REGENERATION AND THE MOULTING CYCLE IN *BLATTELLA GERMANICA* L.

IV. SINGLE AND REPEATED REGENERATION AND METAMORPHOSIS

By A. F. O'Farrell*, A. Stock*, and J. Morgan*

[*Manuscript received February* 24, 1956]

Summary

Single or successive regenerations of metathoracic legs during the fifth instar in *Blattella germanica* L. at 29°C, 70 per cent. R.H., show the same time relations with moulting as previously described for the first instar, and do not affect the duration of development, number of moults, or other characteristics of the resulting adults.

At 70 per cent. R.H. and constant temperatures of 18, 25, or 30°C the capacity fully to regenerate a metathoracic leg persists until the onset of the last moult (metamorphosis), independent of the number of preceding moults, or regenerations of the same leg, or of age. Repeated regeneration of the same leg prolongs development and causes additional moults (supermoults), but the resulting adults are normal in size and appearance. When initiated early in development it produces up to six supermoults and prolongs duration of development by up to 300 per cent. Initiated later, it produces fewer supermoults at 25°C, whereas most animals metamorphose without supermoults at 30°C. Supermoulting has a much higher incidence in males than in females. Irregular increases in duration of the later instars, with a reduction in the amount of visible adult differentiation occurring at each moult, characterize supermoulting animals.

The indirect evidence provided by these results and other recent work suggests that, in cockroaches, the growth and differentiation hormone is quite distinct from the moulting hormone.

I. Introduction

The dependence of regeneration of the leg in *Periplaneta americana* L. on the endocrine glands has recently been demonstrated by Bodenstein (1955), who has also shown that the capacity to produce a fully differentiated regenerate persists throughout development. Other workers have reported that leg regeneration may be associated with supernumerary moults, thus involving postponement of metamorphosis, in *Blattella germanica* L. (Seamans and Woodruff 1939) and in *P. americana* (Zabinski 1936). Study of the time relations between regeneration and moulting in first instar *B. germanica* has revealed a regular interaction suggesting that regeneration both influences, and is influenced by, the endocrine mechanisms controlling the moult (O'Farrell and Stock 1953, 1954; Stock and O'Farrell 1954). The time relations between regeneration and moulting towards the end of the developmental period, the effect of regeneration on metamorphosis, and the degree of persistence of regeneration potency in face of repeated removal of the same leg each time it regenerates are all questions of interest in relation to the physiology of

* Department of Zoology, University of New England, Armidale, N.S.W.

development and the mechanics of regeneration, but have so far received little attention in insects.

The present paper describes experiments designed to elucidate the following points:

(i) Do single or successive regenerations of one or both metathoracic legs in *B. germanica* follow the same general pattern, and have similar time relations with operations and ecdyses towards the end of development as they do in the first instar?

(ii) If the same metathoracic leg is removed each time it regenerates, how often can this process be repeated, and how does it affect the size and differentiation of the regenerate at various constant temperatures?

(iii) Does regeneration influence the number of moults or the total time necessary for development, or have any other effects on metamorphosis?

II. Material and Methods

Mass cultures and experimental animals were maintained as described previously (O'Farrell and Stock 1953). Humidity at each temperature was controlled at approximately 70 ± 10 per cent. R.H. The work on single or successive regenerations was done at a temperature of $29 \pm 0{\cdot}5$°C, while the experiments on repeated regeneration were done at 18, 25, and 30°C (± 1°C in each case). Experimental animals were the progeny of females from cultures kept for several generations at the experimental temperature, except at 18°C, where the parent cultures were kept at 25°C.

In the work on repeated regeneration each experimental batch was hatched from one oötheca, kept until the required age, and then subjected to removal of the left metathoracic leg at the proximal "autotomy" plane, between trochanter and femur. Thereafter, the batch was observed regularly (but not daily) at intervals appropriate to the duration of the intermoult period at the various temperatures. Regenerated legs were removed as soon as observed, and preserved, together with any animals which had died, in numbered vials. All accidentally damaged individuals were eliminated. This routine was followed until all surviving individuals in the batch were adult. The adults were preserved in separate vials according to the number of moults and regenerations they had undergone. This provided a record in material of the number of regenerations and moults, the nature of the regenerates produced at each, and the morphology of the resulting adults. Control batches were observed according to the same schedules as the corresponding experimental animals.

The routine of repeated operations was initiated at several different ages so that different numbers of repetitions could be observed at each temperature even if metamorphosis were uniform. Instars one to six were covered at 18°C, and instars two to five at 30°C. At 25°C, however, all animals underwent the first operation in the first or second instar. It was desirable to cover one of the later instars, but a shortage of animals now made it necessary to use for fourth instar coverage individuals which had already undergone operation and regeneration during their first instar. Data from these animals have been marked "†" in tabulation.

The very wide variation in timing of the later moults, even in the progeny of single inbred females, necessitated a special procedure for establishing experimental

and control batches for the accurate study of time relations. Groups of egg-bearing females were isolated from the mass cultures, and a single dated subculture was established each day by pooling all progeny hatched during the preceding 24 hr. Each subculture was then observed regularly until the first fourth instar animals were seen; thereafter, daily observation was maintained to provide a supply of individuals whose age at the fourth ecdysis was known within 24 hr. Although almost 2000 animals were reared in this way during a 4-months period, it was rarely possible to obtain a substantial number of individuals entering the fifth instar not only on the same day but also at the same age. As a compromise, therefore, each experimental batch comprised individuals undergoing the fourth moult on the same day and not deviating by more than 3 days from an age of 25 days. In subdividing each such batch into experimental and control groups, care was taken to ensure that the age distribution of the 12 or fewer individuals in each group was as far as possible the same. Similar methods were used to provide a few batches of sixth and seventh instar animals of known age, but the bulk of the work was done with fifth instar material.

Removal of the left metathoracic leg between the trochanter and femur was carried out on each of the fourth, fifth, and sixth days of the fifth instar, using a total of about 400 experimental and 250 control animals. Two further series, each of about 90 animals, were subjected to successive operations, the left metathoracic leg being removed on either the fourth or the sixth day, and the right metathoracic leg on the ninth day of the fifth instar. Daily observations were continued on each batch, with appropriate recording and preservation of material, until all the animals had undergone the fifth moult. Some 200 experimental animals and 120 controls were kept under regular (but not daily) observation after the fifth moult until they were all adult.

III. Experimental Results

(*a*) *Single and Successive Regenerations Resulting from Operations Performed During the Fifth Instar*

Removal of one metathoracic leg during the fifth instar resulted in the appearance at the fifth ecdysis of a rounded papilla, usually containing a blastema-like mass of cells, or else of a fully differentiated hypotypical leg with tetramerous tarsus, usually about two-thirds of the length of the corresponding normal leg. Nothing resembling an intermediate stage in differentiation between a papilla and a regenerate was seen among the very few instances of abnormal regeneration observed. Where a papilla appeared, the fifth ecdysis was not delayed; where a regenerate appeared, it was delayed by a period approximating the time interval which had elapsed between the fourth ecdysis and the operation. As Table 1 shows, operations performed 3–4 days after the fourth ecdysis resulted in the appearance of regenerates at the fifth ecdysis, which was delayed by about 3 days; operations performed 4–5 and 5–6 days after the fourth ecdysis and leading to the appearance of regenerates at the fifth were responsible for an average delay in ecdysis of 4 and 5 days respectively. So far, the results corresponded remarkably closely to those described by O'Farrell and Stock (1953) for single operations in the first instar.

O'Farrell and Stock (1954) further showed that there was a "critical period" about half way through the first instar, and that regeneration "put back the clock"

to establish a secondary moulting cycle with its own "secondary critical period". In the fifth instar, the work on single regenerations suggested that the critical period should occur in most animals about 5–6 days after the fourth ecdysis, and that any secondary critical period established by removing a leg earlier than 6 days after the fourth ecdysis should begin about 5 days after the operation. On these assumptions, then, successive operations separated by an interval of less than 5 days should give a preponderance of animals with two regenerates, while those separated by an interval of about 5 days should give a preponderance of animals with a regenerate on the side of the first operation and a papilla on the side of the second. As Table 2 shows, the results conformed well with this expectation. Animals which had already reached the critical period at the first operation moulted without any delay and produced two

TABLE 1

DURATION OF FIFTH INSTAR IN RELATION TO TIME OF REMOVAL OF LEFT METATHORACIC LEG AND NATURE OF REGENERATE PRODUCED AT FIFTH ECDYSIS AT 29°C, 70% R.H.

P, rounded papilla only; R, fully differentiated regenerate with tetramerous tarsus

Interval between Fourth Ecdysis and Operation (days)	Number and Type of Regenerates Observed at Fifth Ecdysis when Occurring at Stated Number of Days after Fourth Ecdysis											
	5–6	6–7	7–8	8–9	9–10	10–11	11–12	12–13	13–14	14–15	15–16	16–17
No operation (controls)	10	29	40	47	34	41	22	15	6	4	0	0
3–4	0	0	0	0	0	23R	48R	34R	11R	0	0	0
4–5	0	2P	2P	6P	2P	1R	34R	41R	25R	2R	0	0
5–6	0	1P	3P	13P	8P	4P	0	1R	35R	49R	16R	7R

papillae; those producing a regenerate and papilla had the fifth ecdysis delayed by a period approximating to the time between the fourth ecdysis and the first operation, and those producing two regenerates had their fifth ecdysis delayed by a period approximating to the time between the fourth ecdysis and the second operation.

Morphologically, too, the papillae and regenerates produced after successive operations in the fifth instar were quite comparable with those arising after similar operations in the first instar. The two papillae of a pair were rather small and erratically asymmetrical in size and shape. The regenerate and papilla combination usually had large and well-differentiated regenerates, while the papillae were variable in size and blastema content. The two-regenerates combination always showed asymmetry in size and usually also in details of differentiation, the larger regenerate appearing on the side of the first operation. Only one intermediate was observed; this was on the side of the second operation.

Significant differences in the timing of the sixth and seventh ecdyses were not detected in the 183 experimental and 109 control animals followed through to metamorphosis. By analogy with the results from first instar animals, such differences would not be expected to be large, and might well be obliterated by the very wide variations in the timing of later moults; the data for the fifth moult of controls in Table 1 indicate the magnitude of these variations. Animals producing a papilla at the fifth ecdysis had a well-differentiated regenerate at the sixth, irrespective of whether metamorphosis occurred then or was postponed until the seventh ecdysis. Similarly, animals producing two papillae, or a regenerate and a papilla, at the fifth

TABLE 2

DURATION OF FIFTH INSTAR IN RELATION TO TIMES OF REMOVAL OF LEFT AND RIGHT METATHORACIC LEGS IN SUCCESSION AND NATURE OF REGENERATES PRODUCED AT FIFTH ECDYSIS AT 29°C, 70% R.H.

PP, two small, asymmetrical papillae; RP, fully differentiated regenerate on left side and papilla on right side; RI, fully differentiated regenerate on left side and an intermediate stage between papilla and regenerate on right side; RR, fully differentiated regenerates on both sides, that on the right side being smaller

Interval (days) between Fourth Ecdysis and Operation on:		Number and Nature of Regenerates Observed at Fifth Ecdysis when Occurring at Stated Number of Days after Fourth Ecdysis								
Left Side	Right Side	9–10	10–11	11–12	12–13	13–14	14–15	15–16	16–17	17–18	18–19
3–4	8–9	0	26RP	36RP	4RP	1RP	1RI	2RR	6RR	1RR	0
5–6	8–9	7PP	6PP	0	0	3RP	3RP	7RR	36RR	18RR	6RR

ecdysis had an almost symmetrical pair of large regenerates at the sixth, whether metamorphosis occurred or not. Those which had one or two regenerates at the fifth ecdysis showed the usual increase of symmetry at the sixth, again independently of metamorphosis.

Finally, a few animals were observed, without attempting a detailed study of time relations, after suffering removal of the left metathoracic leg early or late in the sixth instar. Early operations yielded a well-formed regenerate at the sixth ecdysis, independently of metamorphosis. Late operations gave a typical papilla at the sixth ecdysis, which was the last for most males and some females. Those which did not metamorphose until the seventh ecdysis then produced a regenerate at that time. These results suggested that no change in the mechanism controlling regeneration in relation to moulting had taken place up to the beginning of the critical period in the penultimate instar. This conclusion was supported by the more precise work on fifth instar material, where the whole picture conformed very closely to that of first instar animals despite the fact that major changes in the direction of metamorphosis (e.g. development of gonads, elaboration of genitalia and wing rudiments) were already

well under way at the time of operation. At all events, the capacity to regenerate certainly persisted up to the last possible moment before metamorphosis.

(*b*) *Influence of Repeated Regeneration on Regenerative Capacity*

In this work, the regenerated leg was always removed as soon as seen, in order to ensure the appearance of a regenerate rather than a papilla at the following ecdysis. Prolonged sequences of repetition of regeneration were observed in substantial

TABLE 3

SEQUENCES OF REPEATED REGENERATION OBSERVED AT THREE CONSTANT TEMPERATURES AND 70% R.H.

Temp. (°C)	Instar in which the Sequence Began	Number of Individuals Observed to Undergo Each Number of Repetitions of Regeneration Shown											
		1	2	3	4	5	6	7	8	9	10	11	12
18	First	60	59	34	19	3	—	—	—	—	—	—	—
	Second	35	27	27	22	13*	—	—	—	—	—	—	—
	Third	76	49	40	35	20	—	—	—	—	—	—	—
	Fourth	46	44	37	25*	—	—	—	—	—	—	—	—
	Fifth	42	38*	28	8*	—	—	—	—	—	—	—	—
	Sixth	25*	14*	5*	—	—	—	—	—	—	—	—	—
	Total, all instars	284*	231*	171*	109*	36*	—	—	—	—	—	—	—
25	First	76	42	38	28	23	21	21*	17*	15*	8*	3*	1*
	Second	18	16	15	14	14	12*	11*	3*	0	0	0	0
	Fourth	0†	23	22	22	22	21*	8*	2*	1*	0	0	0
	Total, all instars	94	81	75	64	59	54*	40*	22*	16*	8*	3*	1*
30	Second	52	50	49	48	44	41*	34*	21*	10*	4*	2*	0
	Third	35	35	34	34*	18*	8*	1*	0	0	0	0	0
	Fourth	24	22	20*	7*	1*	0	0	0	0	0	0	0
	Fifth	36	36*	18*	8*	5*	2*	0	0	0	0	0	0
	Total, all instars	147	143*	121*	97*	68*	51*	35*	21*	10*	4*	2*	0

* Total includes one or more adults. † See text, Section II.

numbers of animals at each temperature used, as shown in Table 3. Work at 18°C had to be suspended after observing a maximum of only five repetitions, because the only available cool incubator burned out and destroyed the animals. At 25°C, however, and also at 30°C, uninterrupted sequences of up to 11 repetitions were observed (associated, of course, with supernumerary ecdyses); in one instance at 25°C, a female animal produced nine regenerates in succession, failed entirely to regenerate

at its tenth ecdysis, and then produced three more regenerates to make a total of 12 regenerations in 13 moults and 14 instars. About 96 per cent. of all the regenerates examined at each temperature were indistinguishable in both relative and absolute size and level of differentiation from those resulting from single operations. No serious decline in regenerative capacity with age or with frequency of repetition, therefore, appeared to be present in most of the material.

About 4 per cent. of all regenerates examined were classed arbitrarily as "defective", without attempting to distinguish degrees of defect. At 18°C, almost all the defects were minor imperfections in the tarsus, although one or two gross deformities of the whole leg occurred. The animals which had undergone seven or more moults produced about 20 per cent. of defective regenerates against the overall incidence of less than 5 per cent. Similarly, at 30°C, where the total incidence of defects was 4·3 per cent., 12 per cent. of animals undergoing eight or more moults showed defects; these included spectacular deformities such as hypertrophy, duplication of the tarsus, and one instance of total failure to regenerate. The situation at 25°C was somewhat different. Two instances of total failure to regenerate were recorded, both at the tenth ecdysis; but no gross abnormalities or even clearly-recognizable minor imperfections were observed. A rather subjective impression that the tarsi were somewhat less well differentiated in many of the animals undergoing nine or more moults remains, but it is clear that the extent of defect occurring at 25°C was substantially less than at the other two temperatures. Probably, the capacity for normal differentiation did decline somewhat, either with the approach of metamorphosis or with an increased number of repetitions of regeneration (the two factors are difficult to distinguish). This effect was not very pronounced and seems to have been almost negligible at 25°C.

Failure to regenerate was not associated with the appearance of any structure resembling a papilla; the condition rather suggested a twisting and general abortion of the trochanter. Likewise, none of the gross abnormalities observed resembled either a papilla or an intermediate stage in differentiation between papilla and normal regenerate. Among the minor defects, failure to produce a large, well-defined pretarsus with fully developed claws might perhaps be regarded as a very late "intermediate" stage in proximo-distal differentiation. On the whole, then, repeated regeneration apparently did not cause any serious suspension of the "all or nothing" principle, at least for operations performed early in any instar.

(c) *Influence of Regeneration on the Number of Moults Preceding Metamorphosis*

(i) *Effect of Single and Successive Regeneration During the Fifth Instar.*—In all, 183 experimental and 109 control animals were followed through to metamorphosis at 29°C from the time relations experiments on fifth instar material. Table 4 shows that there was no marked difference between regenerating animals and controls in the number of moults preceding metamorphosis. The proportion of control females undergoing the extra (seventh) moult in different batches varied between 25 and 75 per cent. Hence no significance was attached to the differences in this respect between regenerating animals undergoing different treatments (Table 4). The rather high proportion of males undergoing the seventh moult after producing a papilla at the

fifth and a regenerate at the sixth ecdysis may have been significant, since very few control or other males had this extra moult. Two females also had an eighth moult after producing two regenerates at the fifth ecdysis; these moults were not paralleled in any of the other animals, and were therefore true supernumerary moults.

In none of the experimental animals of Table 4 was the age at metamorphosis outside the range of variation observed in the controls (viz. 40–52 days in animals undergoing six moults and 50–65 days in animals undergoing seven moults). The last moult of experimental animals tended to begin a little later than in controls, presumably because of the still uncompensated delay in the fifth moult caused by

TABLE 4

EFFECT OF REMOVAL OF ONE OR BOTH METATHORACIC LEGS DURING THE FIFTH INSTAR ON THE NUMBER OF MOULTS PRECEDING METAMORPHOSIS AT 29°C, 70% R.H.

Regenerates Produced at the Fifth Ecdysis	Number and Sex of Individuals Metamorphosing at:								
	Sixth Ecdysis			Seventh Ecdysis			Eighth Ecdysis		
	♂	♀	Total	♂	♀	Total	♂	♀	Total
None (controls)	46	16	62	3	44	47	0	0	0
One papilla	2	3	5	5	5	10	0	0	0
One regenerate	50	33	83	5	20	25	0	0	0
Two regenerates	25	9	34	1	25	26	0	2	2

regeneration, but a significant difference between experimental animals and controls in average age at metamorphosis was not demonstrated. It was therefore concluded that, apart from the obviously exceptional individuals mentioned above, neither the number of moults nor the duration of development was influenced by regeneration during the fifth instar.

(ii) *Effect of Repeated Regeneration.*—At all the experimental temperatures, the great majority of control males metamorphosed at the sixth ecdysis. The proportion of control females undergoing an extra (seventh) moult again varied between 25 and 75 per cent. in different batches. As regenerates can appear only at an ecdysis, it will be clear from Table 3 that many of the experimental animals had more than seven moults (the maximum observed was, in fact, 13). The seventh moult, being absent in almost all control males and many control females, is effectively a supernumerary moult, but it has nothing to do with regeneration. Additional moults associated with regeneration, beginning with the seventh in males and the eighth in females, will therefore be termed "supermoults" rather than "supernumerary moults" in this paper (see Table 6). Tables 5 and 6 taken together provide a summary of the data obtained on the relationship between repeated regeneration and supermoulting.

TABLE 5

SURVIVAL AND METAMORPHOSIS OF ANIMALS UNDERGOING VARIOUS SEQUENCES OF REPEATED REGENERATION AND SIX OR MORE ECDYSES AT THREE CONSTANT TEMPERATURES AND 70% R.H.

Temp. (°C)	Instar in which the Sequence Began	Number of Adults and Non-Adults Undergoing Number of Ecdyses Shown:														
		6		7		8		9		10		11		12		13
		Adult	Non-Adult	Adult	Non-Adult	Adult	Non-Adult	Adult	Non-Adult	Adult	Non-Adult	Adult	Non-Adult	Adult	Non-Adult	Adult only
18	First	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Second	3	10	0	0	0	0	0	0	0	0	0	0	0	0	0
	Third	0	35	0	20	0	0	0	0	0	0	0	0	0	0	0
	Fourth	0	37	1	24	0	0	0	0	0	0	0	0	0	0	0
	Fifth	1	37	0	28	1	7	0	0	0	0	0	0	0	0	0
	Sixth	2	23	6	8	1	4	0	0	0	0	0	0	0	0	0
	Total, all instars	6	142	7	80	2	11	0	0	0	0	0	0	0	0	0

TABLE 5 (*Continued*)

Temp. (°C)	Instar in which the Sequence Began	Number of Adults and Non-Adults Undergoing Number of Ecdyses Shown:														
		6		7		8		9		10		11		12		13
		Adult	Non-Adult	Adult	Non-Adult	Adult	Non-Adult	Adult	Non-Adult	Adult	Non-Adult	Adult	Non-Adult	Adult	Non-Adult	Adult only
25	First	0	21	3	18	2	15	6	9	3	5	2	1*	0	1*	1*
	Second	0	14	1	11	8	3	3	0	0	0	0	0	0	0	0
	Fourth	0†	22	0	22	13	8	6	2	1	1	1	0	0	0	0
	Total, all instars	0	57	4	51	23	26	15	11	4	6	3	1	0	1	1
30	Second	0	44	3	38	10	24	6	15	5	5	2	2	2‡	0	0
	Third	16	18	10	8	7	1	1	0	0	0	0	0	0	0	0
	Fourth	12	8	6	1	1	0	0	0	0	0	0	0	0	0	0
	Fifth	18	18	8	10	1	7	2	3	1	1	1?§	0	0	0	0
	Total, all instars	46	88	27	57	19	32	9	18	6	6	3	2	2	0	0

* Failed to regenerate at the tenth ecdysis only.

† See text, Section II.

‡ "Nymphoid" female included (see text, Section III (*e*)).

§ Died in ecdysis, too shrivelled to dissect, but certainly male and probably metamorphosing.

Table 5 shows the number of animals, including non-adults, surviving at each ecdysis and temperature. Only 11 males and 4 females metamorphosed at 18°C before the breakdown of the incubator. Eight males supermoulted but no females did so, 80 eighth instar and 11 ninth instar non-adults were observed at 18°C, and some of these would have undergone at least three supermoults had they lived to metamorphose. At 25 and 30°C numerous adults were reared and the data on non-adults given in Table 5 are of less interest in relation to supermoulting; they do, however, show that mortality was much lower at the higher temperatures.

TABLE 6

NUMBER OF SUPERMOULTS ASSOCIATED WITH REPEATED REGENERATION AT 25 AND 30°C, 70% R.H.
The first supermoult is assumed to be the seventh moult for males and the eighth for females.

Temp. (°C)	Instar in which the Sequence Began	Number and Sex of Animals Metamorphosing After the Number of Supermoults Shown:														Total, All Ecdyses
		0		1		2		3		4		5		6		
		♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	
25	First	0	1	2	0	2	5	1	2	1	2	0	0	0	1*	17
	Second	0	1	0	5	3	1	2	0	0	0	0	0	0	0	12
	Fourth†	0	0	0	5	8	4	2	1	0	1	0	0	0	0	21
	Total, all instars	0	2	2	10	13	10	5	3	1	3	0	0	0	1*	50
30	Second	0	0	3	6	4	3	3	3	2	2	0	1‡	1	0	28
	Third	11	10	5	3	4	1	0	0	0	0	0	0	0	0	34
	Fourth	6	8	4	1	0	0	0	0	0	0	0	0	0	0	19
	Fifth	9	14	3	0	1	1	1	0	1	0	1?§	0	0	0	31
	Total, all instars	26	32	15	10	9	5	4	3	3	2	1?§	1	1	0	112

* Failed to regenerate at the tenth ecdysis only.

† See text, Section II.

‡ "Nymphoid" female included (see text, Section III (*e*)).

§ Died in ecdysis, too shrivelled to dissect, but certainly male and probably metamorphosing.

The data on supermoulting of animals reaching metamorphosis, presented in Table 6, were derived from 21 males and 29 females reared at 25°C and from 59 males and 53 females reared at 30°C. All but two (both females) had at least one supermoult at 25°C, but at 30°C 44 per cent. of males and 60 per cent. of females metamorphosed without a supermoult. The sequence initiated in the second instar at 30°C, however, yielded supermoults in all the 13 males and 15 females reared: the general pattern in this group broadly resembled that of the 6 males and 11 females reared at

25°C from the sequence initiated in the first instar, with many animals undergoing three or more supermoults, up to a maximum of six. At 25°C, sequences initiated in the second and fourth instars had a lower incidence of supermoults than that initiated in the first instar, and the maximum number recorded was only four (one case). In these sequences all males had at least two supermoults, but females with two or more were limited to one in the second instar sequence, and to six (55 per cent. of the number of females reared) with the fourth instar sequence. The latter figure may be influenced by the fact that the animals had already regenerated in the first instar before the sequence was started in the fourth (see Section II). With the first instar sequence, however, the incidence of two or more supermoults was relatively higher in females than in males, in the ratio of about 7:5. The otherwise similar second instar sequence at 30°C had this ratio reversed (4:3 in favour of males). In other sequences at 30°C the preponderance of supermoults in males was very marked. About 15–16 per cent. of the adults of both sexes reared at 30°C from the third and fifth instar sequences had two or more supermoults, but none of those from the fourth instar sequence had more than one. Thus it appeared that the effect of repeated regeneration on the number of moults may have fallen to a minimum for sequences initiated about the middle of the developmental period (i.e. the fourth instar). The same principle appeared to operate to some extent at 25°C, but comparable sequences began one moult earlier than at 30°C. Without further data this interpretation can only be regarded as provisional. But the following definite conclusions can be drawn from the data:

(1) Repeated regeneration is associated with supermoults at 18, 25, and 30°C. The incidence of supermoults is greater at 25 than at 30°C.

(2) Sequences of repeated regeneration initiated in the first instar at 25°C and in the second instar at 30°C produce a greater incidence of supermoults than those begun in later instars.

(3) The number of supermoults undergone by individuals is similar at 25 and 30°C, rarely exceeding three and exceptionally exceeding four.

(4) Except in sequences begun in the first instar at 25°C, males tend to undergo supermoults more readily than females, and do so much more frequently in sequences begun in the third, fourth, or fifth instars at 30°C.

(*d*) *Influence of Regeneration on Duration of Postembryonic Development*

The duration of development was normal in animals regenerating only in the fifth instar at 29°C (see Section III (*c*) (i)). For those undergoing repeated regeneration, no satisfactory information was obtained at 18°C, but the data for 25 and 30°C are presented in Table 7. The age of metamorphosis became more and more variable with an increased number of moults, and wide overlaps occurred. Table 7 therefore shows the range of maximum and minimum ages observed, as well as the average age at metamorphosis after each number of ecdyses. The table takes no account of the instar in which the sequence was initiated nor of the sex of the animals, because no consistent differences were recognized in relation to these points.

Evidently the duration of development was hardly increased in animals metamorphosing at the sixth or seventh ecdysis, although at 30°C the average duration

at the seventh ecdysis of experimental animals approximated to the maximum duration recorded for controls. As the number of ecdyses increased beyond seven, the duration of development became more and more prolonged, reaching about three times the control average in the slowest animal at 25°C, and four times the control average in two animals at 30°C. This prolongation resulted not only from the increased number of instars but also from an increase in their average duration. In controls, the average duration of the first instar was about 10 days at 25°C and 5 days at 30°C. The mean duration of an instar over the whole developmental period, however, was

TABLE 7

AGE AT METAMORPHOSIS AFTER VARIOUS NUMBERS OF MOULTS IN ANIMALS UNDERGOING REPEATED REGENERATION AT 25 AND 30°C, 70% R.H.

Ages are given in days from hatching, correct to the nearest 3 days. For controls at 25°C, the range was 66–105 days, average 84 days. For controls at 30°C, the range was 32–56 days, average 42 days. Controls undergoing seven ecdyses tended towards the upper figure at each temperature. N.R., not recorded

Temp. (°C)	Range and Average of Ages (days) Recorded at Metamorphosis at Each Ecdysis Shown:														
	Sixth		Seventh		Eighth		Ninth		Tenth		Eleventh		Twelfth		Thir-teenth
	Range	Av.	Range	Av.	Range	Av.	Range	Av.	Range	Av.	Range	Av.	Range	Av.	Range
25	N.R.	—	74–98	92	94–143	112	119–185	150	144–175	160	186–203	196	N.R.	—	247*
30	39–54	44	46–75	56	56–100	70	69–102	88	88–137	98	117–134	122	168–190	180	N.R.

* One animal only.

about 13 days at 25°C and 7 days at 30°C. This was due to a general slowing down in the rhythm of moulting, such that one or more of the later instars approached or exceeded twice the duration of the first. A similar process apparently continued with the supermoults of regenerating animals, so that instars with a duration of the order of 30 days occurred in some supermoulting animals at 30°C.

It unfortunately proved impossible to devise a satisfactory method of following through the development of individual animals, except where they had some peculiarity of facies or were the sole survivors of an experimental batch. The five slowest growing animals at 30°C (not all in one batch) had a peculiar facies which made them recognizable from the seventh moult onwards (see Section III (*e*)). At the seventh ecdysis their ages ranged from 75 to 97 days, and the four which survived metamorphosed at the eleventh and twelfth ecdyses, being the only animals to attain such a number. This suggested the possibility that some animals might be "predestined" to a large number of supermoults by slow growth at quite an early stage. Table 8 was therefore constructed from the data of the second instar sequence at 30°C, the only one with sufficient numbers undergoing several supermoults to permit this sort of analysis. Small numbers and wide variations in the later moults made analysis beyond the ninth ecdysis pointless.

Clearly there was a sharp bimodal distribution in the ages of non-adult animals at the sixth to ninth ecdyses. Animals of group A completed a given moult several days before any of group B began it. Metamorphosis at a given ecdysis conformed

to group A timing rather than group B. Nevertheless, groups A and B did not comprise the same individuals from one moult to the next. At the ninth ecdysis, 16 adults and group A non-adults were recorded, yet only 11 group A non-adults were present at the eighth ecdysis. Thus the group B of the eighth ecdysis must have contributed some of the adults or group A non-adults recorded at the ninth. On mean ages this seems difficult, but the earliest-moulting group B individuals of the eighth ecdysis could have produced the latest-moulting group A or adult individuals of the ninth ecdysis, given a rather short intermoult period of the order of 12–14 days. Thus intermoult periods did not undergo a regular increase with the number of ecdyses; animals undergoing large numbers of supermoults had some exceptionally long intermoult

TABLE 8

AGES AT SIXTH TO NINTH ECDYSES OF ANIMALS UNDERGOING REPEATED REGENERATION SEQUENCE INITIATED IN SECOND INSTAR AT 30°C, 70% R.H.

Means are given in days from hatching, standard errors to the nearest day

Animal Groups	Age Statistics (days) for:							
	Sixth Ecdysis		Seventh Ecdysis		Eighth Ecdysis		Ninth Ecdysis	
	No. of Animals	Mean ± S.E.	No. of Animals	Mean ± S.E.	No. of Animals	Mean ± S.E.	No. of Animals	Mean ± S.E.
Non-adults, group A	30	44 ± 1	22	56 ± 1	11	66 ± 2	10	87 ± 4
Non-adults, group B	14	56 ± 1	14	75 ± 3	13	91 ± 5	5	120 ± 4
Adult males	0	—	3	55 ± 3	3	67 ± 3	3	87 ± 12
Adult females	0	—	0	—	6	65 ± 3	3	92 ± 7

periods (classifying them in group B) followed by a restoration of normal periods one or more moults before metamorphosis. In the abnormal animals with 12 moults, however, restoration of a more normal moulting rhythm at the ninth or tenth ecdysis was followed by a very long intermoult period immediately before metamorphosis (30 days for the male, 28 days for the female). Thus repeated regeneration in the animals of Table 8 seems to have been associated with a rather irregular fluctuation rather than a consistent progressive increase in the duration of intermoult periods. The occurrence of at least one very long intermoult period in supermoulting animals seems to be established, however, although it can not be precisely related to the physiological onset of metamorphosis.

(e) *General Effects of Regeneration*

Both adults and non-adults appeared to be morphologically and functionally normal after undergoing single or successive regenerations in the fifth instar at 29°C.

In the animals undergoing repeated regeneration, supermoulting was not accompanied by gross morphological peculiarities in the resulting adults, which were distinguishable from controls only by the presence of the regenerate. "Super-differentiation", like that described by Bodenstein (1953) for *Periplaneta* adults was not observed in any

of the experimental animals; these, of course, had undergone their supermoults before and not after metamorphosis. At 18°C, the few adults reared showed several instances of failure of the wings to expand normally, both in controls and experimental animals; this effect was not observed at higher temperatures and seems unlikely to be related to regeneration.

The only striking abnormality observed was that of the single female undergoing 12 moults at 30°C. This was very small, with imperfectly developed wings and genitalia, but a predominantly adult appearance, somewhat recalling the "nymphoids" described by Scharrer (1946) in *Leucophaea*. It died 15 days after the twelfth ecdysis without showing any sign of further moulting. The ovaries were somewhat deformed, containing white crystalline deposits which were also unusually abundant in the fat-body and even occurred in the coxal musculature of the regenerated leg. The internal organs otherwise appeared normal.

Non-adult animals destined to undergo several supermoults were recognizable at the sixth or seventh ecdysis; they were obviously abnormally small and had less differentiation of the wing buds and external genitalia than normal. An animal destined for 10 moults did not present at its seventh ecdysis the appearance of a normal fourth instar animal, but of something intermediate in size and characteristics between the normal fourth instar and the normal pre-adult. The appearance in supermoulting animals of the morphological changes leading towards metamorphosis was, therefore, not delayed in its onset, but merely retarded by being spread over an increased number of moults. Animals undergoing more than 10 moults at 30°C, however, were grossly abnormal in appearance from at least the seventh ecdysis. They were very small, although not resembling the pathological first instar "dwarfs" observed by O'Farrell and Stock (1953). The cuticle was pale, nut-brown rather than the shining black or dark brown of normal non-adults, and the pale appearance was accentuated by unusually heavy white crystalline deposits in the fat-body, visible through the translucent cuticle. Some other experimental animals and a few controls, especially at 30°C, showed similar but less marked peculiarities. These were associated with lowered viability, an increased number of moults, and some deformity of the gonads, especially in females. Apparently these peculiarities resulted from some sporadically occurring metabolic disturbance, possibly related to the intracellular symbionts (Brooks and Richards 1955).

IV. Discussion

Regeneration may sometimes be a local or largely localized phenomenon, as in the crustacean *Asellus* (Needham 1949); there is in this animal a decline and recovery of regeneration potency extending over a series of repetitions of regeneration of the same appendage. In *B. germanica*, however, the metathoracic leg retains its regeneration potency almost unimpaired over a series of repeated regenerations far exceeding the normal number of moults. This continues up to the physiological onset of metamorphosis, which is often delayed by a number of supermoults, suggesting that regeneration of the metathoracic leg in *B. germanica* is a systemic rather than a local phenomenon. Such decline in regeneration potency as is observed is qualitative (e.g. imperfectly formed pretarsi) rather than quantitative (e.g. reduction in size of

regenerates). It is also possible that the adult expectation of life may be influenced by the slowing down of development associated with repeated regeneration, but this has not been investigated.

The present work shows that the overall course of events in regeneration and moulting is the same in the fifth instar as in the first. It is, therefore, unnecessary to postulate a special interaction between repeated regeneration and adult differentiation; the results should be explicable in terms of the relationships already demonstrated between regeneration and the moulting cycle (O'Farrell and Stock 1953, 1954; Stock and O'Farrell 1954). The effects of repeated regeneration on metamorphosis can be interpreted as an extension of the already postulated disturbance of endocrine balance. The virtual absence of nymphoids, of gigantism, and of super-differentiation in animals, whose adult differentiation was spread over a greatly increased number of moults, suggests that competition between the regenerate and the organism as a whole for the differentiation promoting hormone(s) is a self-regulating mechanism (compare the "compensation" of delay in moulting due to a single regeneration discussed by O'Farrell and Stock 1953). The hormone balance required for development to proceed normally may be most critical in the early instars, since it is then that the initiation of repeated regeneration has the greatest effect in prolonging development and inducing supermoults, presumably through the draining off of differentiation hormone(s) by the regenerate. Maturation of the testes in the male, however, may make additional demands on the hormone(s) concerned during the middle of the larval period; this may explain the higher incidence of supermoulting seen in males in which repeated regeneration was initiated comparatively late in development. Given a shortage of hormone(s), the prolongation of one or more intermoult periods may be explained by the necessity for restoring concentrations to a level sufficient to permit moulting.

Novak (1951*a*, 1951*b*) regards metamorphosis as the outcome of balanced competition between adult differentiation, due to an intracellular factor in the tissues, and larval differentiation under the influence of the juvenile hormone of the corpus allatum in the presence of a moulting hormone which is not responsible for promoting growth and differentiation. On this hypothesis repeated regeneration might be expected to produce additional moults; but these would occur, if anything, in a shortened developmental period, and at least some of the resulting adults should be abnormal. The overall time relations between single and successive regenerations and moulting are also difficult to explain on Novak's theory.

The majority of workers (see Wigglesworth 1954) consider that metamorphosis results from changes in the balance between two antagonistic hormone systems, one a "juvenile" hormone and the other promoting both moulting and growth (including adult differentiation). This theory can explain the observed time relations between single and successive regenerations and moulting, as well as the absence of premature metamorphosis and aberrant adults in repeatedly regenerating animals, by reference to the idea of a balanced system. If, however, the postponement of metamorphosis in repeatedly regenerating animals is due to a deficiency in a growth and differentiation hormone also responsible for moulting, the observed results are difficult to interpret; numerous supermoults occur during the very period when the hormone deficiency is

preventing metamorphosis. This difficulty is resolved by assuming that (at least in *B. germanica*) two linked but distinct hormones are responsible for moulting on the one hand and for growth and adult differentiation on the other. Utilization of the growth hormone in regeneration might abolish the competence of the tissues to respond to the moulting hormone until a normal balance was restored, as suggested by O'Farrell and Stock (1953) in explaining the time relations of single regeneration and moulting. With repeated regeneration, a moult would then occur each time the level of growth hormone became sufficient to restore tissue competence towards the moulting hormone; this level of growth hormone would not necessarily be high enough to bring about metamorphosis.

The difficulty of identifying by direct methods the many elements probably present in the hormone complex of insects seems to have led to caution in suggesting additions to their number. The indirect evidence provided by the present work seems to justify the suggestion that in *B. germanica* there may be a moulting hormone entirely distinct from, but functionally connected with, the growth and differentiation hormone.

V. Acknowledgments

We are indebted to the Research Committee, University of New England, and to the New South Wales State Cancer Council for providing funds to assist this work.

VI. References

Bodenstein, D. (1953).—Studies on the humoral mechanisms in growth and metamorphosis of the cockroach, *Periplaneta americana*. I. Transplantation of integumental structures and experimental parabiosis. *J. Exp. Zool.* **123**: 189–232.

Bodenstein, D. (1955).—Contributions to the problem of regeneration in insects. *J. Exp. Zool.* **129**: 209–24.

Brooks, M. A., and Richards, A. G. (1955).—Intracellular symbiosis in cockroaches. I. Production of aposymbiotic cockroaches. *Biol. Bull.* **109**: 22–39.

Needham, A. E. (1949).—Depletion and recuperation of the local factors during repeated regeneration. *J. Exp. Zool.* **112**: 207–32.

Novak, V. J. A. (1951*a*).—New aspects of the metamorphosis of insects. *Nature* **167**: 132–3.

Novak, V. J. A. (1951*b*).—The metamorphosis hormones and morphogenesis in *Oncopeltus fasciatus* Dal. *Mém. Soc. Zool. Tchécosl.* **15**: 1–48.

O'Farrell, A. F., and Stock, A. (1953).—Regeneration and the moulting cycle in *Blattella germanica* L. I. Single regeneration initiated during the first instar. *Aust. J. Biol. Sci.* **6**: 485–500.

O'Farrell, A. F., and Stock, A. (1954).—Regeneration and the moulting cycle in *Blattella germanica* L. III. Successive regeneration of both metathoracic legs. *Aust. J. Biol. Sci.* **7**: 525–36.

Scharrer, B. (1946).—The role of the corpora allata in the development of *Leucophaea maderae* (Orthoptera). *Endocrinology* **38**: 35–45.

Seamans, L., and Woodruff, L. C. (1939).—Some factors influencing the number of molts of the German roach. *J. Kans. Ent. Soc.* **12**: 73–6.

Stock, A., and O'Farrell, A. F. (1954).—Regeneration and the moulting cycle in *Blattella germanica* L. II. Simultaneous regeneration of both metathoracic legs. *Aust. J. Biol. Sci.* **7**: 302–7.

Wigglesworth, V. B. (1954).—"The Physiology of Insect Metamorphosis." 152 pp. (Cambridge Monographs in Experimental Biology No. 1). (Cambridge Univ. Press.)

Zabinski, J. (1936).—Inconstancy of the number of moults during the post-embryonic development of certain Blattidae. *Ann. Mus. Zool. Polon.* **11** (12).