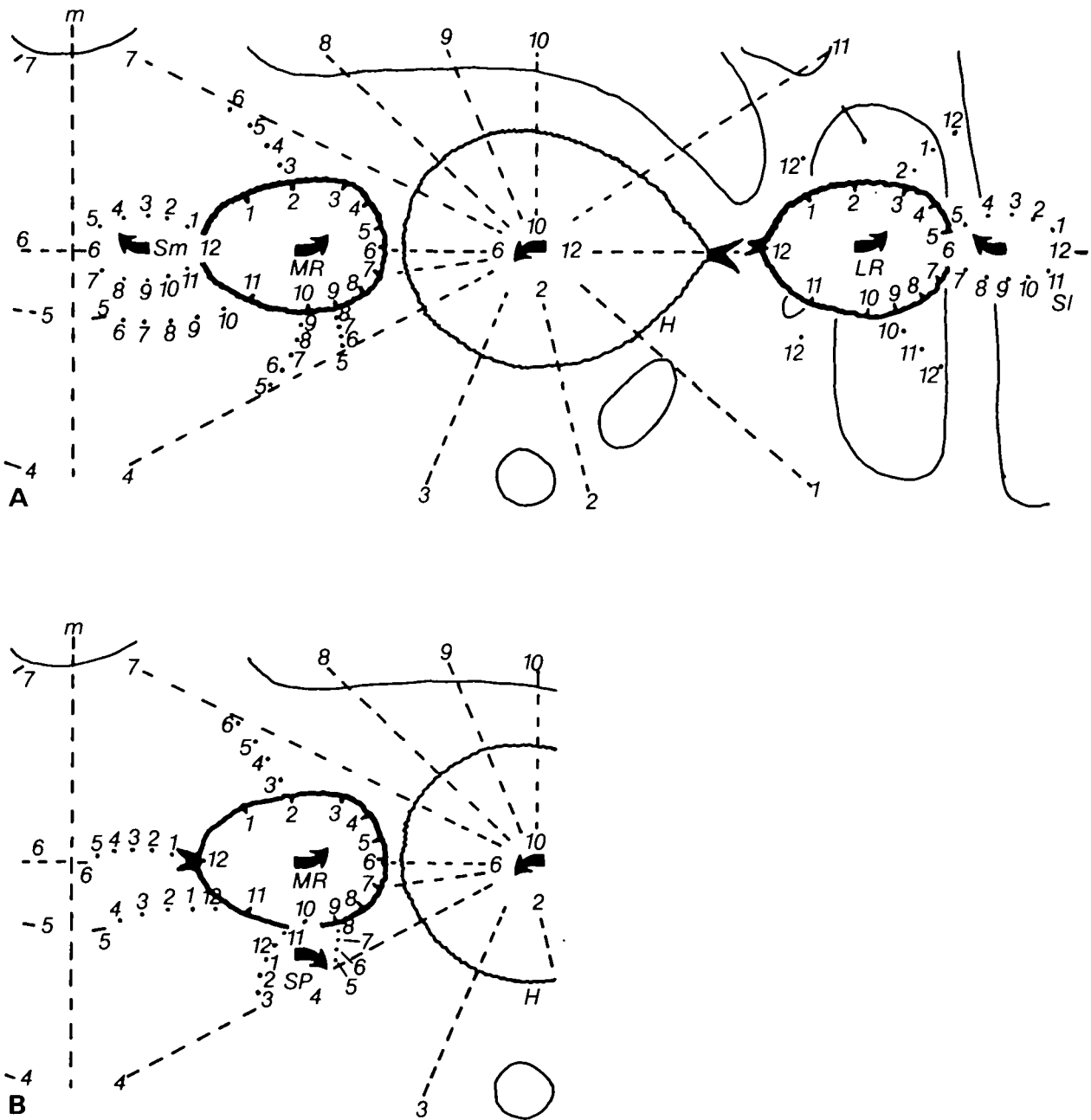


Fig. 8. Interpretation of the results of experiments LR and MR in terms of a Polar Coordinate Model with unequal spacing of circumferential positional values (1–12). See Fig. 5 for notations and abbreviations. In experiment LR (left leg grafted, 180° rotated, lateral to the host left leg) the pattern of intercalation between the grafted leg (LR) and the surrounding thorax will generate a supernumerary left leg (Sl) lateral to the graft, as is the case with equal spacing of values. In experiment MR (left leg grafted, 180° rotated, medial to the host left leg), two different patterns of intercalation are shown in A and B. Interaction between graft 2 and host 7 and between graft 9 and host 4 will give unambiguous shortest-route intercalation but either half circumference could form at the 12/6, 11/5 and 10/4 confrontations. If this sector intercalates *via* anterior values, one right supernumerary (Sm in A) will form medial to the graft, but if it intercalates *via* posterior values, the supernumerary will form in a posterior position (Sp in B). The unequal spacing of values does not influence predictions for experiments M1–M4 and L1 and L2.

thoracic tissue from close to the midline is confronted with opposite M/L polarity and, in this case also, the two regions of epidermis need not come from opposite sides of the body.

Effects on lateral sclerites

In experiments L1 and LR where a leg is grafted lateral to the leg site, a supernumerary is formed and there are associated abnormalities in the sclerites

lateral and anterior to the legs (Figs 4, 7). The origin of these adult sclerites can be deduced from the extent of adult deletion/duplication patterns resulting from excisions of transverse strips of the larval segment (French & Rowlands, 1986). The area just lateral to the larval leg (used as the site for L1 and LR grafts) normally forms the posterior part of adult sclerite LP3, while the region immediately anterior to it forms the posterior part of L3 (see fig. 12 of French & Rowlands, 1986).

After grafts L1 and LR, the tissue removed from the host site would be expected to regenerate between the supernumerary leg and the more lateral region of the host, and the fact that it has the character of LP2 when the grafted leg is mesothoracic (Fig. 4) argues that regeneration occurs largely from the graft tissue. If pattern regulation involves re-establishing positional continuity, the two extra leg bases (graft and supernumerary) will be accompanied by triplication of immediately adjacent regions. After M/L reversal of the grafted leg (graft L1), this would involve regions posterior and anterior to the leg base (e.g. the posterior tip of adult sclerite L3), while the 180° rotation leads to more extensive intercalation involving triplication of tissue immediately lateral to the leg base (e.g. value 12 in Fig. 8A – compare also the extent of medial intercalation after grafts M2 and MR in Fig. 5Aii,Bii). This could explain why the posterior tip of sclerite LP3 is frequently triplicated after graft LR but not L1 (Figs 4, 7).

Conclusion

When a leg is grafted medial or lateral to the normal leg site, a supernumerary leg is formed at the confrontation between its base and the noncorresponding ventral thorax. This demonstrates clearly that pattern regulation can occur across the medial-lateral axis of the segment, just as it occurs following excisions that confront different anterior-posterior levels of the segment. The supernumerary leg is often accompanied by duplications of thoracic sclerites and, whereas those involving lateral sclerites are readily explicable in terms of current models of pattern regulation, the regeneration of midline structures is unexpected. For most of the present grafts, the two major current models both predict the characteristics of the supernumerary legs. In the two rotated grafts, where the predictions differ, the number, position and orientation of the legs are in substantial accord with a modified Polar Coordinate Model, but there remain features of these and other

experiments on the ventral thorax which are not satisfactorily explained.

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