

Proximodistal leg regeneration in *Carausius morosus*: growth, intercalation and proximalization

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Summary

The proximodistal epidermal organization of the regenerated insect leg has been studied by grafting between corresponding and noncorresponding levels of the pro- and metathoracic femur and tibia. The results have been studied quantitatively (growth rates of the associated parts and of unsegmented intercalary structures) and qualitatively (nature, length, polarity of intercalary structures). In grafts between equivalent levels, no intercalary structure is formed, but a differential growth has been observed, the distal one fifth of segments growing about 1.5 to 2 times more than the proximal one. In grafts between different levels, unsegmented intercalary structures are formed from the distal part which thereby acquires proximal

characteristics (proximalization). However, distal tibial cells do not form femur in this process under an hypothetical femoral influence: there is no 'dominance' of femur over tibia. Some segmented intercalary structures have also been observed, but their formation cannot be related clearly to differences in the healing process. To explain proximalization, an hypothesis is presented suggesting that distal cells, which grow faster, would be the first to enter a period when positional value becomes labile and would then adapt to the proximal cells' value.

Key words: insect regeneration, leg growth, proximodistal intercalation, proximalization.

Introduction

Studies of cellular differentiation along the proximodistal axis in the larval legs of cockroaches led Bohn (1966, 1970a, 1971) to conclude that there is a gradient of differentiation repeated in the successive segments (segmental gradient). Grafting together the same levels of different segments such as femur and tibia does not trigger any morphogenesis, while, on the contrary, association of different levels of the same segment or of different segments promotes a morphogenesis whose characteristics are as follows.

(1) Following removal of tissue, a shortened segment recovers its normal length after two moults.

(2) The intercalary regenerate is polarized proximodistally in accordance with the initial positions of proximal and distal levels. In particular, when a proximal level is grafted onto a distal one, the intercalary regenerate has a polarity which is reversed when compared to the rest of the leg.

(3) The intercalary regenerate is derived in most cases from the epidermis of the distal level and, in contravention of the general rule of distal transformation (distalization) prevailing in regeneration following an amputation, forms structures more proximal than its level of origin (proximalization – Bohn, 1970a,b, 1971, 1976).

Experiments similar to those of Bohn have been performed in other Blattidae (Bullière, 1970, 1971; Bullière & Sengel, 1970a,b; French, 1976). The results mainly agree with Bohn's but in most cases proximalization could not be directly demonstrated. To explain proximalization, Bullière proposed an interpretation based on the idea of dominance. For any segment (e.g. femur) that dominated a more distal one (e.g. tibia), its cells could impose to the others, not only a polarity, but also a segmental determination (e.g. femoral cells could transform tibial cells into femoral ones). In Hemiptera, Shaw & Bryant (1975) also obtained intercalary regenerates.

Although they had no means of determining which part of a segment this intercalary regenerate represented, they concluded that 'each level in the appendage has the developmental capacity to produce only more distal structures'. In larval legs of the phasmid *Carausius morosus*, our results (Bart, 1972) partially agree with Bohn's results. However, intercalary structures are always much shorter than the missing part of a segmental gradient and we could not observe an overt proximalization of the distal epidermis.

Considering all these facts and different interpretations, we studied intercalary regeneration in regenerating legs of *Carausius*. They have convenient features that enhance operation success and they grow considerably through larval life. Therefore, we hoped that intercalary structures would be large enough to show if proximalization and dominance occur. Besides qualitative and quantitative studies on these structures we have also studied, as controls, the normal growth of parts of the femur and tibia. Our results show that: (1) the normal growth rate of a distal part of a segment is greater than that of a

proximal part; (2) in intercalary regeneration, proximalization, usually, occurs within the distal part; (3) femoral tissues do not exert a dominance (in the sense of Bullière) over tibial ones.

Together, these results suggest a model for proximalization.

Materials and methods

Because prothoracic (L1) and metathoracic (L3) legs differ in morphology, length of segments, pigmentation, setae and cuticular details in femur (F) (Figs 1, 2) and tibia (T), we used these two legs in the experiments. Grafts were usually made by exchanging parts of segments between ipsilateral regenerates of L1 and L3 (Figs 3, 4) of the same animal in the second larval instar (sometimes in the third one) during the first 2–3 days of the stage. In every case, the anteroposterior and dorsoventral axes were in register at the level of grafting. Considering a segment base as level 100 and the apex as level 100, sections were made at three main levels located, respectively, at about 15–20%, 50% and 80–85% of the segment length. For convenience, these

Figs 1–12. Proximodistal polarity of legs is from left to right (as indicated by black arrows in 8 and 11B), except in 9 and 10 where it is from up to down. Bars, 2 mm except in Figs 3 and 4, 0.5 mm. F, femur; T, tibia; 1, prothoracic; 3, metathoracic.

Fig. 1. Anterior view of normal prothoracic femur with tibia base. Proximal femur is red and dorsal ridges (arrow) are pronounced.

Fig. 2. Anterior view of normal metathoracic femur and tibia base.

Figs 3, 4. Prothoracic (3) and metathoracic (4) leg regenerates as they appear at the beginning of the second larval instar after an autotomy performed during the first 2 days of the first instar. For the femur and tibia, the differences are very minor. The prothoracic femur is simply a little longer than prothoracic tibia, metathoracic femur and tibia which all three have nearly the same length.

Fig. 5. Adult legs resulting from F1P–F3P and F3P–F1P grafts in the same animal. Exchanges were performed at stage 2 at about the one fifth proximal level. The initial proximal one fifth of each femur has grown less than the rest and is about one tenth of the total leg length.

Fig. 6. Adult leg resulting from F3P–F1D graft. Initially of about the same length, the two parts have grown very differently. Moreover, close to F3 stump, the F1 part shows red pigmentation typical of proximal F1 (large arrow). The small arrows indicate the boundary between trochanter and femur F3 (autotomy level).

Fig. 7. Adult leg resulting from T3P–F1D graft. The F1 part has grown considerably and, close to T3, displays an overtly proximalized part (arrow).

Fig. 8. T3P–F1D graft. Here, proximally, F1 has

regenerated a structure (between white arrows) with reversed polarity indicated by black arrowheads. It comprises a femoral apex, a femorotibial joint (arrowhead) and a tibia base. At the junction level with the T3 stump, ventrally, a small supernumerary structure has developed.

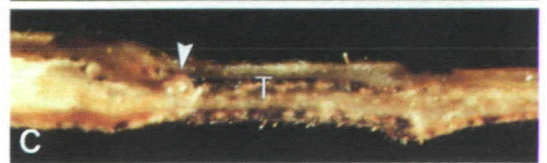
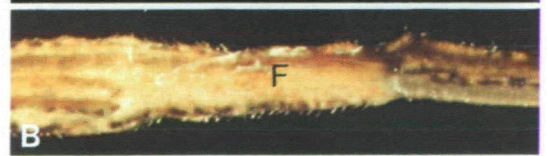
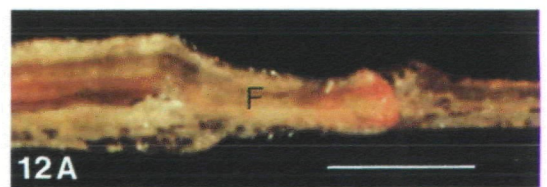
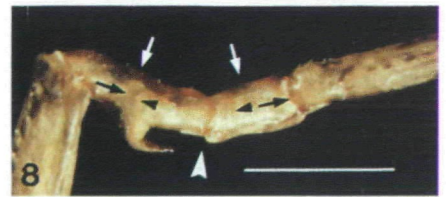
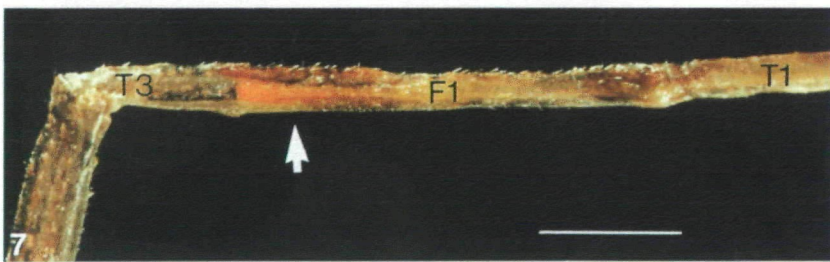
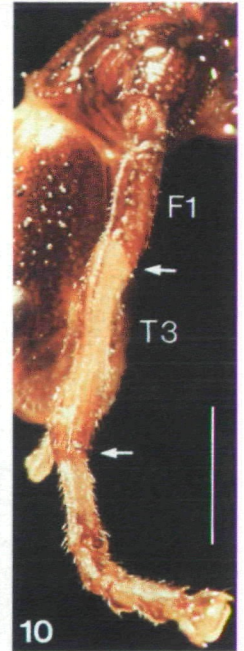
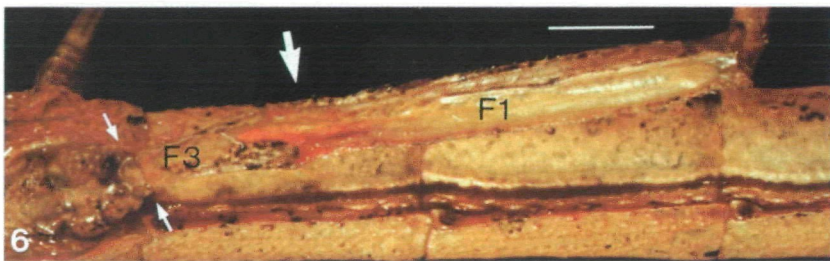
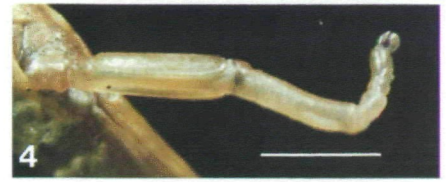
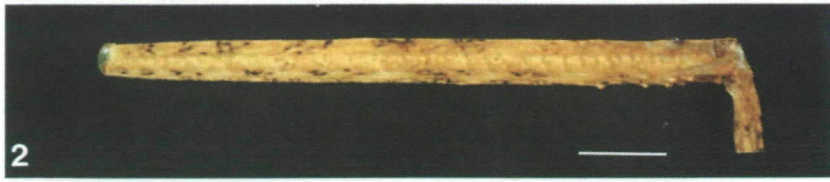
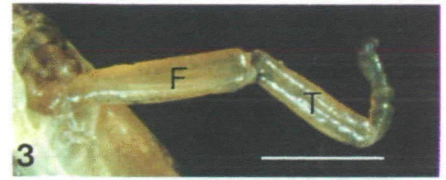
Fig. 9. T3P–T1D graft; resulting adult leg. The junction level is revealed by pigmentation differences (arrow).

T1D, initially of the same length as T3P, has grown about four times as much as T3P. Presumably, a proximalization has occurred within T1D.

Fig. 10. F1P–T3D graft; result at stage 6. The length difference between F1 and T3 (between arrows) parts is less than in T3P–T1D (Fig. 9) shown in stage 7. However, T3 has grown to an abnormal extent.

Fig. 11. F1D–T3P. An intercalary structure (IS, between arrowheads), with reversed proximodistal polarity (black arrowheads directed toward left in B) has grown between F1 and T3. (A) Anterior view. The pigmentation and setae are femoral and, close to T3, proximal differentiation is indicated by red pigmentation: proximalization has occurred in F1 (arrow). (B) Posterior view. A partial, discontinuous dorsal ridge (small white arrows) is present close to F1 with normal polarity. The proximalized part, close to T3 is incompletely differentiated with no setae or ridges.

Fig. 12. F1D–T3P. Three resulting adult legs to show variability. (A,B) Intercalary structures are femoral ones (F) and have nearly same length, but A shows a well-pigmented proximal region whereas B is very lightly coloured close to T3. (C) The intercalary structure is essentially tibial (T). However, F1 in this case has regenerated a small partial apex (arrowhead) with a piece of articular membrane (not visible here).



levels will simply be called proximal (P), mid (M) and distal (D) levels. The levels of section were measured with an eyepiece graticule with reference to the details shown in Fig. 13. Growth rates were calculated for each part of an association as ratios of the lengths in successive stages. A mean growth rate with standard deviation was established for each animal of each experimental series for periods of two to five consecutive stages. Statistical comparisons of growth rates were made using the Student's *t* test. According to the concept of the segmental gradient, the associations fall into two groups: those assumed to have no discontinuity, which serve as controls, and those with a discontinuity. For simplicity, the different associations will be described in terms of the host-graft junctions. For instance, an association between a proximal level (P) of the metathoracic (3) femur (F) as stump and a distal level (D) of the prothoracic (1) tibia (T) as graft will be named: F3P-T1D.

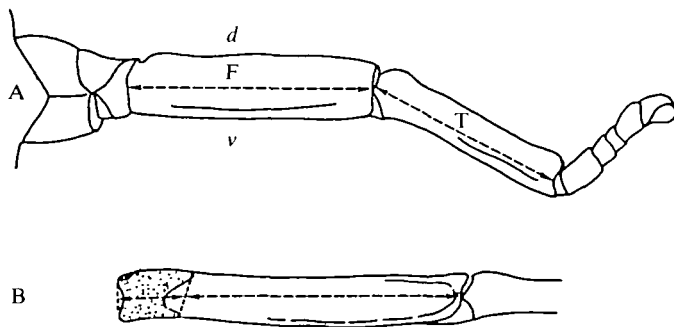


Fig. 13. (A) Leg regenerate in second larval instar before a grafting operation. Measurements in the femur (F) and tibia (T) were taken between arrows in the middle of the anterior or posterior sides. (B) Composite segment resulting from a control graft at proximal femur level. In cases with an irregular boundary, measurements were taken with respect to a line joining ventral and dorsal extremities of the boundary. *d*, dorsal; *v*, ventral.

Results

(A) *Growth of whole femur and tibia of normal regenerates*

Mean growth rates of regenerate segments following leg autotomy performed either at the beginning of the first or of the second larval instar are shown in Table 1 (growth between successive instars) and in Fig. 14 (cumulative growth).

For leg 1, the tibia grows more than the femur and differences in growth rate are generally significant or highly significant. For leg 3, the tibia grows a little more than the femur, but a significant difference is seen only between stages 2 and 3 (autotomy in stage 1) or 3 and 4 (autotomy in stage 2).

The growth rates are similar after an autotomy performed either in stage 1 or in stage 2 although values are higher in the latter case. Comparison between the growth of femurs 1 and 3 between stages 2 and 4 or between stages 2 and 6, shows no significant difference, and this is also true for tibiae (although tibia 1 grows a little more than tibia 3).

(B) *Associations with no discontinuity*

(1) *Growth of femoral parts (associations F1-F3)*

Six series of grafts were performed: F1P-F3P, F3P-F1P (Fig. 5), F1M-F3M, F3M-F1M, F1D-F3D, F3D-F1D. Mean growth rates between stages 2 and 4 and stages 2 and 6 were calculated for the proximal and the distal parts which had each been associated with a complementary part of the other leg. We also evaluated the combined growth of the sum of these two parts and compared it to the growth of the whole segment. In all cases, the total mean growth of the two parts does not differ significantly from that of a normal femur (Fig. 15), and the distal part always grows more than the proximal one and

Table 1. Mean growth rates (GR) and standard deviation (S.D.) of femurs (F) and tibiae (T) of prothoracic (1) and metathoracic (3) legs regenerated between consecutive stages

Grafts	2-3		3-4		4-5		5-6		6-7	
	GR	S.D.	GR	S.D.	GR	S.D.	GR	S.D.	GR	S.D.
<i>Autotomy in S1</i>										
F1	2.87	0.22 (9)	1.62	0.06 (9)	1.42	0.04 (9)	1.32	0.02 (9)	1.37	0.05 (8)
T1	3.31	0.26 (9)	1.68	0.06 (9)	1.47	0.03 (9)	1.35	0.02 (9)	1.45	0.05 (8)
F3	3.02	0.23 (9)	1.61	0.08 (10)	1.41	0.03 (10)	1.34	0.02 (10)	1.41	0.03 (8)
T3	3.29	0.29 (9)	1.62	0.09 (9)	1.43	0.04 (9)	1.36	0.03 (9)	1.40	0.06 (8)
<i>Autotomy in S2</i>										
F1			3.19	0.35 (10)	1.65	0.05 (10)	1.41	0.05 (9)	1.42	0.04 (8)
T1			3.55	0.32 (10)	1.72	0.08 (10)	1.43	0.03 (9)	1.51	0.05 (8)
F3			3.16	0.15 (9)	1.63	0.06 (9)	1.38	0.03 (9)	1.47	0.03 (7)
T3			3.41	0.16 (9)	1.65	0.07 (9)	1.40	0.03 (9)	1.47	0.03 (7)

The number of measured cases is indicated in parentheses.

differences, clear at stage 4, are significant by stage 6 (Fig. 16). For instance, the growth of the distal half of both femurs is about 1.5 times that of the corresponding proximal half (Figs 16, 17A) and furthermore, the distal one fifth grows about two times more than the proximal fifth.

(2) *Growth of tibial parts (associations T1-T3)*

The results (Table 2) are very similar to those obtained for femurs. The distal half grows more than the proximal half (Fig. 17B) and there is a highly significant difference in growth between the proximal one fifth and the distal one fifth. The total growth of proximal and distal parts does not differ significantly from the growth of the normal segment except in the case of associations performed at the proximal level. In this case, the separated parts of L1 have a significantly greater growth. For an unknown reason, the animals of this series show an abnormal growth, the mean growth of their femurs (not operated) being significantly greater than the growth of femurs of control animals.

(3) *Growth in F-T associations*

Associations were performed on the leg 1 regenerate by cutting at a proximal level in femur and tibia and grafting the distal part of tibia onto proximal part of femur. Healing was generally very good and, in most cases, no morphogenesis occurred between the associated parts. Growth rates were calculated between stages 4 and 7 and compared with those of equivalent parts of femur and tibia in the previous experiments (Fig. 18). No significant differences were found.

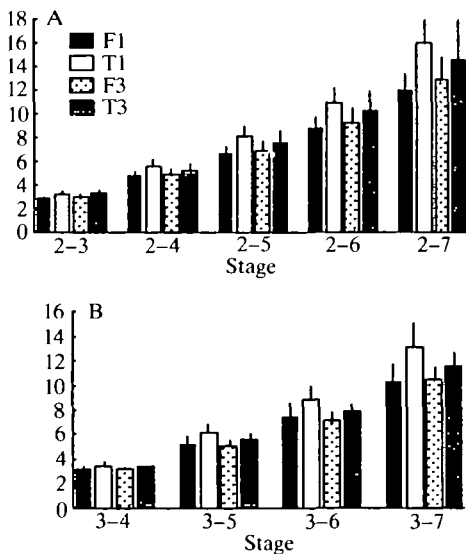


Fig. 14. Growth rates (on ordinate) with standard deviation (bar) of whole femurs (F) and tibias (T) of prothoracic (1) and metathoracic (3) legs from stage 2 (A) or 3 (B) until the adult stage (7). These rates are shown after 1 (2-3, 3-4) or 2 (2-4, 3-5) etc. moults.

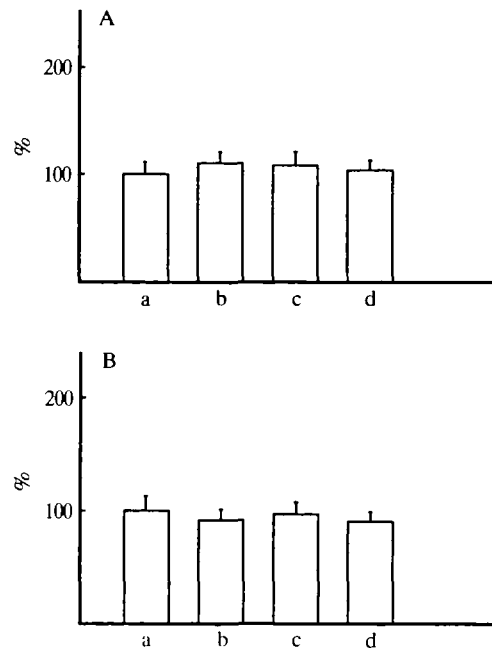


Fig. 15. (A) Comparison of growth rates between stages 2 and 6 of normal F1 (a) and of the sum of proximal and distal parts of F1 following exchanges with F3 at the same proximal (b), mid (c) or distal (d) levels. Growth rates are calculated relative to that of the normal F1 (= 100% on ordinate). No difference is significant. (B) Same comparison for F3.

In summary, the results for femur and tibia are very consistent: the distal half apparently has a greater growth rate than the proximal one.

(C) *Associations with discontinuity*

(1) *Stump level, proximal (F or T); graft level, distal F*

Two different series were performed: FP-FD, T3P-F1D (Fig. 19).

(a) *Growth and differentiation of the graft.*

(i) *FP-FD grafts.* From 58 successful grafts, 14 were lost at the first postoperative moult (stage 3) and 16 additional ones at the second moult (stage 4). The junction between stump and graft was usually narrow, poorly differentiated and easily broken. At stage 3, the graft was sometimes a little longer than the stump and the proximal one quarter to one third of its length had an unpigmented epidermis and no ridges. At stage 4, the length of this part was about two fifths of the graft length which was now clearly greater than the stump length (Fig. 19). Later in development, in this zone, femoral ridges were regularly present anterodorsally and posterodorsally and sometimes along the ventral side but, except in 2 out of 15 cases,

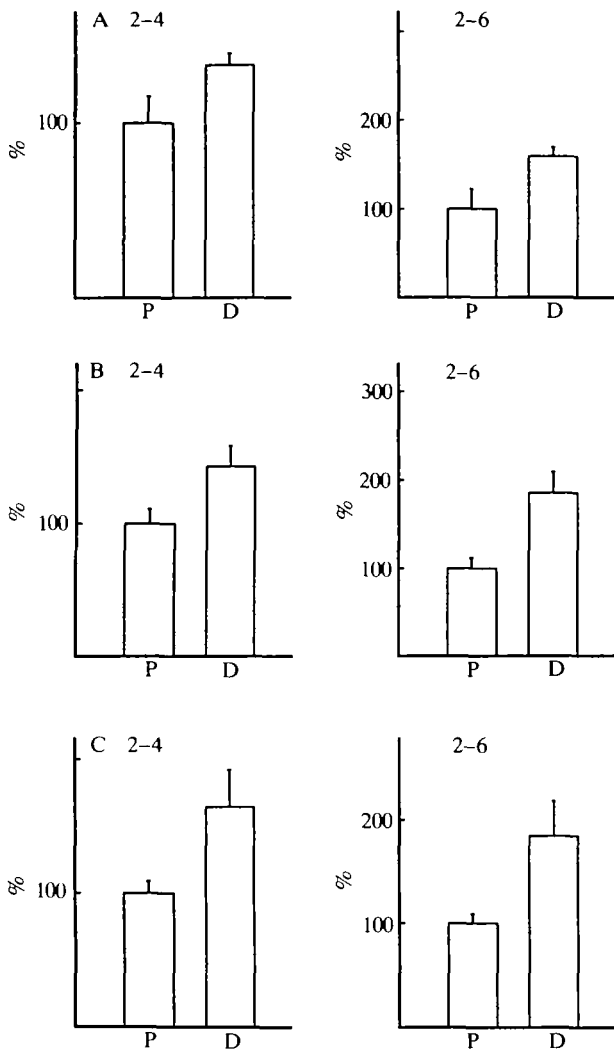


Fig. 16. Growth rates of the two parts of F1 between stages 2 and 4 (2-4) and stages 2 and 6 (2-6) following exchange at the same level with F3. The proximal part (P) serves as reference (100%). D, distal part. Exchanges at (A): proximal level, (B): mid level, (C): distal level. Differences are significant at the 1% level.

not anteroventrally or posteroventrally, where they are very pronounced on a normal femur.

Growth of this graft was studied between stages 2 and 4, 4 and 6, and for some cases between 3 and 7 or 4 and 7. Compared to F1D grafted onto F3D, these grafts showed significantly greater growth (Fig. 19). Moreover, in seven cases of F3P-F1D grafts which developed up to stage 7, the anterior part of the graft, close to the stump, had red pigmentation as in the normal proximal F1 (Fig. 6). This red part, representing a proximalization, extended over 10-28% of the grafted F1, whereas on an F1 regenerate it occupies 40-50% of the length. Even when one adds the length of the F3 stump, which represented about 10% of a F3 regenerate in S7, to this 10-28%, we get a value less than the 40-50%.

(ii) *T3P-F1D grafts.* In 31 out of 45 successful grafts, no segmented structures formed at the junction level and the grafted femur developed similarly (Fig. 19) to those in the previous series (FP-FD). In the seven cases that reached the adult stage, it displayed a red pigmentation in its proximal part and formed dorsal ridges (Fig. 7). The length of the red part varied between 5% and 25% of the femoral length with no clear correlation with the tibial stump length.

(b) *Growth and differentiation of the stump.* Both the femur and tibia stumps usually had greater growth (Fig. 20) than in the previous series where they were associated with a proximal level. However, there is no morphological character or pigmentation which could demonstrate some distalization.

(c) *Segmented structures.* Without any clear abnormality of healing just after grafting, joints and/or segmented structures may form at the junction level from the graft, graft and stump or only from the stump.

(i) *Joint structures formed from the graft.* This produces a proximally directed structure which consists of a femoral apex and articular membrane (one case, series F3P-F1D) or of a femoral apex and a tibial base (six cases, series T3P-F1D) which heals with the tibial stump. In two cases of this last series, there was a small spike located ventrally at the level of the junction (Fig. 8).

(ii) *Jointed structures formed from the graft and stump.* This occurs in one case of the F3P-F1D series, but only in the posteroventral position where F3P forms a long piece of femur with an apex and F1D regenerates an apex with articular membrane. In the T3P-F1D series, stump and graft both form tibia and some tarsal segments.

(iii) *Independent regulation of the stump.* In one case in each of the two series, the stump completed a femur (F3P-F1D) or a tibia (T3P-F1D) without any morphogenesis in the graft.

(2) *Stump level, proximal (F or T); graft level, distal T*

Four different series were performed: F1P-T3D, F3P-T1D, T1P-T3D, T3P-T1D. Formation of an articulated junction is not unfrequent (e.g. 9 out of 47 initial cases in FP-TD associations). However, we shall consider here only the cases where the junction did not form an articular membrane. The main results are that the stump, frequently (F1P, F3P, T1P), grew more than in grafts with no discontinuity (Fig. 21) and that the distal graft in every case grew much more than the stump (Figs 9, 10 and Fig. 22).

Table 2. Exchanges at the same level between tibias 1 and 3

	Proximal part (a)		Distal part (b)		Difference (a)-(b)	Proximal + distal parts (c)		Normal tibia (d)		Difference (c)-(d)
	GR	s.d.	GR	s.d.		GR	s.d.	GR	s.d.	
<i>Leg 1</i>										
Exchanges at										
P Level	6.25	0.77 (8)	14.02	1.08 (8)	xx	12.74	0.99 (8)			xx
M Level	9.82	0.98 (9)	12.27	1.48 (10)	xx	10.80	0.92 (9)			0
D Level	11.58	1.26 (9)	12.28	2.48 (11)	0	11.92	1.40 (9)			0
Control: no exchange								10.84	1.25 (9)	
<i>Leg 3</i>										
Exchanges at										
P Level	6.48	1.16 (9)	11.70	0.99 (9)	xx	10.74	0.70 (9)			0
M Level	9.07	0.71 (10)	11.19	0.84 (8)	xx	10.14	0.60 (8)			0
D Level	9.86	1.18 (12)	11.58	2.55 (9)	0	9.90	0.81 (9)			0
Control: no exchange								10.21	1.70 (8)	

Mean growth rates GR, standard deviation s.d. and (number of cases) for each of the two parts, proximal and distal, and of their sum between stages 2 and 6. Growth rate differences: 0, not significant; x, significant; or xx, highly significant.

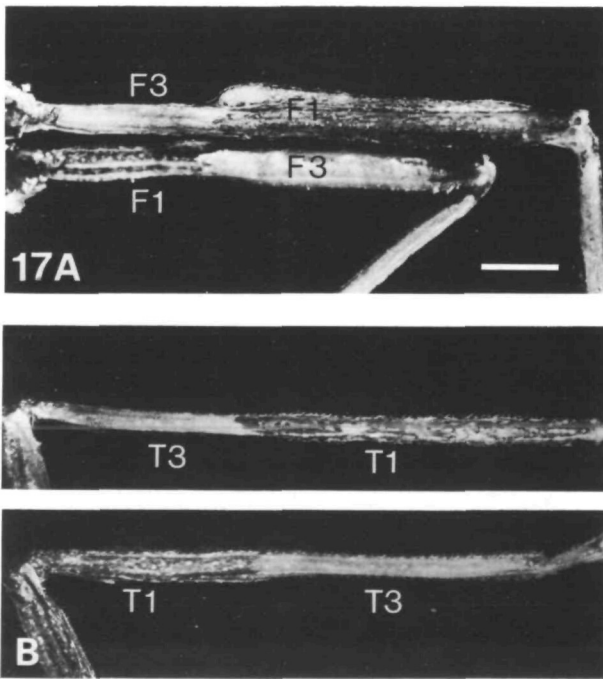


Fig. 17. Adult legs resulting from exchange, in stage 2, at mid level. (A) between F1 and F3 leg regenerates, (B) between T1 and T3 leg regenerates. Differences in morphology and pigmentation mark the boundaries between legs 1 and 3 (arrowhead). Clearly, in each case, the distal half has more grown than the proximal one. Proximodistal polarity: left to right. Bar, 2mm.

(3) Stump level, distal (F or T); graft level, proximal (F or T)

(a) FD-TP grafts. 81 cases were studied, most of them F1D-T3P grafts. In nearly all cases, an intercalary structure appeared with reversed polarity. The

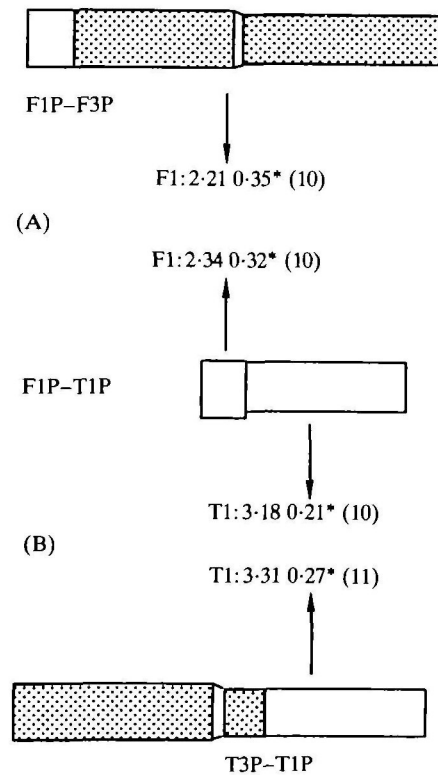


Fig. 18. F-T proximal level associations. (A) F1 proximal one fifth growth rates between stages 4 and 7 in F1P-F3P grafts (control) and F1P-T1P grafts. (B) T1 distal four fifths growth rates between stages 4 and 7 in T3P-T1P grafts (control) and F1P-T1P grafts. In each case, growth rate, standard deviation* and (number of cases) are indicated. Differences in (A) or (B) are not significant.

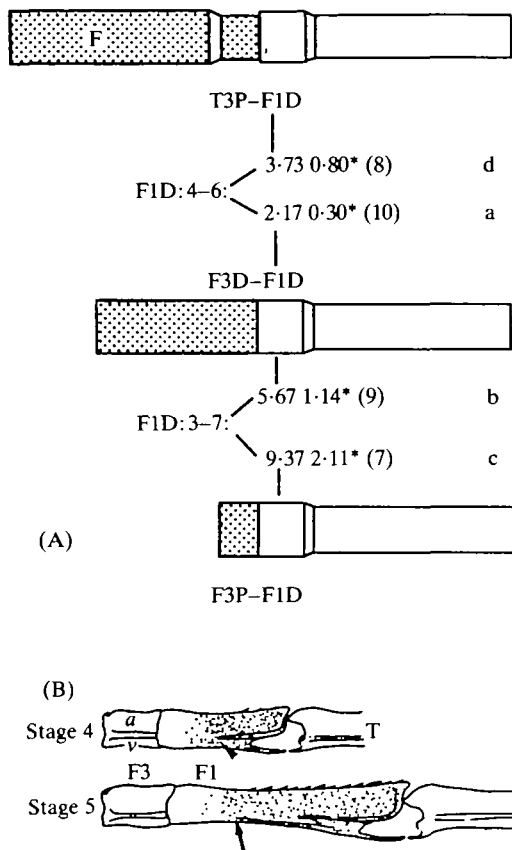


Fig. 19. Growth of F1 distal one fifth. (A) Comparison of growth rates in cases of no discontinuity (a,b, F3D-F1D grafts) and of discontinuity (c, F3P-F1D grafts; d, T3P-F1D grafts) between stage 4 and 6 or 3 and 7. In each case, growth rate, standard deviation* and (number of cases) are indicated. Differences between b and c, and between a and d, are highly significant. (B) Appearance of an F3P-F1D graft at stages 4 and 5 following an association performed at stage 2. At stage 4, the F1 part is already clearly longer than the F3 one and this is accentuated at stage 5. At stage 4, the arrowhead points to the anterior (a)-ventral (v) ridge which is missing from the proximal part of F1. This part is also poorly pigmented and has no setae. At stage 5, this proximal part is still poorly differentiated. The arrow indicates the proximal end of the ventral ridge which has developed proximalwards and bears setae. In the T3P-F1D graft, F1D behaves similarly.

differentiation of the intercalary structure was not complete at the second postoperative moult. Close to the proximal end of the tibia, it bore a thin, glistening cuticle on about one quarter of its length. On the ventral side, setae were present in some cases after the third postoperative moult (fifth larval instar) but some intercalary structures still had no setae in the adult.

The intercalary regenerate usually exhibits clear femoral characters such as dorsal and ventral ridges or parts of ridges differentiated close to the femoral

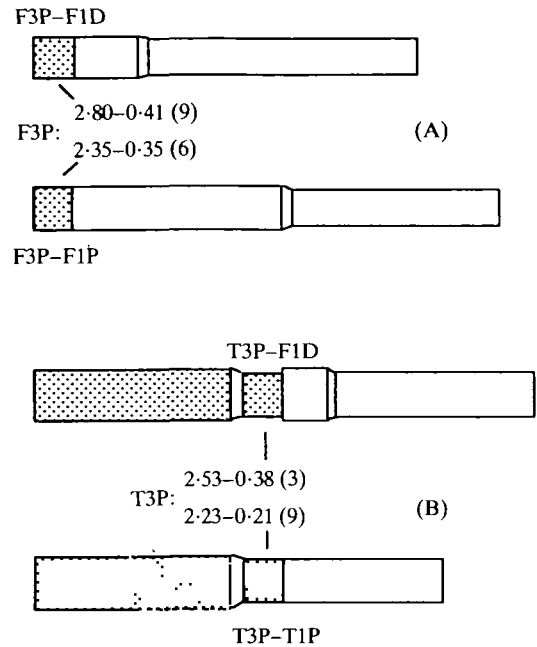


Fig. 20. (A) Comparison of proximal F3 growth rates in cases of no discontinuity; F3P-F1P grafts, and of discontinuity, F3P-F1D grafts. There is a significant difference (at the 1% level) in growth rate between the two classes of experiments. (B) Comparison of proximal T3 growth rates in T3P-T1P grafts (no discontinuity) and T3P-F1D grafts (discontinuity). There is no significant difference. Figures show growth rates (between stages 4 and 7), standard deviations * and (number of cases).

stump, bearing femoral setae and symmetrical with the femoral ridges. However, in most of F1D-T3P cases, no clear anterodorsal ridge is found. In this last series, on the anterior side, close to the grafted tibia, the intercalary structure has a red spot or patch (Fig. 11A). The pigmentation is more or less pronounced and its extent varies greatly, without any clear relationship with the total length of the intercalary structure (Fig. 12A,B). In other associations such as F3D-T1P, the intercalary structures never display prothoracic features. The mean initial discontinuity corresponds to about 60% of a segment length, but this large difference of levels triggers the formation of only a rather short structure. It is femoral in nature and its length expressed relative to the femoral stump rarely reaches 40% and usually has a value of only 25%. In 21 cases, regeneration, usually from the femur, tends to complete an apex with an articular membrane. It develops rather slowly, only becoming clear after the third postoperative moult. In one case, the femerotibial joint was completely regenerated, but it usually only forms joint structures on a part of the circumference, the rest exhibiting a reversed intercalary structure. In three other cases, the reversed intercalary structure associated with the partial femoral apex is tibial (Fig. 12C). Besides these

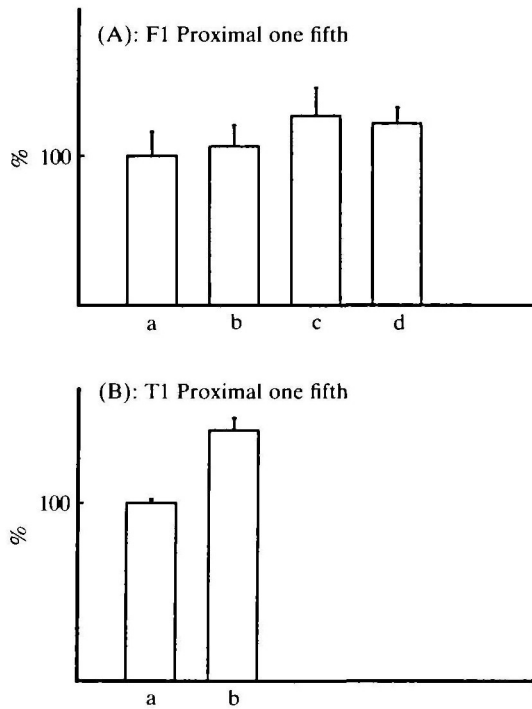


Fig. 21. Comparison of growth rates of (A) proximal F1 one fifth between stages 4 and 7 in the following associations: a, F1P–F3P (9 cases, no discontinuity); b, F1P–T1P (10 cases, no discontinuity); c, F1P–F3D (5 cases, discontinuity); d, F1P–T3D (6 cases, discontinuity). Differences are not significant between a and b or between c and d, but c and d both differ significantly from a and b (at the level of 5%). (B) Proximal T1 one fifth between stages 4 and 6, in case of no discontinuity (a, T1P–T3P; 10 cases) and of discontinuity (b, T1P–T3D grafts; 6 cases). Difference is highly significant.

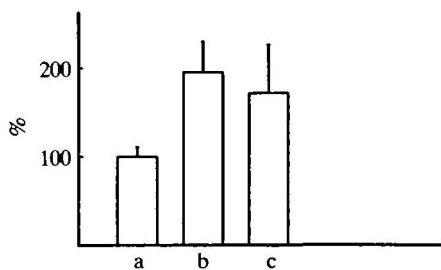


Fig. 22. Growth rates between stages 4 and 7 of the distal T1 one fifth in a, T3D–T1D grafts (10 cases): no discontinuity; b, T3P–T1D grafts (5 cases) and c, F3P–T1D (5 cases): discontinuity. b and c do not differ significantly but both differ significantly from a.

three cases, we frequently observe patches of reversed setae in the base of the grafted tibia, usually on the dorsal side.

(b) *TD–FP grafts.* Two types of associations have been performed: T3D–F1P (Fig. 23) and T1D–F3P.

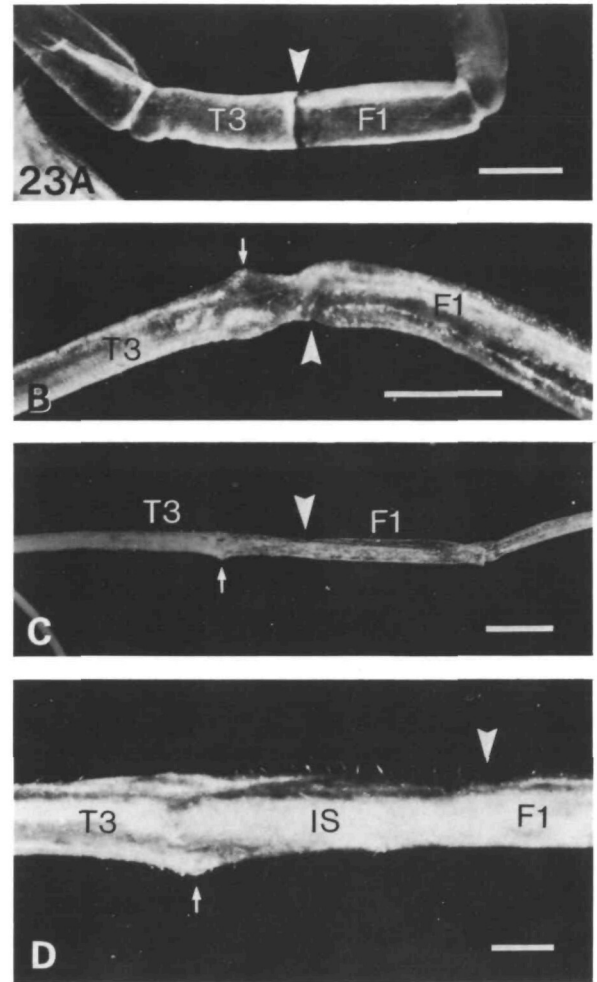


Fig. 23. Results of T3D–F1P grafts. Normal proximodistal polarity is from left to right. Arrowhead, ventral junction level; arrow, dorsal level in T3 where the proximodistal polarity reversal occurs. The intercalary structure (IS) is located between arrowhead and arrow. Bar, 0.5 mm except in C, 2 mm. (A) Appearance of the association at stage 2. (B) Intercalary structure at stage 3. (C) Intercalary structure at stage 6. (D) Enlargement of C. Setae of the intercalary structure are typically tibial ones and have reversed polarity.

An intercalary structure with reversed polarity developed progressively in all the successful cases (seven in stage 5 after three postoperative moults, three in stage 6 and one in the adult stage). Clearly it was of the tibial stump type and was about one third as long as this stump (Fig. 23).

(4) *Stump level, mid femur; graft level, proximal tibia (i.e. FM–TP grafts)*

Three autografts were performed in second instar animals and eight homografts were performed between legs 1 and 3 using femoral stump in stage 2 and tibial grafts from animals in stage 3. In the latter cases, the parts have nearly the same diameter and

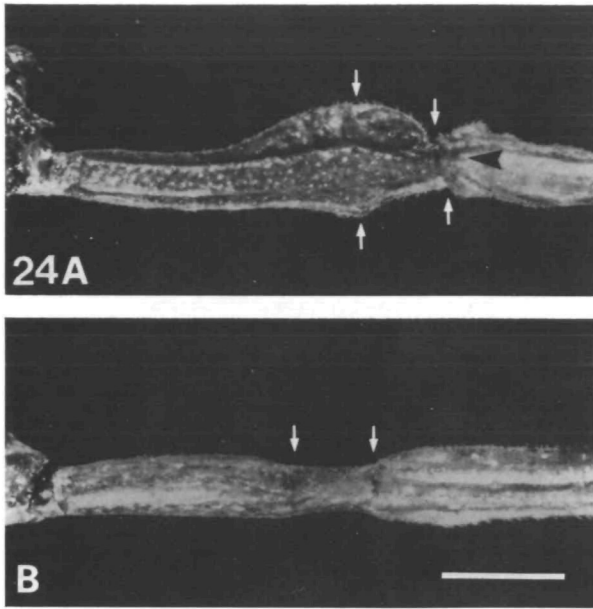


Fig. 24. Results in S7 (adult) of FM-TP grafts performed at stage 2. A femoral intercalary structure (between arrows) with reversed polarity (as indicated by setae on the edge of ridges in A) has been formed at the junction level. (A) F1M-T1P. Posterior view. Notice that tibial ridges converge posterodorsally (black arrowhead). The intercalary structure seems essentially to be a mirror image of the distal femoral stump part. (B) F3M-T1P. Anterior view. Bar, 2 mm.

heal more readily. The difference in levels was about 30%; an intercalary morphogenesis always occurred and was studied until the adult stage. In one case, the femur formed a small outgrowth on the anterior side but this did not differentiate a femoral apex. On this side, the tibia formed a structure with reversed orientation. In the ten other cases, the intercalary structure was always femoral. Only three cases had an F1 stump and in none of them did the intercalary structure show an overt proximalization: it looked like a mirror image of the most distal part of the femoral stump (Fig. 24). The length of the intercalary structure was calculated as a percentage of the normally orientated femoral stump and it varied between 30 and 40%.

Discussion

We shall discuss the cases with no segmental discontinuity, followed by the main characteristics of intercalary morphogenesis and the occurrence of proximalization and so-called 'dominance'. Then we shall propose an hypothesis to account for the results.

(A) Associations with no segmental discontinuity

The respective participations of the associated parts (L1 and L3) are very clear and apparently the distal

parts have a greater relative growth than proximal parts. However, temporarily during the first postoperative intermolt, the boundary is unclear as the healing zone has a smooth undifferentiated cuticle. Could some transdetermination in this region transform the more proximal cells of leg 1 (or 3) into cells of leg 3 (or 1)? This seems unlikely as transdetermination, which occurs in *Carausius* when regeneration proceeds from the two basal antenna segments, has never been observed in legs, in regeneration or in supernumerary formation (Bart, 1971). So we consider that the boundary at the time of healing is maintained in later stages.

There is, of course, some imprecision when two 'identical' levels of different femurs or of femur and tibia are associated. Level can be estimated to about 5%. In the case of discontinuity with some positional values missing, there could be a morphogenesis in the distal part. This could explain a slightly greater growth of this part but could not account for the great difference between growth rates. In the case of discontinuity with overlap of positional values, since the distal part base is slightly more proximal than the proximal part apex, a small intercalary regenerate with reversed polarity could appear. In no case have such regenerates been observed. From this observation, and also from the fact that there is no significant difference between growth of the sum of the two parts and the growth of an unoperated segment, we conclude that the observed differences in proximal and distal parts growth rates indicate that the distal part of a segment always grows more than the proximal ones.

Growth of whole segments of normal legs (*Blatella germanica*: Tanaka *et al.* 1986) or of leg regenerates (*Carausius morosus*: Voy, 1952) has been studied, but growth of parts of segments has not really been investigated in studies on proximodistal intercalary regeneration. For instance, Bohn (1970) established a growth rate for femur and tibia and, for associations of parts of femur and tibia of pro-, meso-, and metathoracic legs, with discontinuities, he assumed that each of these parts had the same growth rate as the corresponding whole segment. Bullière & Bullière (1985) consider their results in the same way. As Bohn and Bullière did not perform the same experiments as ours, it remains to be shown whether there are differences between *Carausius* and cockroaches in the way in which growth proceeds within segments such as femur and tibia.

(B) Intercalary morphogenesis

In the case of discontinuity in the segmental organization, we observed the formation of two kinds of structures, segmented and unsegmented.

(1) *Segmented structures*

They were observed in the following series: FP–FD, TP–FD and FD–TP, on a part or on the whole circumference. Such structures have already been seen following grafts on larval legs, even those with no discontinuity. They have been considered as consequences of healing difficulties, simply due to the operation itself (Bohn, 1970*b*; Bullière, 1971) or provoked by an apolysis occurring in the graft, close to the junction (French, 1976). In our experimental conditions, apart from difficulty of healing, as the operations involved the distal femoral part close to the ventral articular femorotibial membrane, necrosis could have caused this membrane to be involved in the healing process, triggering the regeneration of a complete joint. Alternatively, the reconstruction of an articulation may be a normal result of confronting the extreme ends of a segment, as proposed by Wright & Lawrence (1981*a,b*) for intersegmental membrane reconstitution in abdominal segments of *Oncopeltus*, and by French (1980) for leg articulation reconstitution in cockroaches. It is clear that further experiments are necessary, particularly in order to see if the results are related to the moult cycle as proposed by Mittenthal & Trevarrow (1983).

(2) *Unsegmented structures*

(a) *Growth and length.* When a distal (or a mid) level stump receives a proximal level graft, an intercalary structure, with reversed polarity is visible as early as the first postoperative moult and its boundaries are clearly defined. In the other type of discontinuity, where a proximal level stump is associated to a distal level graft, an intercalary structure can develop with the same polarity as both graft and stump. As the grafted distal part grows in its normal location about twice as much as a proximal part, in order to conclude that an intercalary structure has formed, a careful quantitative comparison of growth in the two situations is necessary. We find that, in the different cases FP–FD, TP–FD, FP–TD, TP–TD, the distal part always grows significantly more than normal (and, in most cases, the differences are highly significant). Near the junction, it presents a zone which shows little differentiation during about three postoperative moults and resembles a normal regenerate developed after one moult. This zone seems to be a persisting growth zone which progressively lengthens the intercalary structure to re-establish approximate continuity of positional values. So, considering the development of the whole intercalary structure with reversed polarity, or of a distal part in the case of a proximal level stump, differentiation proceeds disto-proximally as indicated by the different cuticular features.

Concerning final length of the intercalary structure, in FP–FD grafts, it appears shorter than expected (compare Figs 5 and 6): there is considerable growth in the distal part but, nevertheless, the chimaeric leg is clearly shorter than a chimaeric leg developed without an initial discontinuity. Considering the intercalary structure with reversed polarity, in FD–TP and FM–TP grafts, its mean value is about 30–40 % of the length of the femoral part. So, intercalated length is roughly proportional to the initial discontinuity and we can consider that there is a gradient of proximodistal positional information at least in the femur. Also, if intercalation restores continuity, it needs a length (and presumably a number of cells) less than normally present, particularly in its proximal part. Therefore, it seems that cells can tolerate some differences in positional values before an intercalation occurs. This last result is similar to those we observed with larval legs (Bart, 1972) and differs from the situation in Blattidae where the discontinuity is filled by an intercalary structure of almost the expected length.

(b) *Distal level and proximalization.* In the adult, the distal tissue close to the junction presents typical proximal features: ridges (sometimes) and, most dramatically, often a red pigmentation. The most impressive results are seen in TP–FD grafts where, as Bohn (1976) demonstrated in *Blabera craniifer*, the grafted femur forms a part (the proximal femur) which is more proximal than stump (proximal tibia) or graft tissue (distal femur). It seems that such a proximalization also occurs in the FP–TD and TP–TD grafts where TD grows more than normal, but does not form recognizable proximal markers. The proximalized part is usually incompletely differentiated, lacking some ventral ridges. Does this mean that transverse positional values corresponding to the ridges are lacking or not expressed? At present, it is unresolved. We can only emphasize that, unlike cockroaches (French & Bullière, 1975), *Carausius* does not undergo transverse regeneration after removal of some transverse positional values.

(c) *Proximal level.* In FP–FD and TP–FD grafts, our quantitative studies show a slightly greater growth than normal in the proximal parts and this could possibly indicate some distal regeneration during formation of the intercalary structure.

In FD–TP grafts, in some cases, the tibia has regenerated towards the distal femur to form all or most of the intercalary structure. Simultaneously, the femur on a part of its circumference has built a spike with distal characters and even a small patch of articular membrane. Regularly, the tibia is associated with this spike. The most plausible explanation is that healing difficulty temporarily isolated the two parts.

(d) *Dominance*. From results obtained in TP–FD and TD–FP grafts, Bullière (1971) and Bullière & Bullière (1985) propose the following explanation: when two segments interact at the junction they both contribute cells to the intercalary structure, but the more proximal one dominates the other. They consider that although cells keep their original pro- or metathoracic quality, they lose their segment type. This is respecified, according to the rules of dominance, by cells that remain differentiated in both stump and graft. However, it must be noticed that they admit dominance to be incomplete: ‘So it is probable that at the time of graft association of femur and tibia (different levels or different generatrices) femur character dominates tibia on most parts of the intercalary regenerate, but near the tibia, the tibia character may prevail’ (Bullière & Bullière, 1985, p. 124). Is that actually ‘dominance’ or simply unequal participation of graft and stump, each retaining its own character? In *Carausius*, between proximal tibia 3 and distal femur 1, a femur-type, intercalary regenerate is formed with a pigmentation demonstrating intermediate levels. This is not due to equal contributions and ‘dominance’ because the marker is *prothoracic* (1), demonstrating that the proximal levels of the regenerate derive from the original distal tissue – the graft in some cases (e.g. F3P–F1D, Fig. 6) and the host in others (e.g. F1D–T3P, Fig. 11). Furthermore, in our FP–TD and TD–FP grafts, the distal tibial tissue confronted with the femoral proximal one, gives a *tibial* intercalary structure. So, for *Carausius* femur and tibia, the concept of dominance of femur over tibia as proposed by Bullière is obviously of no value. However, distal level tissue ‘dominates’ proximal tissue in building the intercalary structure.

(C) *Hypothesis relating to the process of proximalization*

To explain why the distal cells obey a rule of proximalization and build the intercalary structure, we consider the following facts. (1) In *Carausius*, distal epidermis has a greater growth potential than proximal epidermis. (2) The importance of growth potential in the development of structures is particularly clear in *Drosophila*. In a mosaic with normal cells, slow-growing mutant cells (*Minutes*) may be eliminated during growth (Morata & Ripoll, 1975; Simpson & Morata, 1981). In intercalary regeneration, in *Drosophila*, Adler & Bryant (1977) demonstrated, using associations of irradiated anterior tissues with normal posterior ones, that ‘the posterior tissue was able to regenerate anterior structures more frequently and more extensively than when two similar nonirradiated fragments were used’. They ‘interpret this as being due to the fact that only the

posterior cells are able to divide and fill in the positional field by intercalation’. (3) Similarly, Kiehle & Schubiger (1985) observed for leg discs ‘that not only did the anterior cells divide more vigorously than the posterior cells, but also that under the same experimental conditions, anterior cells can regenerate whereas cells of the posterior compartment do not’.

Therefore, we consider that the difference of growth potential in *Carausius* results in the preferential participation of distal cells in intercalary regeneration as they become more proximal *before* proximal cells can become more distal.

Such a proposal must however be considered in relation to the apparent distoproximal progressive differentiation within intercalary structures. Does this differentiation mean that proximalization would also be progressive? If it were the case, it would be difficult to imagine how proximal tissues could be prevented from distalizing. So, to explain our results, we prefer to consider that differences in positional information are very diminished (perhaps eliminated) as distal cells become rapidly proximalized, so that proximal cells are surrounded by positional values which do not stimulate distalization. Now, how can this result in distoproximal differentiation? Our present knowledge about the mechanism of intercalation is rather speculative. For example, Anderson & French (1985) consider four possibilities which may all explain present observations on transverse intercalation. Here we just propose that the initial proximalization leaves some gaps in positional values within the small blastema built at the junction. These gaps could be rather small in the most distal part and rather large in the most proximal part. Anyhow, as growth proceeds more quickly in distal parts, intercalation would soon cease in these parts and full differentiation would be expressed rather rapidly. On the contrary, intercalation would persist in the most proximal part and delay differentiation (Fig. 25).

Clearly, in intercalation, positional information is modified in distal cells. It is disputed whether, in insects, respecification requires DNA synthesis (Besson-Lavoignet & Delachambre, 1981), but it is generally accepted that it is linked with mitosis, although this may not be necessary in some cases (Adler, 1981, 1984). Proximalization may occur if distal cells enter into a phase of lability of positional information before proximal cells. Today, available data about some position-related timing of DNA synthesis or mitosis and differential growth rates are rather limited in heterometabolous insects (Guillaume, 1961; Lawrence, 1968). In *Drosophila*, for the wing disk, results are inconsistent: uniformity of spatial distribution of mitosis (James & Bryant, 1981) or of cells in

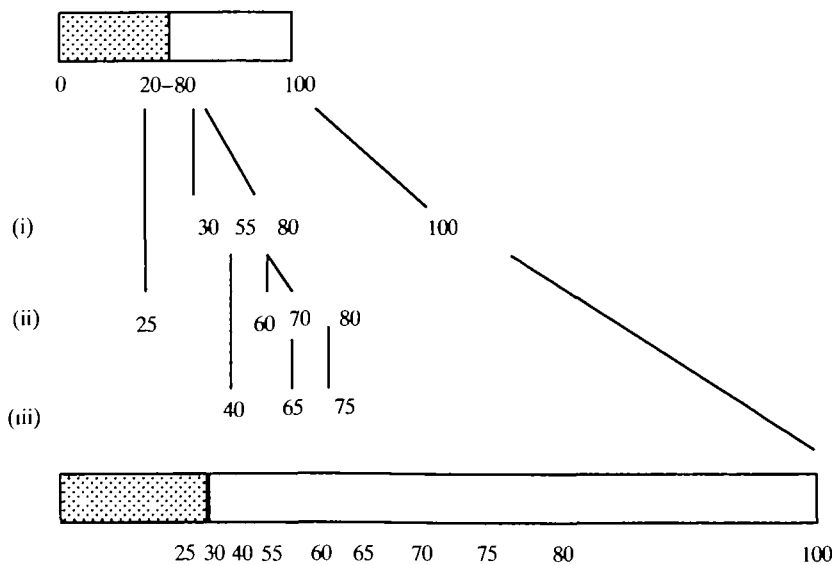


Fig. 25. Suggestion for the gradual formation of an intercalary regenerate following association of a proximal segment level (20) with a distal one (80). (i) Proximalization in the base of the distal part giving positional values 30 and 55 and simultaneously normal growth between 80 and 100. (ii) The proximal part, reacting slowly, undergoes some distalization (positional value 25) and simultaneously, intercalation continues distally (values 60 and 70). (iii) Simultaneous slow intercalation of proximal (40) and more rapid intercalation of distal values (65-75). During (ii) and (iii), normal high levels of growth have occurred between 80 and 100.

S-phase (Adler & MacQueen, 1984) versus nonuniform growth (O'Brochta & Bryant, 1985), a result already observed in *Calliphora* (Vijverberg, 1973, 1974). In larval legs, such studies lack and appear necessary.

Conclusion

(1) In leg regenerates, there is a proximodistal difference in growth rate within the epidermis of the two successive segments, femur and tibia. Particularly, the distal one fifth grows 1.5-2 times faster than the proximal one fifth.

(2) There is also a segmental proximodistal gradient of differentiation in femur and tibia similar to the one known in larval legs. Joining different levels in the gradient triggers an intercalary morphogenesis, usually unsegmented but sometimes segmented. The length of the intercalary unsegmented structure is roughly correlated with the degree of discontinuity.

(3) In most cases, association of a proximal level stump and distal level graft seems to provoke slightly greater growth in the stump and triggers much greater growth in the graft.

(4) In most cases, intercalation occurs by a process of proximalization, the distal cells becoming more proximal, presumably adopting nearly the positional value of the proximal cells that they join.

(5) During proximalization, femoral cells do not impose femoral differentiation on tibial cells: there is no 'dominance'.

(6) To explain the facts, we propose the following hypothesis: distal cells are able to become more proximal before proximal ones can become more distal. This ability would result from distal cells going through a phase of lability of positional information before proximal cells.

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Resume

La différenciation épidermique proximo-distale de régénérats de pattes a été étudiée en associant des niveaux (proximal, moyen, distal) de fémurs et/ou de tibias pro- et métathoraciques, avec ou sans discontinuité. Les résultats ont été étudiés quantitativement (taux de croissance des parties associées et des structures intercalaires non segmentées) et qualitativement (nature, longueur et polarité des structures intercalaires). En absence de discontinuité, aucune structure intercalaire n'est formée mais une croissance différentielle a été observée, le 1/5e distal des segments s'allongeant de 1,5 à 2 fois plus que le 1/5e proximal. En cas de discontinuité, des structures intercalaires non segmentées sont formées à partir du fragment distal. A proximité du fragment proximal, ces structures présentent des caractères proximaux (proximalisation). Dans ce processus, le fémur n'exprime aucune dominance vis à vis du tibia: en aucun cas des cellules tibiales distales ne donnent naissance à des cellules fémorales proximales. Dans un certain nombre de cas, les structures intercalaires sont pourvues d'une articulation partielle ou complète. Le caractère segmenté ou non de la structure intercalaire n'a pas de relation évidente avec le déroulement apparent de la cicatrisation. Pour expliquer la proximalisation, à titre d'hypothèse, nous considérons que les cellules distales deviendraient proximales avant que les proximales ne deviennent distales: en raison d'une croissance plus rapide, elles passeraient les

premières par une période où l'information de position serait labile.

References

- ADLER, P. N. (1981). Growth during pattern regulation in imaginal disc. *Devl Biol.* **87**, 356–373.
- ADLER, P. N. (1984). DNA replication and pattern regulation in the imaginal wing disc of *Drosophila*. *Devl Biol.* **102**, 300–308.
- ADLER, P. N. & BRYANT, P. J. (1977). Participation of lethally irradiated imaginal disc tissue in pattern regulation in *Drosophila*. *Devl Biol.* **60**, 298–304.
- ADLER, P. N. & MACQUEEN, M. (1984). Cell proliferation and DNA replication in the imaginal wing disc of *Drosophila melanogaster*. *Devl Biol.* **103**, 28–37.
- ANDERSON, H. & FRENCH, V. (1985). Cell division during intercalary regeneration in the cockroach leg. *J. Embryol. exp. Morph.* **90**, 57–78.
- BART, A. (1971). Modalités de formation et de développement d'un centre morphogénétique surnuméraire chez *Carausius morosus*. *Wilhelm Roux' Arch. EntwMech. Org.* **168**, 97–124.
- BART, A. (1972). Morphogénèse provoquée par une discontinuité proximo-distale au niveau de la patte de *Carausius morosus* Br. Associations de niveaux différenciés. *Archs Biol., Liège* **83**, 129–166.
- BESSON-LAVOIGNET, M. T. & DELACHAMBRE, J. (1981). The epidermal cell cycle during the metamorphosis of *Tenebrio molitor* L. (Insecta Coleoptera). *Devl Biol.* **83**, 255–265.
- BOHN, H. (1966). Transplantationsexperimente mit interkalärer Regeneration zum Nachweis eines sich segmental wiederholenden Gradienten im Bein von *Leucophaea* (Blattaria). *Verh. Deutsch. Zool. Ges.*, 499–508.
- BOHN, H. (1970a). Interkaläre Regeneration und segmentale Gradienten bei den Extremitäten von *Leucophaea*-Larven (Blattaria) I Femur und Tibia. *Wilhelm Roux' Arch. EntwMech. Org.* **165**, 303–341.
- BOHN, H. (1970b). Interkaläre Regeneration und segmentale Gradienten bei den Extremitäten von *Leucophaea*-Larven (Blattaria) II Coxa und Tarsus. *Devl Biol.* **23**, 355–379.
- BOHN, H. (1971). Interkaläre Regeneration und segmentale Gradienten bei den Extremitäten von *Leucophaea*-Larven III Die Herkunft des interkalären Regenerates. *Wilhelm Roux' Arch. EntwMech. Org.* **167**, 209–221.
- BOHN, H. (1976). Regeneration of proximal tissues from a more distal amputation level in the insect leg (*Blaberus craniifer*, Blattaria). *Devl Biol.* **53**, 285–293.
- BULLIÈRE, D. (1970). Sur le déterminisme de la qualité régionale des régénérats d'appendices chez la Blatte, *Blabera craniifer*. *J. Embryol. exp. Morph.* **23**, 323–335.
- BULLIÈRE, D. (1971). Utilisation de la régénération intercalaire pour l'étude de la détermination cellulaire au cours de la morphogénèse chez *Blabera craniifer*. *Devl Biol.* **25**, 672–709.
- BULLIÈRE, D. & BULLIÈRE, F. (1985). Regeneration. In *Comprehensive Insect Physiology, Biochemistry and Pharmacology*, vol. 2 (ed. G. A. Kerkut & L. I. Gilbert), pp. 371–424. New York: Pergamon Press.
- BULLIÈRE, D. & SENDEL, P. (1970a). Sur le déterminisme de la différenciation qualitative des cellules au cours de la régénération des appendices thoraciques chez *Blabera craniifer* (Insecte Dictyoptère) *C. r. hebd. Séanc. Acad. Sci. Paris* **270**, 1141–1144.
- BULLIÈRE, D. & SENDEL, P. (1970b). Nouvelles données sur la détermination qualitative des cellules au cours de la régénération chez les Insectes. *C. r. hebd. Séanc. Acad. Sci. Paris* **270**, 2556–2559.
- FRENCH, V. (1976). Leg regeneration in the cockroach, *Blattella germanica*. Regeneration from a congruent tibial graft/host junction. *Wilhelm Roux' Arch. devl Biol.* **179**, 57–76.
- FRENCH, V. (1980). Positional information around the segments of the cockroach leg. *J. Embryol. exp. Morph.* **59**, 281–313.
- FRENCH, V. & BULLIÈRE, D. (1975). Nouvelles données sur la détermination de la position des cellules épidermiques sur un appendice de Blatte. *C. r. hebd. Séanc. Acad. Sci. Paris* **280**, 53–56.
- GRAVES, B. J. & SCHUBIGER, G. (1982). Cell cycle changes during growth and differentiation of imaginal leg discs in *Drosophila melanogaster*. *Devl Biol.* **93**, 104–110.
- GUILLAUME, M. (1961). La multiplication cellulaire au cours de l'intermue dans divers tissus du phasme *Clitumnus extradentatus* Br. *Bull. Soc. zool. Fr.* **86**, 2–3, 361–371.
- JAMES, A. A. & BRYANT, P. J. (1981). Mutations causing pattern deficiencies and duplications in the imaginal wing disk of *Drosophila melanogaster*. *Devl Biol.* **85**, 39–54.
- KIEHLE, C. P. & SCHUBIGER, G. (1985). Cell proliferation changes during pattern regulation in imaginal leg discs of *Drosophila melanogaster*. *Devl Biol.* **109**, 336–346.
- LAWRENCE, P. A. (1968). Mitosis and the cell cycle in the metamorphic moult of the milkweed bug, *Oncopeltus fasciatus*. A radioautographic study. *J. Cell Sci.* **3**, 391–404.
- MITTENTHAL, J. E. & TREVARROW, W. W. (1983). Intercalary regeneration in legs of crayfish: central segments. *J. exp. Zool.* **225**, 15–31.
- MORATA, G. & RIPOLL, P. (1975). Minutes: mutants of *Drosophila* autonomously affecting cell division rate. *Devl Biol.* **427**, 211–221.
- O'BROCHTA, D. & BRYANT, P. J. (1985). A zone of non-proliferating cells at a lineage restriction boundary in *Drosophila*. *Nature, Lond.* **313**, 138–141.
- SHAW, V. K. & BRYANT, P. J. (1975). Intercalary leg regeneration in the large milkweed bug *Oncopeltus fasciatus*. *Devl Biol.* **45**, 187–191.
- SIMPSON, P. & MORATA, G. (1981). Differential mitotic rates and patterns of growth in compartments in the *Drosophila* wing. *Devl Biol.* **85**, 299–308.

- TANAKA, A., OGAWA, E. & OHTAKE, M. (1986). Relative growth of leg segments of the german cockroach, *Blatella germanica*. *Growth* **50**, 273–286.
- VIJVERBERG, A. J. (1973). Incorporation of tritiated thymidine in the wing and leg disks of *Calliphora erythrocephala* Meigen. Short term effect of ecdysterone on DNA synthesis during larval and pupal development. *Netherlands J. Zool.* **23**, 189–214.
- VIJVERBERG, A. J. (1974). A cytological study of the proliferation patterns in imaginal disks of *Calliphora erythrocephala* Meigen during larval and pupal development. *Netherlands J. Zool.* **24**, 171–217.
- VOY, A. (1952). Régénération et croissance des pattes atypiques chez le phasme (*Carausius morosus* BR.). *Bull. Biol. Fr. Belg.* **86**, 449–470.
- WRIGHT, D. A. & LAWRENCE, P. A. (1981a). Regeneration of the segment boundary in *Oncopeltus*. *Devl Biol.* **85**, 317–327.
- WRIGHT, D. A. & LAWRENCE, P. A. (1981b). Regeneration of segment boundary in *Oncopeltus*; cell lineage. *Devl Biol.* **85**, 328–333.

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